

Todar's Online Textbook of Bacteriology

Dedication to Hans Zinsser

Welcome to Todar's Online Textbook of Bacteriology <http://www.textbookofbacteriology.net/>. This textbook has evolved from online and live lectures presented in my bacteriology courses at the University of Wisconsin-Madison. Its contents are suitable for reading or presentation in courses or course modules concerning general microbiology and medical bacteriology at the college and advanced high school levels of education. As an electronic text, new material is constantly being added, and current material is constantly being revised and updated. This is an inherent advantage of the web-based text over the tree-burner.

The textbook will never be complete, as the rate of production of new information in microbiology far outruns the author's ability to acquire and properly present it. If you have suggestions, comments or criticisms regarding the textbook or its contents, or the idea of this type of textbook, please send email to me at the address below.

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Kenneth Todar is currently on the teaching faculty of the Department of Bacteriology at the University of Wisconsin-Madison. He received a PhD degree from The University of Texas at Austin in 1972. Since 1970, he has taught microbiology at The University of Texas, University of Alaska, and University of Wisconsin. His main teaching interests are in bacterial diversity, microbial ecology and pathogenic bacteriology. Tutorial materials associated with two of the courses taught at University of Wisconsin are on the web at [Bacteriology 303: Prokaryotic Microbiology](#) and [Bacteriology 330: Host-Parasite Interactions](#).

Lectures and teaching materials being developed for a new course, "The Microbial World" can be found now on the web at [The Microbial World Home Page](#)

WEB TEXT REVIEW (SCIENCE Magazine VOL 304 04 JUNE 2004 1421) "The Good, the Bad, and the Deadly"

"The pearly droplets in this photo are colonies of *Bacillus anthracis*, the bacterium that causes anthrax. The bugs exude a goopy coating that repels immune system assaults and allows them to establish a foothold in the body. Learn more about the tricks bacteria use to prosper almost everywhere on Earth in this Web text from microbiologist Kenneth Todar of the University of Wisconsin, Madison. High school and college students can absorb the basics of bacterial structure,

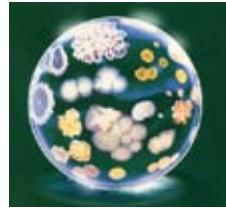


physiology, classification, and ecology. The book emphasizes medical microbiology, exploring how bacteria hitch a ride from host to host, how the body tries to corral invading microbes, and how the bugs elude these defenses. For example, the cholera bacterium releases a toxin that induces intestinal cells to spill ions and water, producing potentially lethal diarrhea."

textbookofbacteriology.net

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[Todar's Online Textbook of Bacteriology](#)

Overview of Bacteriology

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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 eukaryotic 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 "archaeabacteria". This group of procaryotes has since been renamed **Archaea** and has been awarded biological **Domain** status on the level with **Bacteria** and **Eukarya**. 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. Actually, many archaea have been studied as intensively and as long as their bacterial counterparts, but with the notion that they were bacteria.



Figure 1. The cyanobacterium *Anabaena*. American Society for Microbiology. Two (not uncommon) exceptions that procaryotes are unicellular and undifferentiated are seen in *Anabaena*: 1. The organism lives as a multicellular filament or chain of cells. Procaryotes are considered "unicellular organisms" because all the cells in a filament or colony are of the same type, and any one individual cell can give rise to an exact filament or colony; 2. The predominant photosynthetic (bright yellow-green) cells do differentiate into another type of cell: the obviously large "empty" cells occasionally seen along a filament are differentiated cells in which nitrogen fixation, but not photosynthesis, takes place.

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 at least 3 billion years ago. **Anoxygenic photosynthesis** (bacterial photosynthesis, which is anaerobic and does not produce O₂) preceded **oxygenic photosynthesis** (plant-type photosynthesis, which yields O₂). But oxygenic photosynthesis also arose in procaryotes, specifically in the cyanobacteria, which existed millions of years before the evolution of plants. Larger, more complicated eukaryotic cells did not appear until much later, between 1.5 and 2 billion years ago.

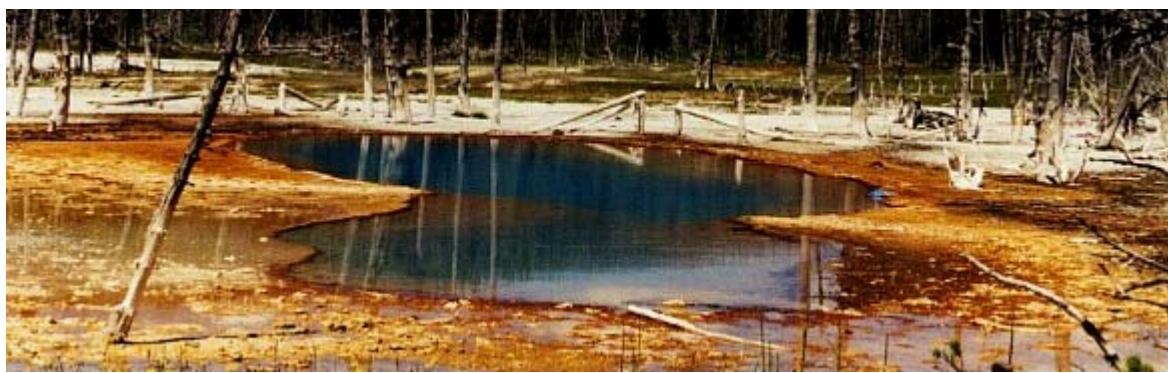


Figure 2. Opalescent Pool in Yellowstone National Park, Wyoming USA. K. Todar. Conditions for life in this environment are similar to Earth over 2 billion years ago. In these types of hot springs, the orange, yellow and brown colors are due to pigmented photosynthetic bacteria which make up the microbial 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 cell structure from eukaryotes, 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.

The Universal Tree of Life

On the basis of **small subunit ribosomal RNA (ssrRNA) analysis** the Woesean tree of life gives rise to **three cellular domains of life: Archaea, Bacteria, and Eukarya** (Figure 3). **Bacteria** (formerly known as **eubacteria**) and **Archaea** (formerly called **archaeabacteria**) share the procaryotic type of cellular configuration, but otherwise are not related to one another any more closely than they are to the eukaryotic domain, **Eukarya**. Between the two procaryotes, **Archaea** are apparently more closely related to **Eukarya** than are the **Bacteria**. **Eukarya** consists of all eukaryotic cell-types, including protista, fungi, plants and animals.

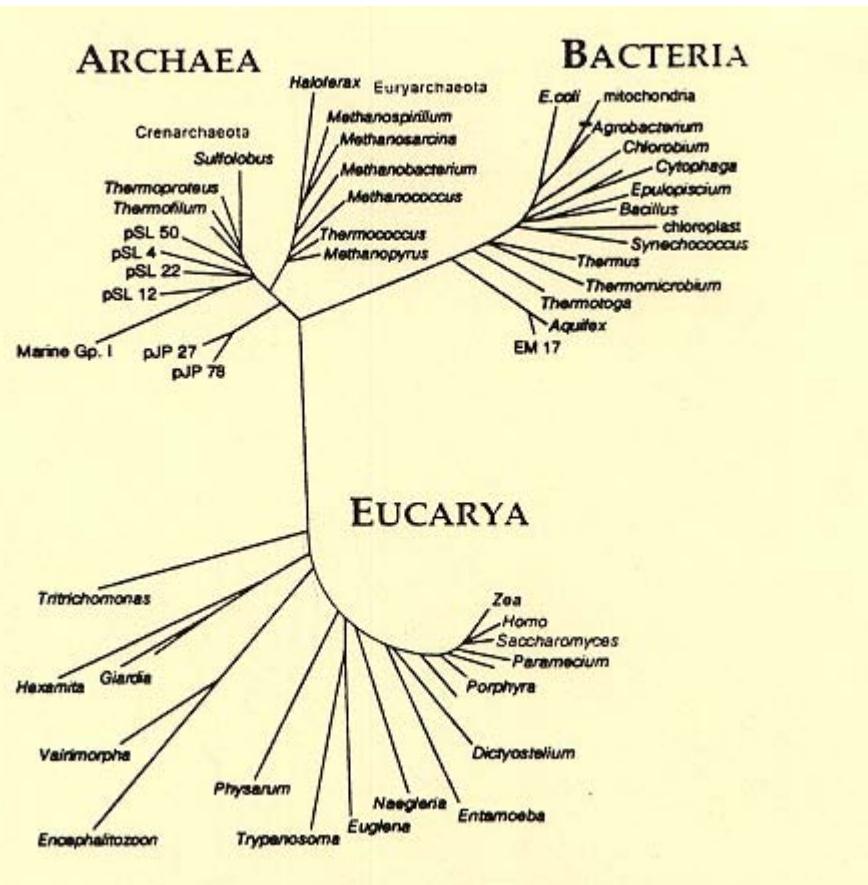


Figure 3. The Universal Tree of Life as derived from sequencing of ssrRNA. N. Pace. Note the three major domains of living organisms: Archaea, Bacteria and Eucarya (Eukarya). The "evolutionary distance" between two organisms is proportional to the measurable distance between the end of a branch to a node to the end of a comparative branch. For example, in Eucarya, humans (*Homo*) are more closely related to corn (*Zea*) than to slime molds (*Dicyostelium*); or in Bacteria, *E. coli* is more closely related to *Agrobacterium* than to *Thermus*.

OFF THE WALL. It is interesting to note several features of phylogeny and evolution that are revealed in the Unrooted Tree.

--Archaea are the least evolved type of cell (they remain closest to the common point of origin). This helps explain why contemporary Archaea are inhabitants of environments that are something like the earth 3.86 billion years ago (hot, salty, acidic, anaerobic, low in organic material, etc.).

--Eucaryotes (Eucarya) are the most evolved type of cell (they move farthest from the common point of origin). However, the eucaryotes do not begin to diversify (branch) until relatively late in evolution, at a time when the Bacteria diversify into oxygenic photosynthesis (*Synechococcus*) and aerobic respiration (*Agrobacterium*).

--Mitochondria and the respiratory bacterium, *Agrobacterium*, are derived from a common ancestor; likewise, chloroplast and the cyanobacterium, *Synechococcus*, arise from a common origin. This is good evidence for the idea of evolutionary endosymbiosis, i.e., that the origin of eukaryotic mitochondria and chloroplasts is in prokaryotic cells that were either captured by, or which invaded, eukaryotic cells, and subsequently entered into a symbiotic association with one cell living inside of the other.

--Diversification in Eucarya is within the Protista (unicellular protozoa, algae, and including fungi). The only multicellular eukaryotes on the Tree are *Zea* (plants) and *Homo* (animals). Since the protists, along with the archaea and bacteria, constitute the microbial ("microorganismal") community of the planet, this helps to substantiate the claim the microorganisms are the predominant and most diverse form of life on Earth.

--Humans (*Homo*) are more closely related to yeast (*Saccharomyces*) than they are to corn (*Zea*). There are more genetic differences between *E. coli* and *Bacillus* than there are between humans and a paramecium. The protozoan *Trichomonas* is more closely related to the archaea than it is to the protozoan *Trypanosoma*. When the tree branches are amplified there many other similar surprises to biologists.

--Most biology and anthropology students have been presented with fossil and other structural evidence that humans (*Homo*) emerged a very short time ago on the evolutionary clock. The Tree confirms this evidence on the basis of comparative molecular genetic analysis.

Genomic Timescale of Prokaryotic Evolution

Comparison of protein sequences whose genes are common to the genomes of several prokaryotes has resulted in a "genomic timescale of prokaryotic evolution" and establishes the following dates for some major events in prokaryotic evolution (Battistuzzi, et al. MC Evol Biol. 2004; 4: 44). The results are consistent with most other phylogenetic schemes that recognize higher-level groupings of prokaryotes.

Table 1. Genomic timescale for some major events in prokaryotic evolution

Origin of life: prior to 4.1 billion years ago (Ga)

Origin of methanogenesis: 3.8 - 4.1 Ga

Origin of phototrophy: prior to 3.2 Ga

Divergence of the major groups of Archaea: 3.1 - 4.1 Ga

Origin of anaerobic methanotrophy: after 3.1 Ga

Colonization of land: 2.8 - 3.1 Ga

Divergence of the major groups of Bacteria: 2.5 - 3.2 Ga

Origin of aerobic methanotrophy: 2.5 - 2.8 Ga.

The time estimates for methanogenesis support the consideration of methane, in addition to carbon dioxide, as a greenhouse gas responsible for the early warming of the Earth's surface.

Divergence times for the origin of anaerobic methanotrophy are compatible with carbon isotopic values found in rocks dated 2.8 - 2.6 Ga.

The origin of phototrophy is consistent with the earliest bacterial mats and structures identified as stromatolites, but a 2.6 Ga origin of cyanobacteria suggests that those structures (if biologically produced) would have been made by anoxygenic photosynthesizers.

A well-supported group of three major lineages of Bacteria (Actinobacteria, Deinococcus, and Cyanobacteria), that have been called "Terrabacteria", are associated with an early colonization of land.

Size and Distribution of Bacteria and Archaea

Most prokaryotic cells are very small compared to eukaryotic cells. A typical bacterial cell is about 1 micrometer in diameter while most eukaryotic cells are from 10 to 100 micrometers in diameter. Eukaryotic cells have a much greater volume of cytoplasm and a much lower surface : volume ratio than prokaryotic cells. A typical prokaryotic cell is about the size of a eukaryotic mitochondrion. Since prokaryotes 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, prokaryotes 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 eukaryotic 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 prokaryotes, far in excess of undiscovered or unstudied plants, are a tremendous reserve of genetic material and genetic information in nature that awaits exploitation.

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

From a metabolic standpoint, the prokaryotes are extraordinarily diverse, and they exhibit several types of metabolism that are rarely or never seen in eukaryotes. For example, the biological processes of **nitrogen fixation** (conversion of atmospheric nitrogen gas to ammonia) and **methanogenesis** (production of methane) are

metabolically-unique to prokaryotes 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 and archaea 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 prokaryotes in soil habitats have an enormous impact on soil fertility that can affect agricultural practices and crop yields. In the global environment, prokaryotes 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 prokaryotes. 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 CO₂, 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.

STRUCTURE AND FUNCTION OF PROKARYOTIC CELLS

Prokaryotic cells have three architectural regions (Figure 4): **appendages** (proteins attached to the cell surface) in the form of **flagella** and **pili**; 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**.

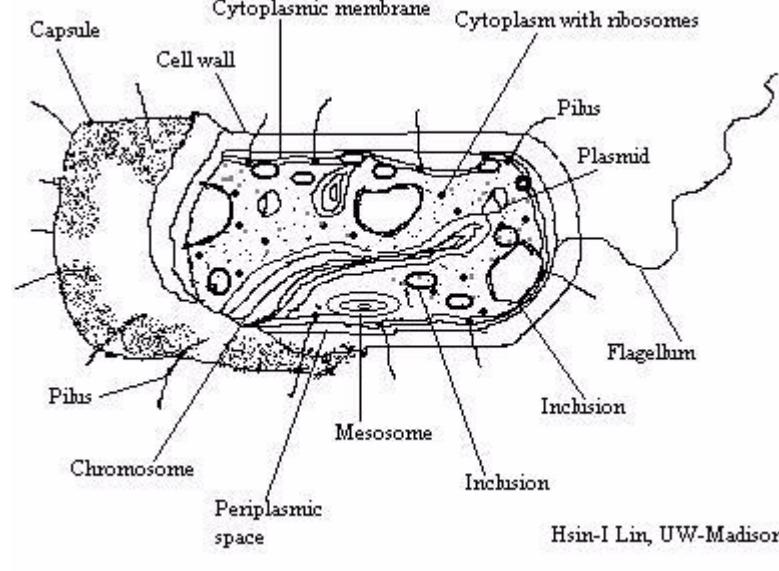


Figure 4. Schematic drawing of a typical bacterium.

Surface Structures-Appendages

Flagella are filamentous protein structures attached to the cell surface that provide swimming movement for most motile prokaryotic cells. 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. Prokaryotes 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 their environment and swim towards them (if they are useful nutrients) or away from them (if they are harmful substances).

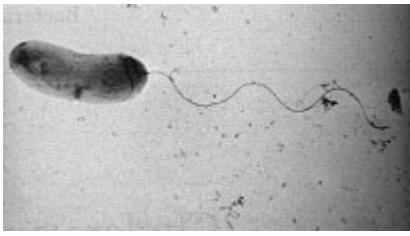


Figure 5.*Vibrio cholerae* has a single polar flagellum for swimming movement. Electron Micrograph of *Vibrio cholerae* by Leodotia Pope, Department of Microbiology, University of Texas at Austin.

Fimbriae and **Pili** are interchangeable terms used to designate short, hair-like structures on the surfaces of prokaryotic cells. Fimbriae are shorter and stiffer than flagella, and slightly smaller in diameter. Like flagella, they are composed of protein. A specialized type of pilus the **F or sex pilus**, mediates the transfer of DNA between mating bacteria, but the function of the smaller, more numerous **common pili** is quite different. Common pili (almost always called **fimbriae**) are usually involved in adherence (attachment) of prokaryotes to surfaces in nature. In medical situations, they are major determinants of bacterial virulence because they allow pathogens to attach to (colonize) tissues and to resist attack by phagocytic white blood cells.

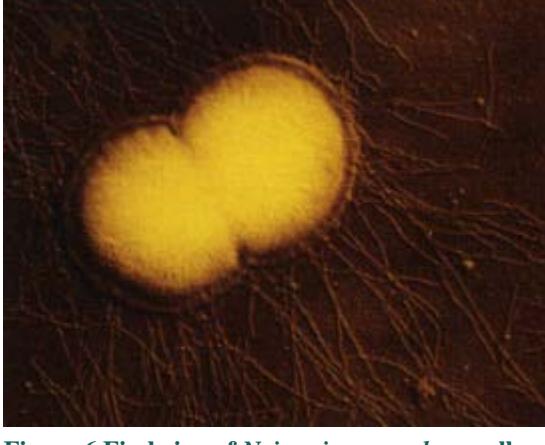


Figure 6.Fimbriae of *Neisseria gonorrhoeae* allow the bacterium to adhere to tissues. Electron micrograph by David M. Phillips, [Visuals Unlimited](#), with permission.

The Cell Envelope

Most prokaryotes have a rigid **cell wall**. The cell wall is an essential structure that protects the delicate cell protoplast from osmotic lysis. The cell wall of Bacteria consists of a polymer of disaccharides cross-linked by short chains of amino acids (peptides). This molecule is a type of **peptidoglycan** which is called **murein**. In the **Gram-positive** bacteria (those that retain the purple crystal violet dye when subjected to the Gram-staining procedure) the cell wall is a thick layer of murein. In the **Gram-negative** bacteria (which do not retain the crystal violet) the cell wall is relatively thin and is composed of a thin layer of murein surrounded by a membranous structure called the **outer membrane**. **Murein** is a substance unique in nature to bacterial cell walls. Also, the outer membrane of Gram-negative bacteria invariably contains a unique component, **lipopolysaccharide (LPS or endotoxin)**, which is toxic to animals. 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.

Although prokaryotes lack any intracellular organelles for respiration or photosynthesis, many species possess the physiologic ability to conduct these processes, usually as a function of the **plasma membrane**. For example, the electron transport system that couples aerobic respiration and ATP synthesis is found in the plasma 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 or photophosphorylation in prokaryotes, analogous to the functions of mitochondria and chloroplasts in eukaryotic cells. The prokaryotic plasma membrane is also a permeability barrier, and it contains a variety of different transport systems that selectively mediate the passage of substances into and out of the cell.

The membranes of Bacteria are structurally similar to the cell membranes of eukaryotes, except that bacterial membranes consist of saturated or monounsaturated fatty acids (rarely polyunsaturated fatty acids) and do not normally contain sterols. The membranes of Archaea form phospholipid bilayers functionally equivalent to bacterial membranes, but archaeal lipids are saturated, branched, repeating isoprenoid subunits that attach to

glycerol via an ether linkage, as opposed to the ester linkage found in glycerides of eukaryotic and bacterial membrane lipids. The structure of archaeal membranes is thought to be an adaptation to their survival in extreme environments.

Most bacteria contain some sort of a polysaccharide layer outside of the cell wall or outer membrane. In a general sense, this layer is called a **capsule** or **glycocalyx**. Capsules, slime layers, and glycocalyx are known to mediate attachment of bacterial cells to particular surfaces. Capsules also protect bacteria from engulfment by predatory protozoa or white blood cells (phagocytes) and from attack by antimicrobial agents of plant or animal origin. Capsules in certain soil bacteria protect them from perennial effects of drying or desiccation.

Importance of Surface Components

All of the various **surface components** of a prokaryotic cell are important in its ecology since they mediate the contact of the cell with its environment. The only "sense" that a prokaryote has results from its immediate contact with its environment. It must use its surface components to assess the environment and respond in a way that supports its own existence and survival in that environment. The surface properties of a prokaryote are determined by the exact molecular composition of its plasma membrane and cell wall, including LPS, and the function of surface structures such as flagella, fimbriae and capsules. Some important ways that prokaryotes use their surface components are (1) as permeability barriers that allow selective passage of nutrients and exclusion of harmful substances; (2) as "adhesins" used to attach or adhere to specific surfaces or tissues; (3) as enzymes to mediate specific reactions on the cell surface important in the survival of the prokaryote; (4) as "sensing proteins" that can respond to temperature, osmolarity, salinity, light, oxygen, nutrients, etc., resulting in a signal to the genome of the cell that will cause a beneficial response to the new environment.

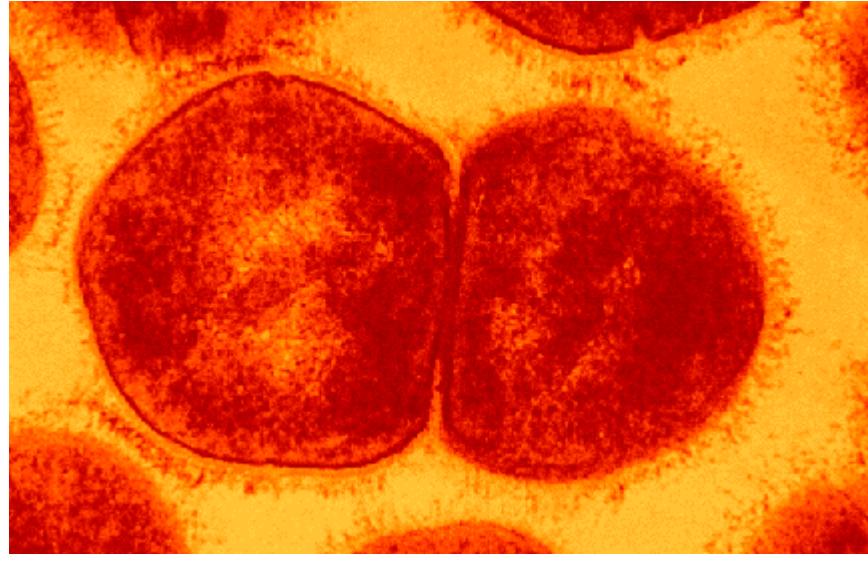


Figure 7. The complex surface of *Streptococcus pyogenes*. Electron micrograph of *Streptococcus pyogenes* by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology, Rockefeller University](#).

Cytoplasmic Constituents

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

The distinct granular appearance of prokaryotic cytoplasm is due to the presence and distribution of **ribosomes**. The ribosomes of prokaryotes are smaller than cytoplasmic ribosomes of eukaryotes. Prokaryotic 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.

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 PO₄ 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.



Figure 8. Bacterial colonies growing in a petri dish containing nutrients.

Hans Knoll Institute, Jena, Germany.

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 **Prokaryotae**). **Protista** remained as a kingdom of unicellular eukaryotic microorganisms. Whittaker refined the system into five kingdoms in 1967, by identifying the **Fungi** as a separate multicellular eukaryotic kingdom of organisms, distinguished by their absorptive mode of 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 eukaryotes. Thus, Woese defined the **three cellular domains of life** as they are displayed in Figure 3 (above): **Eucarya**, **Bacteria** and **Archaea**. Whittaker's Plant, Animal and Fungi kingdoms (all of the multicellular eukaryotes) 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).

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 2). The phylogenetic tree indicates that **Archaea** are more closely related to **Eukarya** than are **Bacteria**. This relatedness seems most evident in the similarities between transcription and translation in the **Archaea** and the **Eukarya**. However, it is also evident that the **Bacteria** have evolved into

chloroplasts and mitochondria, so that these eukaryotic organelles derive their lineage from this group of prokaryotes. Perhaps the biological success of eukaryotic cells springs from the evolutionary merger of the two prokaryotic life forms.

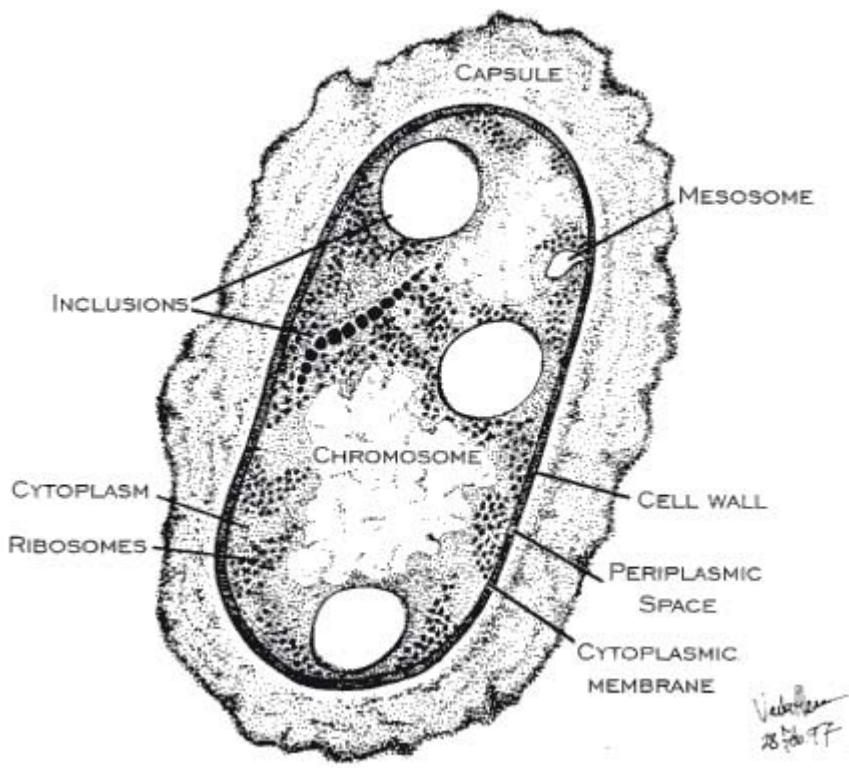


Figure 9 (above) The structure of a typical prokaryotic cell, in this case, a Gram-negative bacterium, compared with (below) a typical eukaryotic cell (plant cell). The prokaryote is about 1 micrometer in diameter and about the size of the eukaryotic chloroplast or mitochondrion. Drawings by Vaike Haas, University of Wisconsin-Madison.

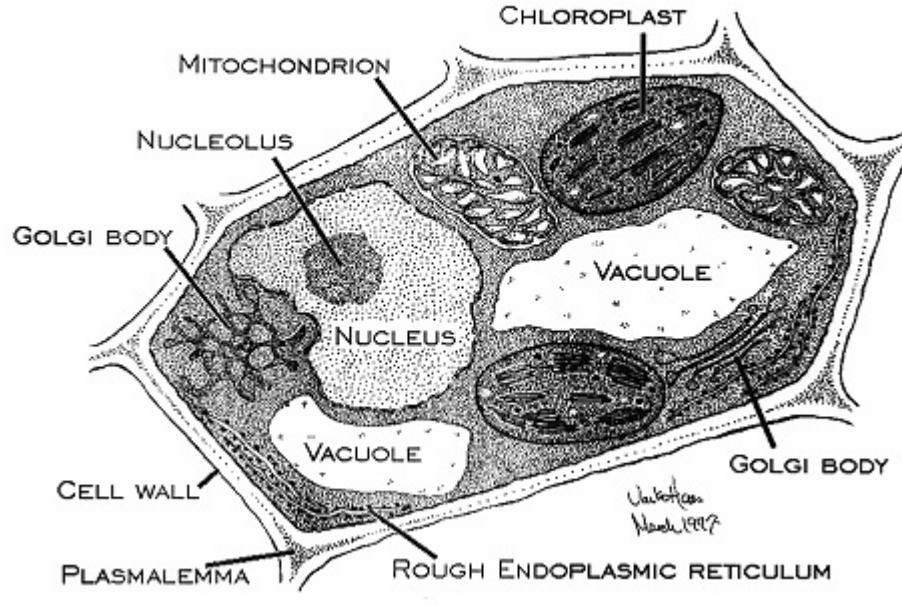


Table 2. Phenotypic properties of Bacteria and Archaea compared with Eukarya.

Property	Biological Domain		
	Eukarya	Bacteria	Archaea
Cell configuration	eukaryotic	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 prokaryotes 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.

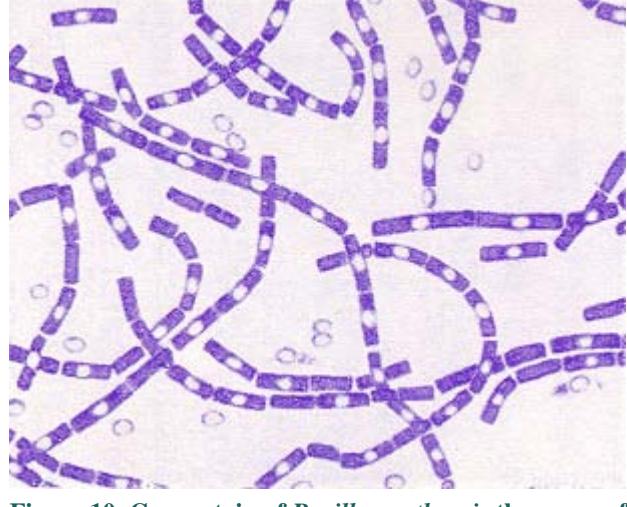


Figure 10. Gram stain of *Bacillus anthracis*, the cause of anthrax. K. Todar.

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.

A modification of the Bergey's criteria for bacterial identification, without a key, is used to organize the groups of prokaryotes for discussion in a companion chapter [Major Groups of Prokaryotes](#)

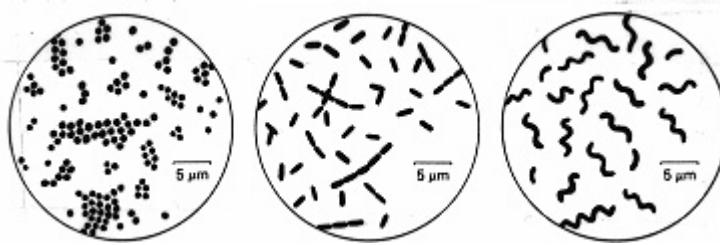


Figure 11. Size and fundamental shapes of prokaryotes revealed by three genera of Bacteria (l to r): *Staphylococcus* (spheres), *Lactobacillus* (rods), and *Aquaspirillum* (spirals).

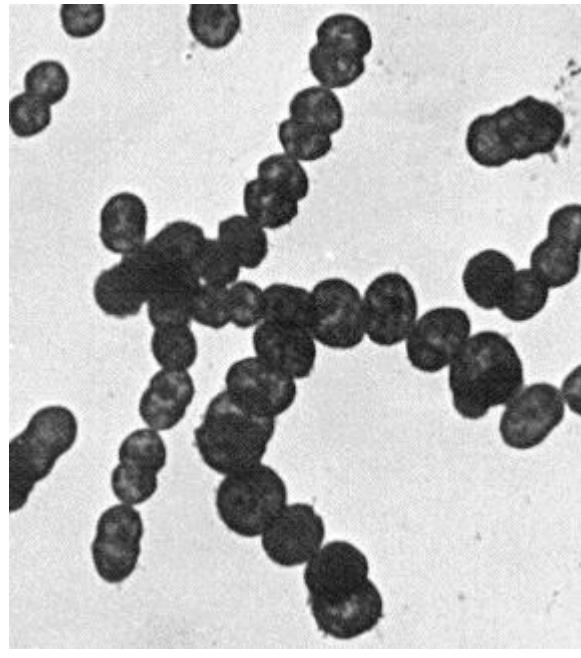


Figure 12. Chains of dividing streptococci. Electron micrograph of *Streptococcus pyogenes* by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology, Rockefeller University](#).

BACTERIAL REPRODUCTION AND GENETICS

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. The process is evidently coordinated by activites associated with the cell membrane. 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 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.

Genetic Exchange in Bacteria

Although prokaryotes do not undergo sexual reproduction, they are not without the ability to exchange genes and undergo **genetic recombination**. Bacteria are known 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 genetic engineering of bacterial cells in the research or biotechnology laboratory is often based on the use of plasmids. The genetic systems of the

Archaea are poorly characterized at this point, although the entire genome of *Methanosaeca* has been sequenced recently which will certainly open up the possibilities for genetic analysis of the group.

Evolution of Bacteria and Archaea

For most prokaryotes, mutation is a major source of variability that allows the species to adapt to new conditions. The mutation rate for most prokaryotic genes is approximately 10^{-8} . This means that if a bacterial population doubles from 10^8 cells to 2×10^8 cells, there is likely to be a mutant present for any given gene. Since prokaryotes 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. The evolution of prokaryotes, driven by such Darwinian principles of evolution (mutation and selection) is called **vertical evolution**.

However, as a result of the processes of genetic exchange described above, the bacteria and archaea can also undergo a process of **horizontal evolution**. In this case, genes are transferred laterally from one organism to another, even between members of different Kingdoms, which may allow immediate experimentation with new genetic characteristics in the recipient. Horizontal evolution is being realized to be a significant force in cellular evolution.

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 prokaryotes.

ECOLOGY OF BACTERIA AND ARCHAEA

Bacteria and Archaea are present in all environments that support life. They may be free-living, or living in associations with eukaryotes (plants and animals), and they are found in environments that support no other form of life. Prokaryotes have the usual nutritional requirements for growth of cells, but many of the ways that they utilize and transform their nutrients are unique.

Nutritional Types of Organisms

In terms of carbon utilization a cell may be heterotrophic or autotrophic. **Heterotrophs** obtain their carbon and energy for growth from organic compounds in nature. **Autotrophs** use CO_2 as a sole source of carbon for growth and obtain their energy from light (**photoautotrophs**) or from the oxidation of inorganic compounds (**lithoautotrophs**).

Most heterotrophic bacteria are **saprophytes**, meaning that they obtain their nourishment from dead organic matter. In the soil, saprophytic bacteria and fungi are responsible for **biodegradation** of organic material. Ultimately, organic molecules, no matter how complex, can be degraded to CO_2 . Probably no naturally-occurring organic substance cannot be degraded by the combined activities of the bacteria and fungi. Hence, most organic matter in nature is converted by heterotrophs to CO_2 , only to be converted back into organic material by autotrophs that die and nourish heterotrophs to complete the carbon cycle.

Lithotrophic prokaryotes have a type of energy-producing metabolism which is unique. Lithotrophs (also called **chemoautotrophs**) use inorganic compounds as sources of energy, i.e., they oxidize compounds such as H_2 or H_2S or NH_3 to obtain electrons to feed into an electron transport system and to produce ATP. Lithotrophs are found in soil and aquatic environments wherever their energy source is present. Most lithotrophs are autotrophs so they can grow in the absence of any organic material. Lithotrophic species are found among the Bacteria and the Archaea. Sulfur-oxidizing lithotrophs convert H_2S to SO_4^{2-} and S° to SO_4^{2-} . Nitrifying bacteria convert NH_3 to NO_2^- and NO_2^- to NO_3^- ; methanogens strip electrons off of H_2 as a source of energy and add electrons to CO_2 to form CH_4 (methane). Lithotrophs have an obvious impact on the sulfur, nitrogen and carbon cycles in the biosphere.

Photosynthetic bacteria convert light energy into chemical energy for growth. Most phototrophic bacteria are autotrophs so their role in the carbon cycle is analogous to that of plants. The planktonic cyanobacteria are the "grass of the sea" and their form of oxygenic photosynthesis generates a substantial amount of O_2 in the biosphere.

However, among the photosynthetic bacteria are types of metabolism not seen in eukaryotes, including **photoheterotrophy** (using light as an energy source while assimilating organic compounds as a source of carbon), anoxygenic photosynthesis, and unique mechanisms of CO₂ fixation (autotrophy).

Photosynthesis has not been found to occur among the **Archaea**, but one archaeal species of employs a light-driven non photosynthetic means of energy generation based on the use of a chromophore called **bacteriorhodopsin**

Responses to Environmental Conditions

Prokaryotes vary widely in their response to O₂ (molecular oxygen). Organisms that require O₂ for growth are called **obligate aerobes**; those which are inhibited or killed by O₂, and which grow only in its absence, are called **obligate anaerobes**; organisms which grow either in the presence or absence of O₂ are called **facultative anaerobes**. Whether or not a particular organism can exist in the presence of O₂ depends upon the distribution of certain enzymes such as superoxide dismutase and catalase that are required to detoxify lethal oxygen radicals that are always generated by living systems in the presence of O₂

Prokaryotes also vary widely in their response to temperature. Those that live at very cold temperatures (0 degrees or lower) are called **psychrophiles**; those which flourish at room temperature (25 degrees) or at the temperature of warm-blooded animals (37 degrees) are called **mesophiles**; those that live at high temperatures (greater than 45 degrees) are **thermophiles**. The only limit that seems to be placed on growth of certain prokaryotes in nature relative to temperature is whether liquid water exists. Hence growing prokaryotic cells can be found in supercooled environments (ice does not form) as low as -20 degrees and superheated environments (steam does not form) as high as 120 degrees. Archaea have been detected around thermal vents on the ocean floor where the temperature is as high as 320 degrees!

Symbiosis

The biomass of prokaryotic cells in the biosphere, their metabolic diversity, and their persistence in all habitats that support life, ensures that the prokaryotes play a crucial role in the cycles of elements and the functioning of the world ecosystem. However, the prokaryotes affect the world ecology in another significant way through their inevitable interactions with insects, plants and animals. Some bacteria are required to associate with insects, animals or plants for the latter to survive. For example, the sex of offspring of certain insects is determined by endosymbiotic bacteria. Ruminant animals (cows, sheep, etc.), whose diet is mainly cellulose (plant material), must have cellulose-digesting bacteria in their intestine to convert the cellulose to a form of carbon that the animal can assimilate. Leguminous plants grow poorly in nitrogen-deprived soils unless they are colonized by nitrogen-fixing bacteria which can supply them with a biologically-useful form of nitrogen.

Bacterial Pathogenicity

Some bacteria are **parasites** of plants or animals, meaning that they grow at the expense of their eukaryotic host and may damage, harm, or even kill it in the process. Such bacteria that cause disease in plants or animals are **pathogens**. Human diseases caused by bacterial pathogens include tuberculosis, whooping cough, diphtheria, tetanus, gonorrhea, syphilis, pneumonia, cholera and typhoid fever, to name a few. The bacteria that cause these diseases have special structural or biochemical properties that determine their virulence or pathogenicity. These include: (1) ability to colonize and invade their host; (2) ability to resist or withstand the antibacterial defenses of the host; (3) ability to produce various toxic substances that damage the host. Plant diseases, likewise, may be caused by bacterial pathogens. More than 200 species of bacteria are associated with plant diseases.



Figure 13. *Borrelia burgdorferi*. This spirochete is the bacterial parasite that causes Lyme disease. CDC.

APPLICATIONS OF BACTERIA IN INDUSTRY AND BIOTECHNOLOGY

Exploitation of Bacteria by Humans

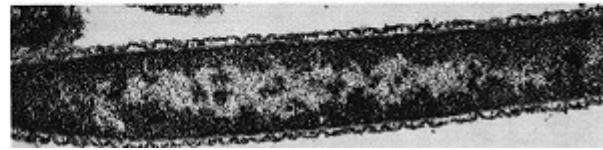
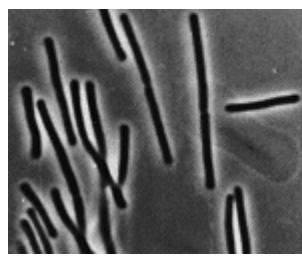
In addition to other ecological roles, prokaryotes, especially bacteria, are used industrially in the manufacture of foods, drugs, vaccines, insecticides, enzymes, hormones and other useful biological products. In fact, through genetic engineering of bacteria, these unicellular organisms can be coaxed to produce just about anything that there is a gene for. The genetic systems of bacteria are the foundation of the biotechnology industry.

In the foods industry, lactic acid bacteria such as *Lactobacillus* and *Streptococcus* are used the manufacture of dairy products such as yogurt, cheese, buttermilk, sour cream, and butter. Lactic acid fermentations are also used in pickling process. Bacterial fermentations can be used to produce lactic acid, acetic acid, ethanol or acetone. In many parts of the world, various human cultures ferment indigenous plant material using *Zymomonas* bacteria to produce the regional alcoholic beverage. For example, in Mexico, a Maguey cactus (*Agave*) is fermented to "cactus beer" or pulque. Pulque can be ingested as is, or distilled into tequila.

In the pharmaceutical industry, bacteria are used to produce antibiotics, vaccines, and medically-useful enzymes. Most antibiotics are made by bacteria that live in soil. Actinomycetes such as *Streptomyces* produce tetracyclines, erythromycin, streptomycin, rifamycin and ivermectin. *Bacillus* species produce bacitracin and polymyxin. Bacterial products are used in the manufacture of vaccines for immunization against infectious disease. Vaccines against diphtheria, whooping cough, tetanus, typhoid fever and cholera are made from components of the bacteria that cause the respective diseases. It is significant to note here that the use of antibiotics against infectious disease and the widespread practice of vaccination (immunization) against infectious disease are two twentieth-century developments that have drastically increased the quality of life and the average life expectancy of individuals in developed countries.

Biotechnology

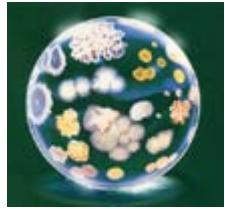
The biotechnology industry uses bacterial cells for the production of human hormones such as insulin and human growth factor (protropin), and human proteins such as interferon, interleukin-2, and tumor necrosis factor. These products are used for the treatment of a variety of diseases ranging from diabetes to tuberculosis and AIDS. Other biotechnological applications of bacteria involve the genetic construction of "super strains" of organisms to perform a particular metabolic task in the environment. For example, bacteria which have been engineered genetically to degrade petroleum products can be used in cleanup efforts of oil spills in seas or on beaches. One area of biotechnology involves improvement of the qualities of plants through genetic engineering. Genes can be introduced into plants by a bacterium *Agrobacterium tumefaciens*. Using *A. tumefaciens*, plants have been genetically engineered so that they are resistant to certain pests, herbicides, and diseases. Finally, the polymerase chain reaction (PCR), a mainstay of the biotechnology industry because it allows scientists to duplicate genes starting with a single molecule of DNA, is based on the use of a DNA polymerase enzyme derived from a thermophilic bacterium, *Thermus aquaticus*.



Thermus aquaticus, the thermophilic bacterium that is the source of taq polymerase.
L wet mount; R electron micrograph. T.D. Brock. [Life at High Temperatures](#).

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STRUCTURE AND FUNCTION OF PROKARYOTIC CELLS

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Prokaryotes are unicellular organisms of relatively simple construction, especially if compared to eukaryotes. Whereas eukaryotic cells have a preponderance of organelles with separate cellular functions, prokaryotes carry out all cellular functions as individual units. A prokaryotic 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 prokaryotic cells are related directly to the structure and organization of the macromolecules in their cell make-up, i.e., DNA, RNA, phospholipids, proteins and polysaccharides. Diversity within the primary structure of these molecules accounts for the diversity that exists among prokaryotes.

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 prokaryotic cell (Figure 1 below) 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 prokaryotic cells in relation to their adaptation, function and behavior in natural environments.

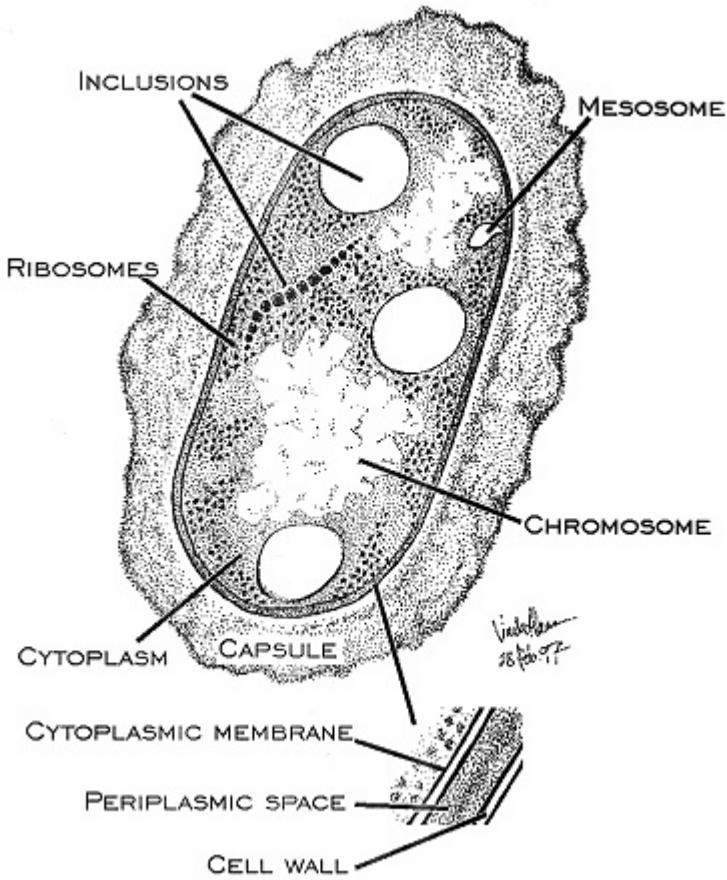


Figure 1. Drawing of a typical prokaryotic cell, by Vaike Haas, University of Wisconsin-Madison. Minimally, a prokaryote is composed of a cell wall and plasma membrane that surrounds its cytoplasm containing a chromosome, ribosomes, enzymes, several classes of RNA, and small molecules (precursors).

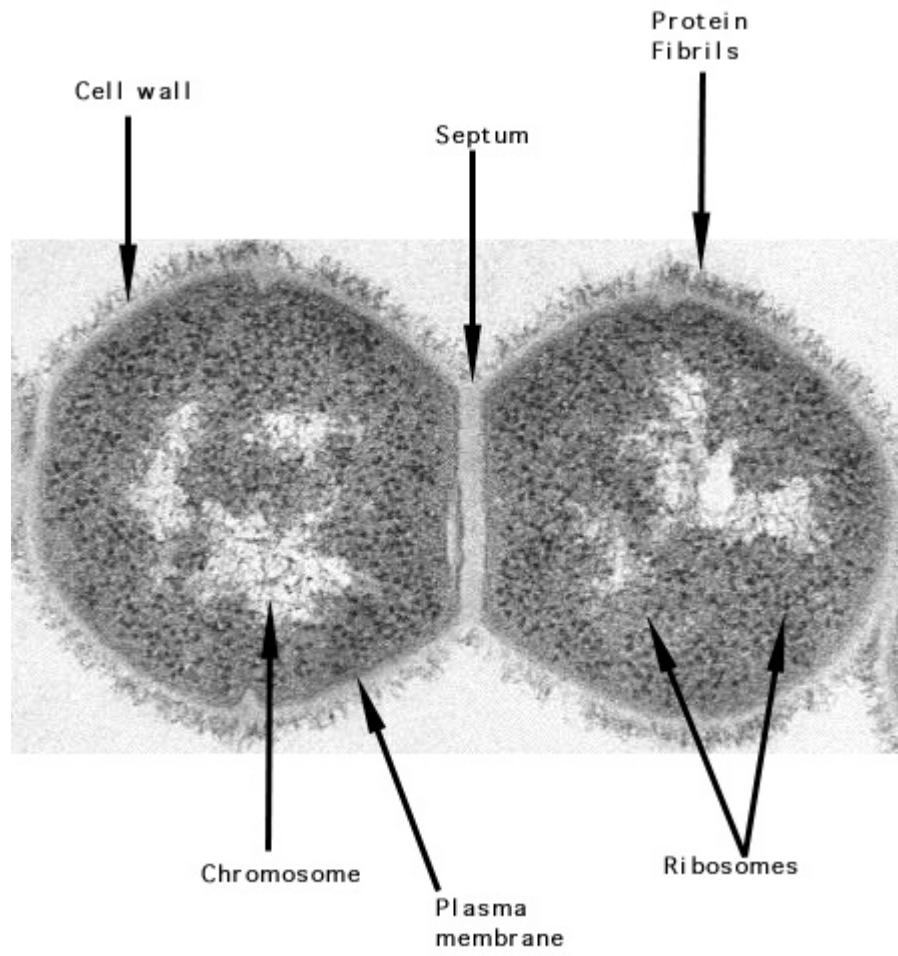


Figure 2. Electron micrograph of an ultra-thin section of a dividing pair of group A streptococci (20,000X). The cell surface fibrils, consisting primarily of M protein, are evident. The bacterial cell wall, to which the fibrils are attached, is also clearly seen as the light staining region between 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* by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology, Rockefeller University](#).

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

Flagella

Flagella are filamentous protein structures attached to the cell surface that provide the swimming movement for most motile prokaryotes. Prokaryotic flagella are much thinner than eukaryotic flagella, and they lack the typical "9 + 2" arrangement of microtubules. The diameter of a prokaryotic 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 4 below (after Dr. Julius Adler of the University of Wisconsin). 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.

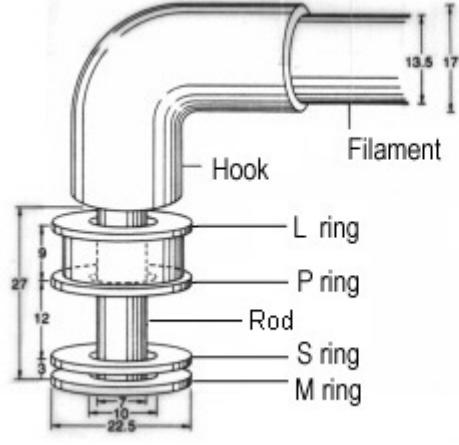


Figure 4. The ultrastructure of a bacterial flagellum (after J. Adler). Measurements are in nanometers. The flagellum of *E. coli* consists of three parts, filament, hook and basal body, all composed of different proteins. The basal body and hook anchor the whip-like filament to the cell surface. The 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.

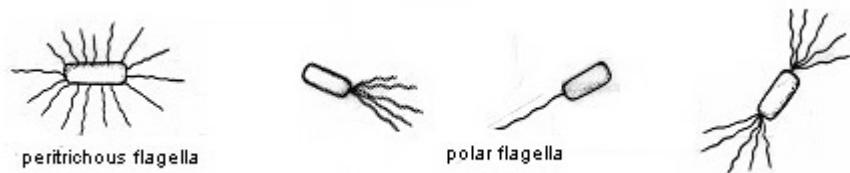


Figure 5. Different arrangements of bacterial flagella. Swimming motility, powered by flagella, occurs in half the bacilli and most of the spirilla. Flagellar arrangements, which can be determined by staining and microscopic observation, may be a clue to the identity of a bacterium. See Figure 6 below.

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).

Prokaryotes 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 prokaryotes include **phototaxis**, **aerotaxis** and **magnetotaxis**. The occurrence of tactic behavior provides evidence for the ecological (survival) advantage of flagella in bacteria and other prokaryotes.

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.

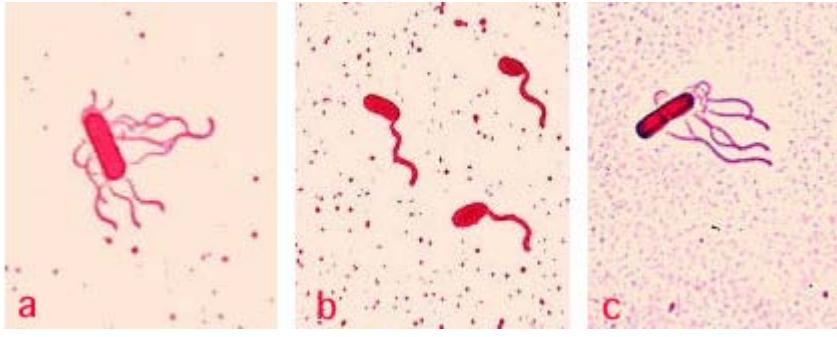


Figure 6. Flagellar stains of three bacteria a. *Bacillus cereus* b. *Vibrio cholerae* c. *Bacillus brevis*. (CDC). Since the bacterial flagellum is below the resolving power of the light microscope, although bacteria can be seen swimming in a microscope field, the organelles of movement cannot be detected. 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 unicellular 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.

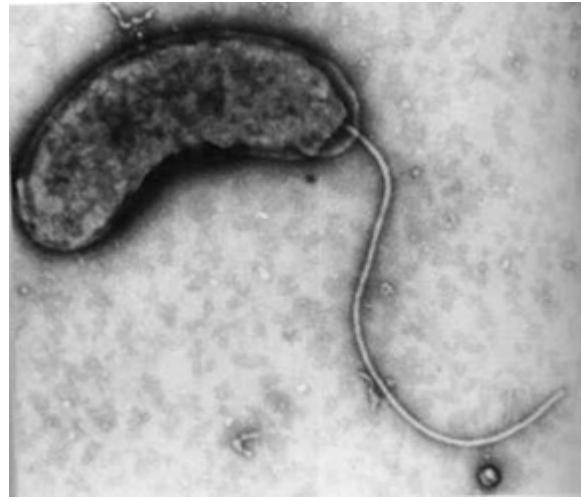


Figure 7. A *Desulfovibrio* species. TEM. About 15,000X. The bacterium is motile by means of a single polar flagellum. Of course, one can determine the presence of flagella by means of electron microscopy. Perhaps this is an alternative way to determine bacterial motility, if you happen to have an electron microscope.

Fimbriae

Fimbriae and **pili** are interchangeable terms used to designate short, hair-like structures on the surfaces of prokaryotic 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 prokaryotes 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 (See Figure 1b above).

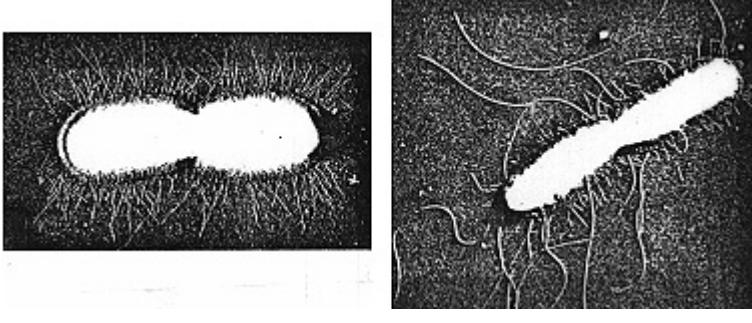


Figure 8. Fimbriae (common pili) and flagella on the surface of bacterial cells. Left: dividing *Shigella* enclosed in fimbriae. The structures are probably involved in the bacterium's ability to adhere to the intestinal surface. Right: dividing pair of *Salmonella* displaying both its peritrichous flagella 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.

Table 2. Some properties of pili and fimbriae.

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

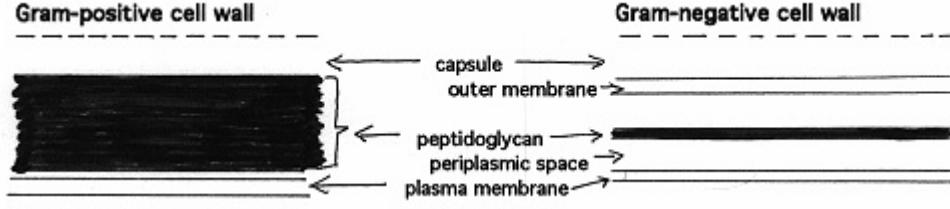


Figure 9. Profiles of the cell envelope the Gram-positive and Gram-negative bacteria. The Gram-positive wall is a uniformly thick layer external to the plasma membrane. It is composed mainly of peptidoglycan (murein). The Gram-negative wall appears thin and multilayered. It consists of a relatively thin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

Capsules

Most prokaryotes 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).

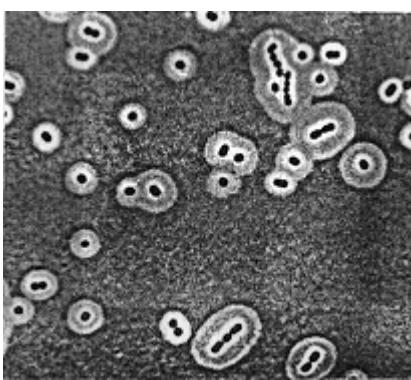


Figure 10. Bacterial capsules outlined by India ink viewed by light microscopy. This is a true capsule, a discrete layer of polysaccharide surrounding the cells. Sometimes bacterial cells are embedded more randomly in a polysaccharide matrix called a slime layer or biofilm. Polysaccharide films that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

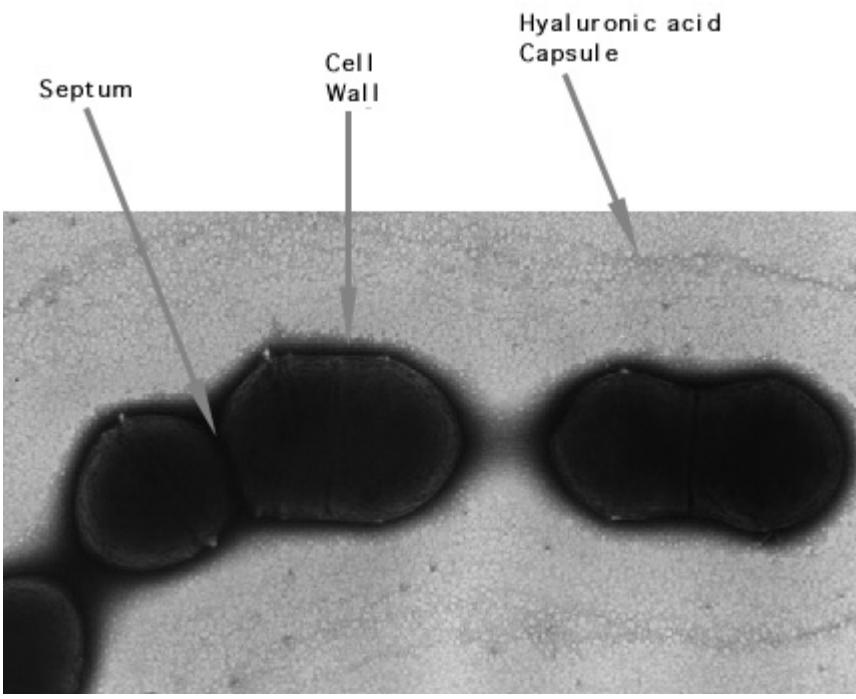


Figure 11. Negative stain of *Streptococcus pyogenes* viewed by transmission electron microscopy (28,000X). The halo around the chain of cells is the hyaluronic acid capsule that surrounds the exterior of the bacteria. The septa between dividing pairs of cells may also be seen. Electron micrograph of *Streptococcus pyogenes* by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology, Rockefeller University](#).

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		
<i>Bacillus anthracis</i>	polypeptide (polyglutamic acid)	D-glutamic acid
<i>Bacillus megaterium</i>	polypeptide and polysaccharide	D-glutamic acid, amino sugars, sugars
<i>Streptococcus mutans</i>	polysaccharide	(dextran) glucose
<i>Streptococcus pneumoniae</i>	polysaccharides	sugars, amino sugars, uronic acids
<i>Streptococcus pyogenes</i>	polysaccharide (hyaluronic acid)	N-acetyl-glucosamine and glucuronic acid
Gram-negative Bacteria		
<i>Acetobacter xylinum</i>	polysaccharide	(cellulose) glucose
<i>Escherichia coli</i>	polysaccharide (colonic acid)	glucose, galactose, fucose glucuronic acid
<i>Pseudomonas aeruginosa</i>	polysaccharide	mannuronic acid
<i>Azotobacter vinelandii</i>	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 prokaryotes 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**. Prokaryotes usually live in relatively dilute environments such that the accumulation of solutes inside the prokaryotic 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.

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**.

The profiles of the cell walls of **Bacteria**, as seen with the electron microscope, are redrawn in Figure 5. 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 beta1,4-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 13 below.

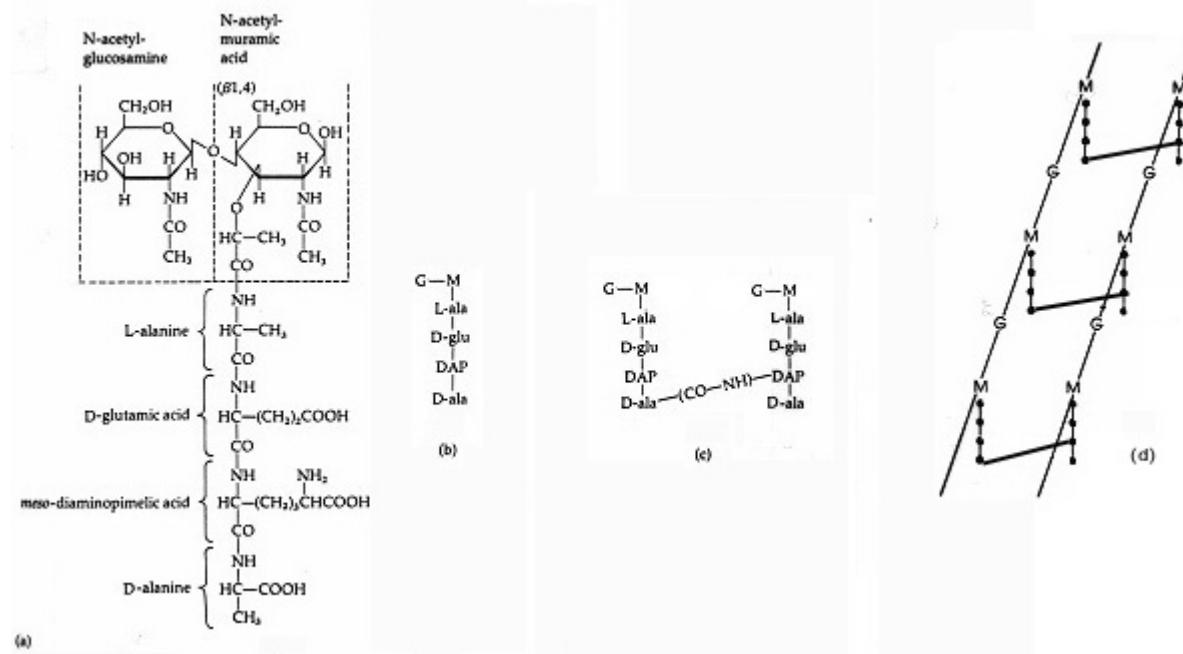


Figure 13. The structure of the muramic acid subunit of the peptidoglycan of *Escherichia coli*. This is the type of murein found in most Gram-negative bacteria. The glycan backbone is a repeat polymer of two amino sugars, N-acetylglucosamine (G) and N-acetylmuramic acid (M). Attached to the N-acetylmuramic 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 14 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.

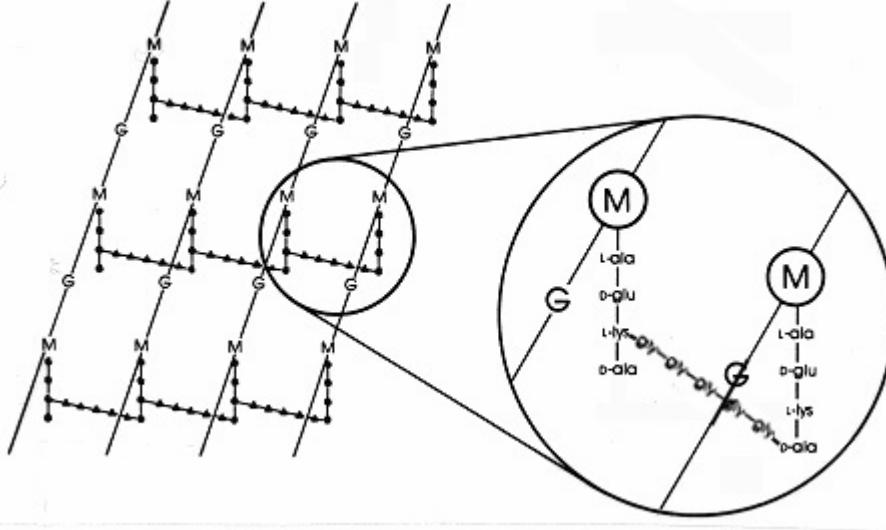


Figure 14. Schematic diagram of the peptidoglycan sheet of *Staphylococcus aureus*. G = N-acetyl-glucosamine; M = N-acetyl-muramic acid; L-ala = L-alanine; D-ala = D-alanine; D-glu = D-glutamic acid; L-lys = L-lysine. This is one type of murein found in Gram-positive bacteria. Compared to the *E. coli* peptidoglycan (Figure 7) 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 15 below). 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.

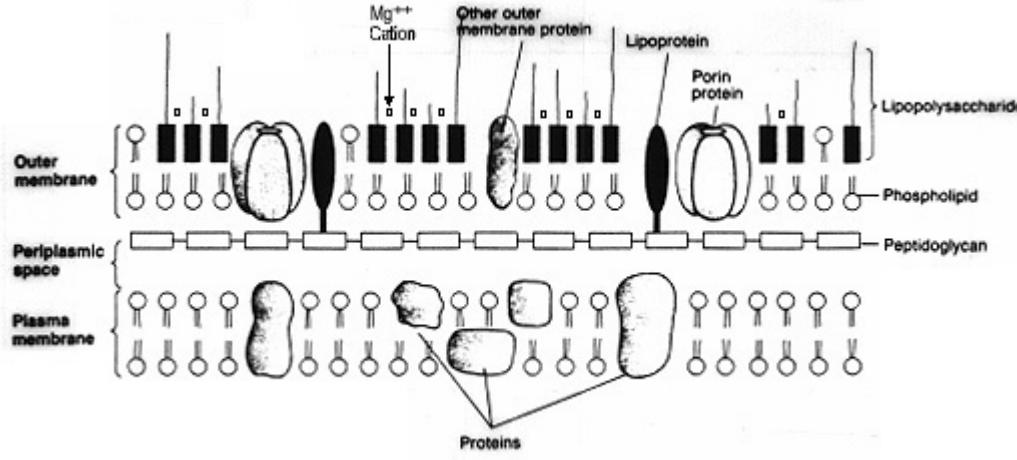


Figure 15. Schematic illustration of the outer membrane, cell wall and plasma membrane of a Gram-negative bacterium. Note the structure and arrangement of molecules that constitute the outer membrane.

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**.

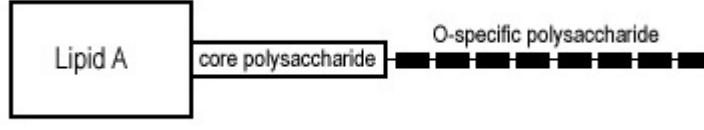


Figure 16. Structure of LPS

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 400,000 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 of 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 prokaryotic cell. Its main function is 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 prokaryotes 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 prokaryotic 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 prokaryotes, 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, prokaryotic 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, CO₂ fixation and ammonia oxidation. The predominant functions of bacterial membranes are listed in the table below.

Table 6. Functions of the prokaryotic 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 17.

The membranes of **Bacteria** are structurally similar to the cell membranes of eukaryotes, except that bacterial membranes consist of saturated or monounsaturated fatty acids (rarely, polyunsaturated fatty acids) and do not normally contain sterols (Figure 11a). The membranes of **Archaea** form bilayers functionally equivalent to bacterial membranes, but archaeal lipids are saturated, branched, repeating isoprenoid subunits that attach to glycerol via an ether linkage as opposed to the ester linkage found in glycerides of eukaryotic and bacterial membrane lipids (Figure 18). The structure of archaeal membranes is thought to be an adaptation to their existence and survival in extreme environments.

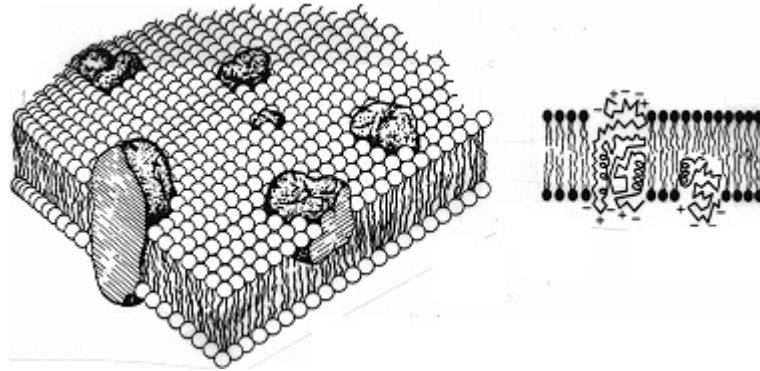


Figure 17. Fluid mosaic model of a biological membrane. In aqueous environments membrane phospholipids arrange themselves in such a way that they spontaneously form a fluid bilayer. Membrane proteins, which may be structural or functional, may be permanently or transiently associated with one side or 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.

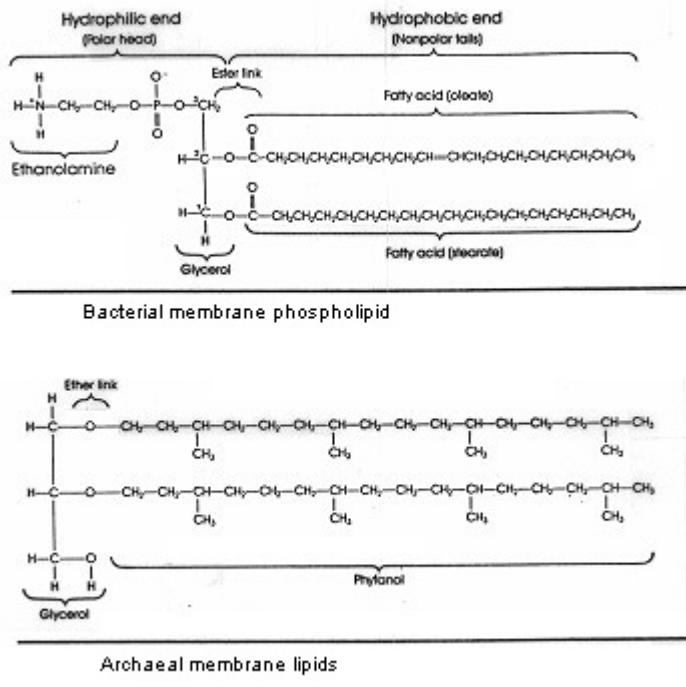


Figure 18. Generalized structure of a membrane lipids. (top). A phospholipid in the membrane of the bacterium *Escherichia coli*. The R1 and R2 positions on glycerol are substituted with saturated or monounsaturated fatty acids, with ester linkages to the glyceride. The R3 position is substituted with phosphatidylethanolamine, the most common substituent in this position in Bacteria. (bottom). An Archaeal membrane lipid. In contrast to bacterial phospholipids, which are glycerol esters of fatty acids, the lipids in membranes of Archaea are diethers of glycerol and long-chain, branched, saturated hydrocarbons called isoprenoids or which are made up of repeating C5 subunits. One of the major isoprenoids is the C20 molecule phytanol. The R3 position of glycerol may or may not be substituted. The structure of archaeal membrane lipids is thought to be an adaptation to extreme environments such as hot and acidic conditions where Archaea prevail in nature.

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.

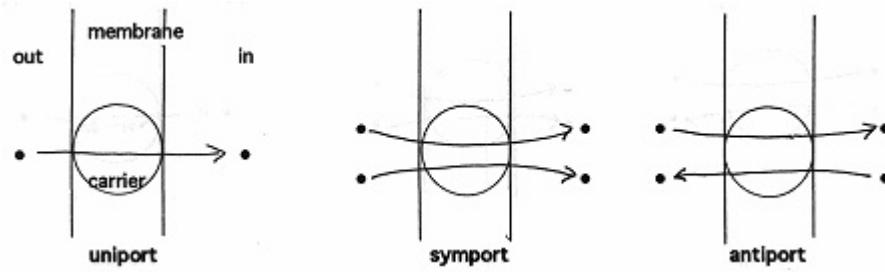


Figure 19. Transport processes in bacterial cells. Solutes enter or exit from bacterial cells by means of one of three processes: uniport, symport (also called cotransport) and antiport (also called exchange diffusion). Transport systems (Figure 13 below) operate by one or another of these processes.

Types of Transport Systems

Bacteria have a variety of types of transport systems which can be used alternatively in various environmental situations. The elaborate development of transport processes and transport systems in prokaryotes probably reflects their need to concentrate substances inside the cytoplasm against the concentration (gradient) of the environment. Concentration of solutes in the cytoplasm requires the operation of an **active transport system**, of which there are two types in bacteria: **ion driven transport systems (IDT)** and **binding-protein dependent transport systems (BPDT)**. The definitive feature of an active transport system is the accumulation of the solute in the cytoplasm at concentrations far in excess of the environment. According to the laws of physical chemistry, this type of process requires energy.

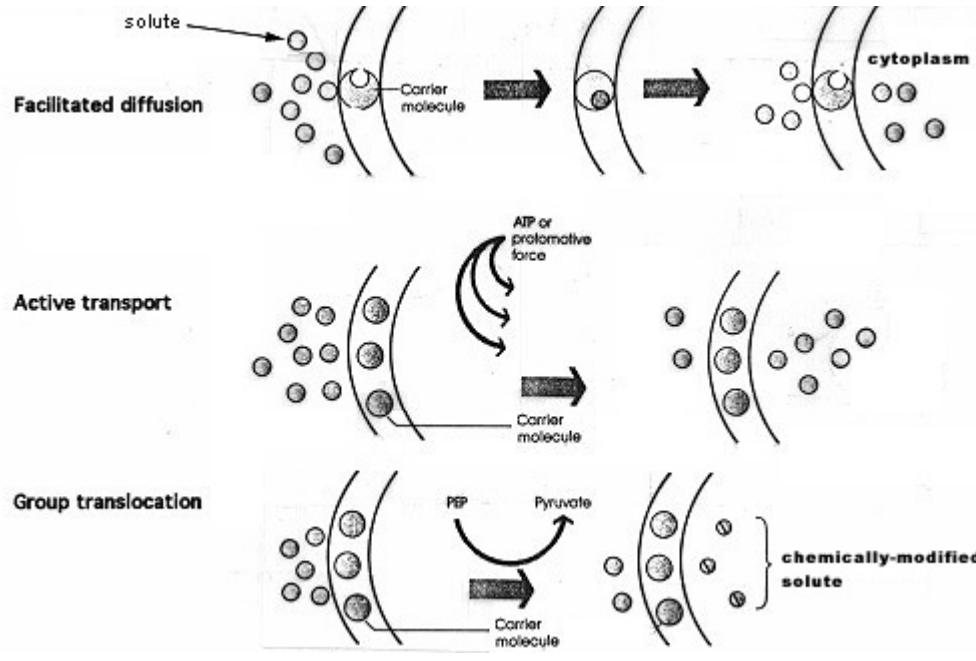


Figure 20. Operation of bacterial transport systems. Bacterial transport systems are operated by transport proteins (sometimes called carriers, porters or permeases) in the plasma membrane. Facilitated diffusion is a carrier-mediated system that does not require energy and does not concentrate solutes against a gradient. Active transport systems such as Ion-driven transport and Binding protein-dependent transport, use energy and concentrate molecules against a concentration gradient. Group translocation systems, such as the phosphotransferase (pts) system in *Escherichia coli*, use energy during transport and modify the solute during its passage across the membrane.

There are four types of carrier-mediated transport systems in prokaryotes. The **carrier** is a protein (or group of proteins) that functions in the passage of a small molecule from one side of a membrane to the other side. A transport system may be a single transmembrane protein that forms a channel that admits passage of a specific solute, or it may be a coordinated system of proteins that binds and sequentially passes a small molecule through

the membrane. Transport systems have the property of **specificity for the solute** transported. Some transport systems transport a single solute with the same specificity and kinetics as an enzyme. Some transport systems will transport (structurally) related molecules, although at reduced efficiency compared to their primary substrate. Most transport systems transport specific sugars, amino acids, anions or cations that are of nutritional value to the bacterium.

Facilitated diffusion systems (FD) are the least common type of transport system in bacteria. Actually, the glycerol uniporter in *E. coli* is the only well known facilitated diffusion system. FD involves the passage of a specific solute through a carrier that forms a channel in the membrane. The solute can move in either direction through the membrane to the point of equilibrium on both sides of the membrane. Although the system is carrier-mediated and specific, no energy is expended in the transport process. For this reason the glycerol molecule cannot be accumulated against the concentration gradient.

Ion driven transport systems (IDT) and **Binding-protein dependent transport systems (BPDT)** are **active transport systems** that are used for transport of most solutes by bacterial cells. IDT is used for accumulation of many ions and amino acids; BPDT is frequently used for sugars and amino acids. IDT is a symport or antiport process that uses a hydrogen ion (H^+) i.e., proton motive force (pmf), or some other cation, i.e., chemiosmotic potential, to drive the transport process. IDT systems such as the lactose permease of *E. coli* utilize the consumption of a hydrogen ion during the transport of lactose. Thus the energy expended during active transport of lactose is in the form of pmf. The lactose permease is a single transmembrane polypeptide that spans the membrane seven times forming a channel that specifically admits lactose.

Binding-protein dependent transport systems (BPDT), such as the histidine transport system in *E. coli*, are composed of four proteins. Two proteins form a membrane channel that allows passage of the histidine. A third protein resides in the periplasmic space where it is able to bind the amino acid and pass it to a forth protein which admits the amino acid into the membrane channel. Driving the solute through the channel involves the expenditure of energy, which is provided by the hydrolysis of ATP.

Group translocation systems (GT), more commonly known as the **phosphotransferase system (PTS)** in *E. coli*, are used primarily for the transport of sugars. Like binding protein-dependent transport systems, they are composed of several distinct components. However, GT systems specific for one sugar may share some of their components with other group transport systems. In *E. coli*, glucose may be transported by a group translocation process that involves the phosphotransferase system. The actual carrier in the membrane is a protein channel fairly specific for glucose. Glucose specifically enters the channel from the outside, but in order to exit into the cytoplasm, it must first be phosphorylated by the phosphotransferase system. The PTS derives energy from the metabolic intermediate phosphoenol pyruvate (PEP). PEP is hydrolyzed to pyruvate and glucose is phosphorylated to form glucose-phosphate during the process. Thus, by the expenditure of a single molecule of high energy phosphate, glucose is transported and changed to glucose-phosphate.

Table 7. Distinguishing characteristics of bacterial transport systems.

PD = passive diffusion

FD = facilitated diffusion

IDT = ion-driven transport

BPDT = binding protein dependent transport

GT = group translocation

Property	PD	FD	IDT	BPDT	GT
carrier mediated	-	+	+	+	+
conc. against gradient	-	-	+	+	NA
specificity	-	+	+	+	+
energy expended	-	-	pmf	ATP	PEP
solute modified during transport	-	-	-	-	+

The plasma membrane of prokaryotes may invaginate into the cytoplasm or form stacks or vesicles attached to the inner membrane surface. These structures are sometimes referred to as **mesosomes**. Such internal membrane systems may be analogous to the cristae of mitochondria or the thylakoids of chloroplasts which increase the surface area of membranes to which enzymes are bound for specific enzymatic functions. The photosynthetic

apparatus (light harvesting pigments and ATPase) of photosynthetic prokaryotes is contained in these types of membranous structures. Mesosomes may also represent specialized membrane regions involved in DNA replication and segregation, cell wall synthesis, or increased enzymatic activity. Membrane foldings and vesicles sometimes appear in electron micrographs of prokaryotic cells as artifacts of preparative techniques. These membranous structures, of course, are not mesosomes, but their existence does not prove that mesosomes are not present in prokaryotes, and there are several examples of prokaryotic membrane topology and appearance that are suggestive of mesosomes.

There are a few antibiotics (e.g. polymyxin), hydrophobic agents (e.g. bile salts), and proteins (e.g. complement) that can damage bacterial membranes.

Table 8. Representative periplasmic proteins in *E. coli*.

Binding proteins

- For amino acids (e.g. histidine, arginine)
- For sugars (e.g. glucose, maltose)
- For vitamins (e.g. thiamine, vitamin B12)
- For ions (e.g. phosphate, sulfate)

Biosynthetic enzymes

- For murein assembly (e.g. transglycosylases, carboxypeptidases, transpeptidases)
- For fimbrial subunit secretion and assembly (e.g. chaperonins)

Degradative enzymes

- phosphatases
- proteases

Detoxifying enzymes

- Beta-lactamases (e.g. penicillinase)
 - Aminoglycoside-phosphorylating enzymes
-

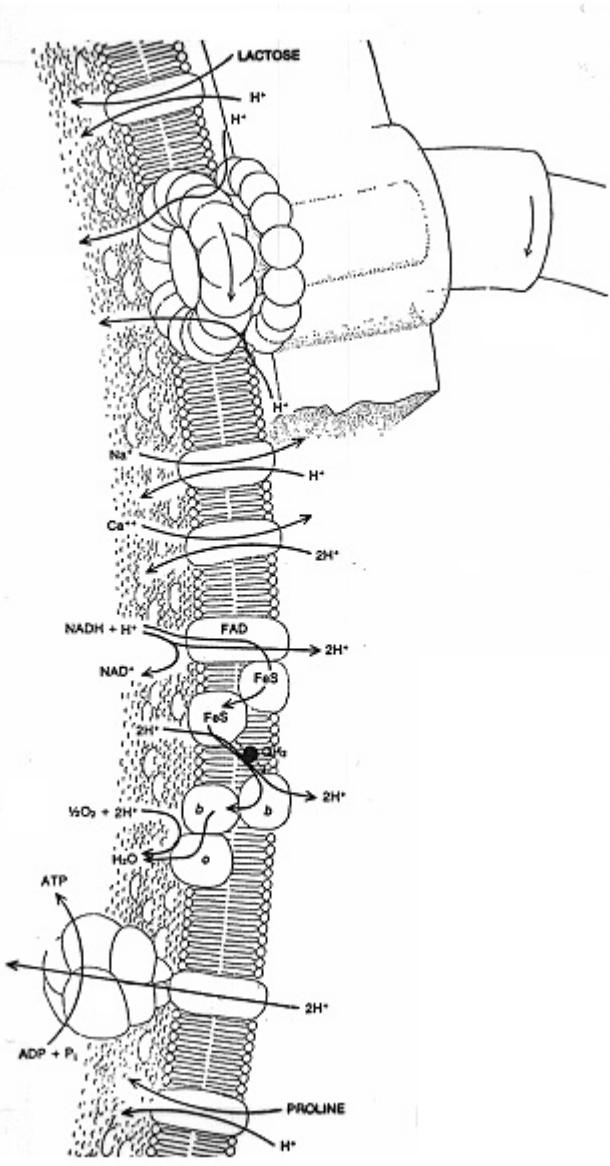


Figure 21. Schematic view of the plasma membrane of *Escherichia coli*. The S and M rings which constitute the flagellar motor are shown. The motor ring is imbedded in the phospholipid bilayer. It is powered by pmf to rotate the flagellar filament. The electron transport system is shown oxidizing NAD 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 prokaryotic cells invariably include the **prokaryotic chromosome** and **ribosomes**. The chromosome is typically one large circular molecule of **DNA**, more or less free in the cytoplasm. Prokaryotes sometimes possess smaller extrachromosomal pieces of DNA called **plasmids**. The total DNA content of a prokaryote is referred to as the cell **genome**. During cell growth and division, the prokaryotic chromosome is replicated in the usual semi-conservative fashion before distribution to progeny cells. However, the eukaryotic processes of meiosis and mitosis are absent in prokaryotes. Replication and segregation of prokaryotic DNA is coordinated by the membrane, possibly by mesosomes.

The distinct granular appearance of prokaryotic cytoplasm is due to the presence and distribution of **ribosomes**. The ribosomes of prokaryotes are smaller than cytoplasmic ribosomes of eukaryotes. Prokaryotic 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 prokaryotes.

Table 9. Molecular composition of *E. coli* under conditions of balanced growth.

Molecule	Percentage of dry weight
Protein	55
Total RNA	20.5
DNA	3.1
Phospholipid	9.1
Lipopolysaccharide	3.4
Murein	2.5
Glycogen	2.5
Small molecules: precursors, metabolites, vitamins, etc.	2.9
Inorganic ions	1.0
Total dry weight	100.0

Table 10. Small molecules present in a growing bacterial cell.

Molecule	Approximate number of kinds
Amino acids, their precursors and derivatives	120
Nucleotides, their precursors and derivatives	100
Fatty acids and their precursors	50
Sugars, carbohydrates and their precursors or derivatives	250
quinones, porphyrins, vitamins, coenzymes and prosthetic groups and their precursors	300

Table 11. 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 prokaryotic 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 PO₄ and possibly energy; elemental sulfur (sulfur globules) are stored by some phototrophic and some lithotrophic prokaryotes 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 12. Some inclusions in bacterial cells.

Cytoplasmic inclusions	Where found	Composition	Function
glycogen	many bacteria e.g. <i>E. coli</i>	polyglucose	reserve carbon and energy source
polybetahydroxybutyric acid (PHB)	many bacteria e.g. <i>Pseudomonas</i>	polymerized hydroxy butyrate	reserve carbon and energy source
polyphosphate (volutin granules)	many bacteria e.g. <i>Corynebacterium</i>	linear or cyclical polymers of PO ₄	reserve phosphate; possibly a reserve of high energy phosphate
sulfur globules	phototrophic purple and green sulfur bacteria and lithotrophic colorless sulfur bacteria	elemental sulfur	reserve of electrons (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 <i>Bacillus</i>)	protein	unknown but toxic to certain insects
magnetosomes	certain aquatic bacteria	magnetite (iron oxide) Fe ₃ O ₄	orienting and migrating along geo-magnetic field lines
carboxysomes	many autotrophic bacteria	enzymes for autotrophic CO ₂ fixation	site of CO ₂ 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.

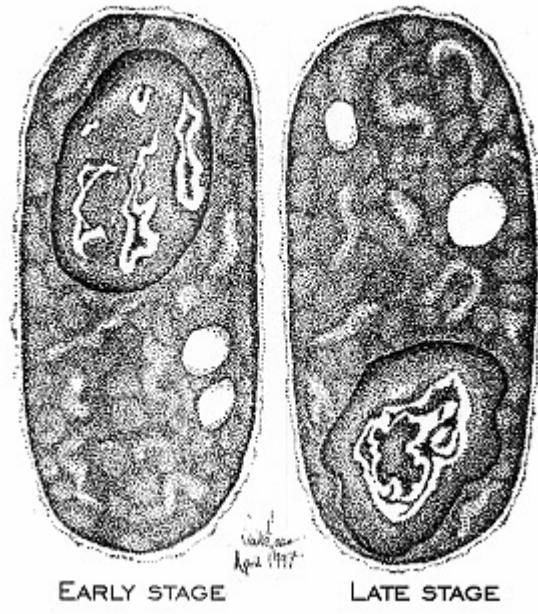


Figure 22. Early and late stages of endospore formation. Drawing by Vaike Haas, University of Wisconsin Madison. During endospore formation, a vegetative cell is converted to a heat-resistant spore. There are eight stages, O,I-VII, in the sporulation cycle of a *Bacillus* species, and the process takes 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 13. 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

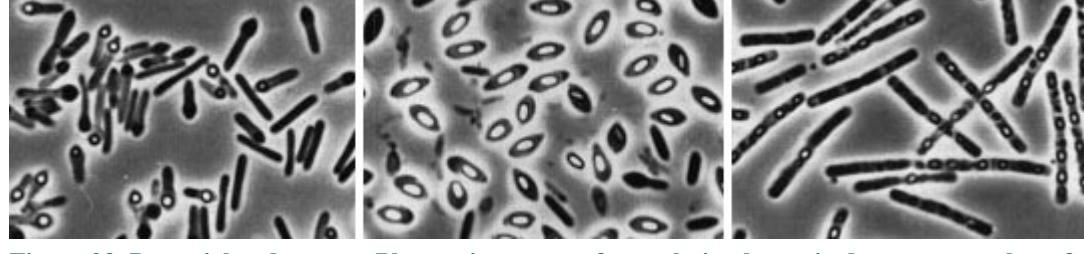


Figure 23. Bacterial endospores. Phase microscopy of sporulating bacteria demonstrates the refractivity of endospores, as well as characteristic spore shapes and locations within the mother cell.

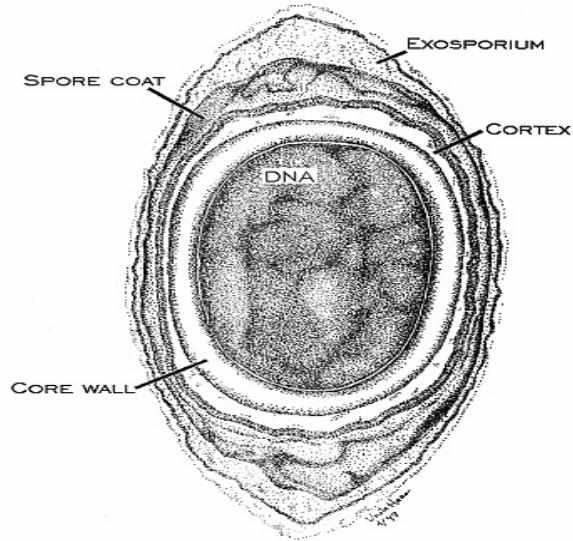
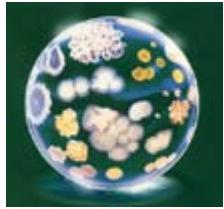


Figure 24. Electron micrograph of a bacterial endospore. The spore has a core wall of unique peptidoglycan surrounded by several layers, including the cortex, the spore coat and the exosporium. The dehydrated core contains the bacterial chromosome and a few ribosomes and enzymes to jump-start protein synthesis and metabolism during germination.

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NUTRITION AND GROWTH OF BACTERIA

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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 ₂ O, 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 ₂ O, organic compounds, H ₂	Main constituent of organic compounds and cell water
Phosphorus	3	inorganic phosphates (PO ₄)	Constituent of nucleic acids, nucleotides, phospholipids, LPS, teichoic acids
Sulfur	1	SO ₄ , H ₂ S, S ⁰ , 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 O₂ 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 CO₂. Organisms that use organic carbon are **heterotrophs** and organisms that use CO₂ 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 prokaryotes may be defined (Table 2).

Table 2. Major nutritional types of prokaryotes

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 ₂ S	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 prokaryotes 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 CO₂, 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-*.



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Figure 1. Cross-feeding between *Staphylococcus aureus* and *Haemophilus influenzae* growing on blood agar. © Gloria J. Delisle and Lewis Tomalty, Queens University, Kingston, Ontario, Canada. Licensed for use by ASM Microbe Library <http://www.microbelibrary.org/>. *Haemophilus influenzae* was first streaked on to the blood agar plate followed by a cross streak with *Staphylococcus aureus*. *H. influenzae* is a fastidious bacterium which requires both hemin and NAD for growth. There is sufficient hemin in blood for growth of *Haemophilus*, but the medium is insufficient in NAD. *S. aureus* produces NAD in excess of its own needs and secretes it into the medium, which supports the growth of *Haemophilus* as satellite colonies.

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 racemization 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 aqueous 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** (Table 4a) 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).

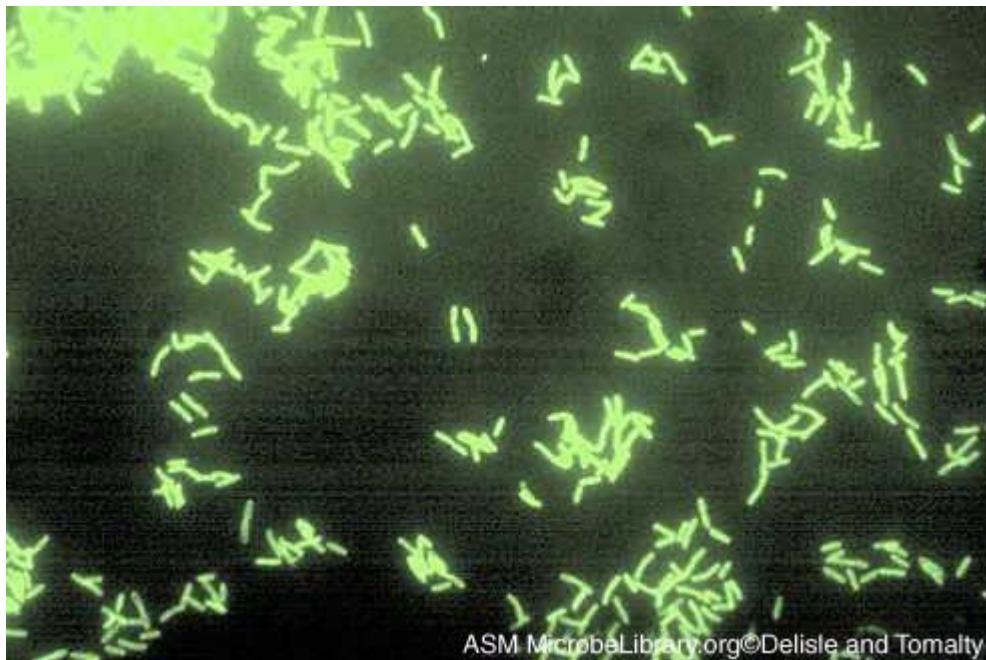


Figure 2. *Legionella pneumophila*. Direct fluorescent antibody (DFA) stain of a patient respiratory tract specimen. © Gloria J. Delisle and Lewis Tomalty. Queens University, Kingston, Ontario, Canada. Licensed for use by ASM Microbe Library <http://www.microbelibrary.org/>. In spite of its natural occurrence 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 Legionnaire's disease in Philadelphia in 1977. Had fluorescent 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 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 it 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 N₂. 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 prokaryotes.

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 ₂ HPO ₄	2.5 g	pH buffer; P and K source
KH ₂ PO ₄	2.5 g	pH buffer; P and K source
(NH ₄) ₂ HPO ₄	1.0 g	pH buffer; N and P source
MgSO ₄ 7H ₂ O	0.20 g	S and Mg ⁺⁺ source
FeSO ₄ 7H ₂ O	0.01 g	Fe ⁺⁺ source
MnSO ₄ 7H ₂ O	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 ₄ Cl	0.52 g	N source
KH ₂ PO ₄	0.28 g	P and K source
MgSO ₄ 7H ₂ O	0.25 g	S and Mg ⁺⁺ source
CaCl ₂ 2H ₂ O	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% CO₂.

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 ₂ O	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 prokaryotes exist in nature under an enormous range of physical conditions such as O₂ 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 prokaryote, 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 prokaryotes 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 H₂O. However, prokaryotes display a wide range of responses to molecular oxygen O₂ (Table 6).

Obligate aerobes require O₂ for growth; they use O₂ as a final electron acceptor in aerobic respiration.

Obligate anaerobes (occasionally called **aerophobes**) do not need or use O₂ as a nutrient. In fact, O₂ is a toxic substance, which either kills or inhibits their growth. Obligate anaerobic prokaryotes 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 O₂) they grow by fermentation or anaerobic respiration, but in the presence of O₂ 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 O₂. They live by fermentation alone whether or not O₂ is present in their environment.

Table 6. Terms used to describe O₂ Relations of Microorganisms.

Group	Environment		
	Aerobic	Anaerobic	O₂ 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 O₂ in its environment depends upon the occurrence and distribution of various enzymes which react with O₂ and various oxygen radicals that are invariably generated by cells in the presence of O₂. All cells contain enzymes capable of reacting with O₂. For example, oxidations of flavoproteins by O₂ invariably result in the formation of H₂O₂ (peroxide) as one major product and small quantities of an even more

toxic free radical, superoxide or O_2^- . 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 3 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.

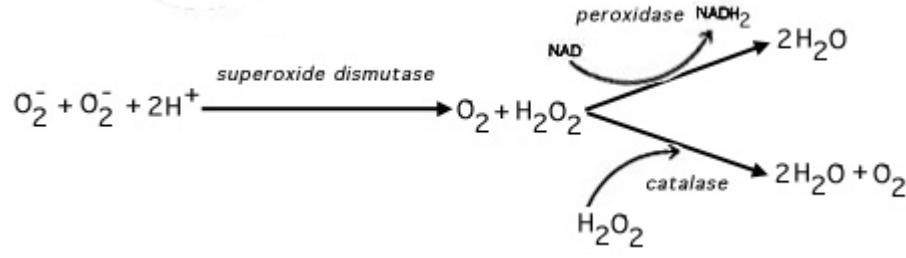


Figure 3. The action of superoxide dismutase, catalase and peroxidase. These enzymes detoxify oxygen radicals that are inevitably generated by living systems in the presence of O_2 . The distribution of these enzymes in cells determines their ability to exist in the presence of O_2

Table 7. Distribution of superoxide dismutase, catalase and peroxidase in prokaryotes 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 prokaryotes 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 4).

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.

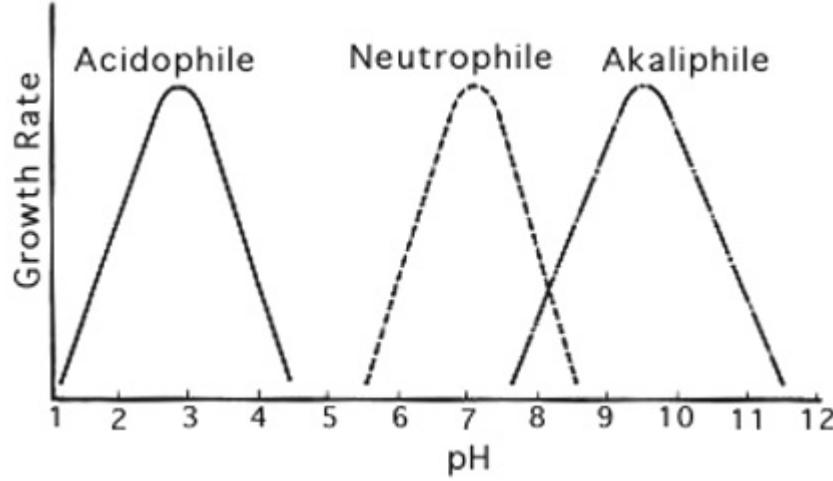


Figure 4. Growth rate vs pH for three environmental classes of prokaryotes. Most free-living bacteria grow over a pH range of about three units. Note the symmetry of the curves below and above the optimum pH for growth.

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

Organism	Minimum pH	Optimum pH	Maximum pH
<i>Thiobacillus thiooxidans</i>	0.5	2.0-2.8	4.0-6.0
<i>Sulfolobus acidocaldarius</i>	1.0	2.0-3.0	5.0
<i>Bacillus acidocaldarius</i>	2.0	4.0	6.0
<i>Zymomonas lindneri</i>	3.5	5.5-6.0	7.5
<i>Lactobacillus acidophilus</i>	4.0-4.6	5.8-6.6	6.8
<i>Staphylococcus aureus</i>	4.2	7.0-7.5	9.3
<i>Escherichia coli</i>	4.4	6.0-7.0	9.0
<i>Clostridium sporogenes</i>	5.0-5.8	6.0-7.6	8.5-9.0
<i>Erwinia caratovora</i>	5.6	7.1	9.3
<i>Pseudomonas aeruginosa</i>	5.6	6.6-7.0	8.0
<i>Thiobacillus novellus</i>	5.7	7.0	9.0
<i>Streptococcus pneumoniae</i>	6.5	7.8	8.3
<i>Nitrobacter</i> 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. Brock's discovery of thermophilic bacteria, archaea and other "extremophiles" in Yellowstone is summarized for the general public in an article at this web site. See [Life at High Temperatures](#).

Subsequently, prokaryotes 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 H₂O 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 6, cf. Figure 4). Considering the total span of temperature where liquid water exists, the prokaryotes 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 prokaryotes. 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.

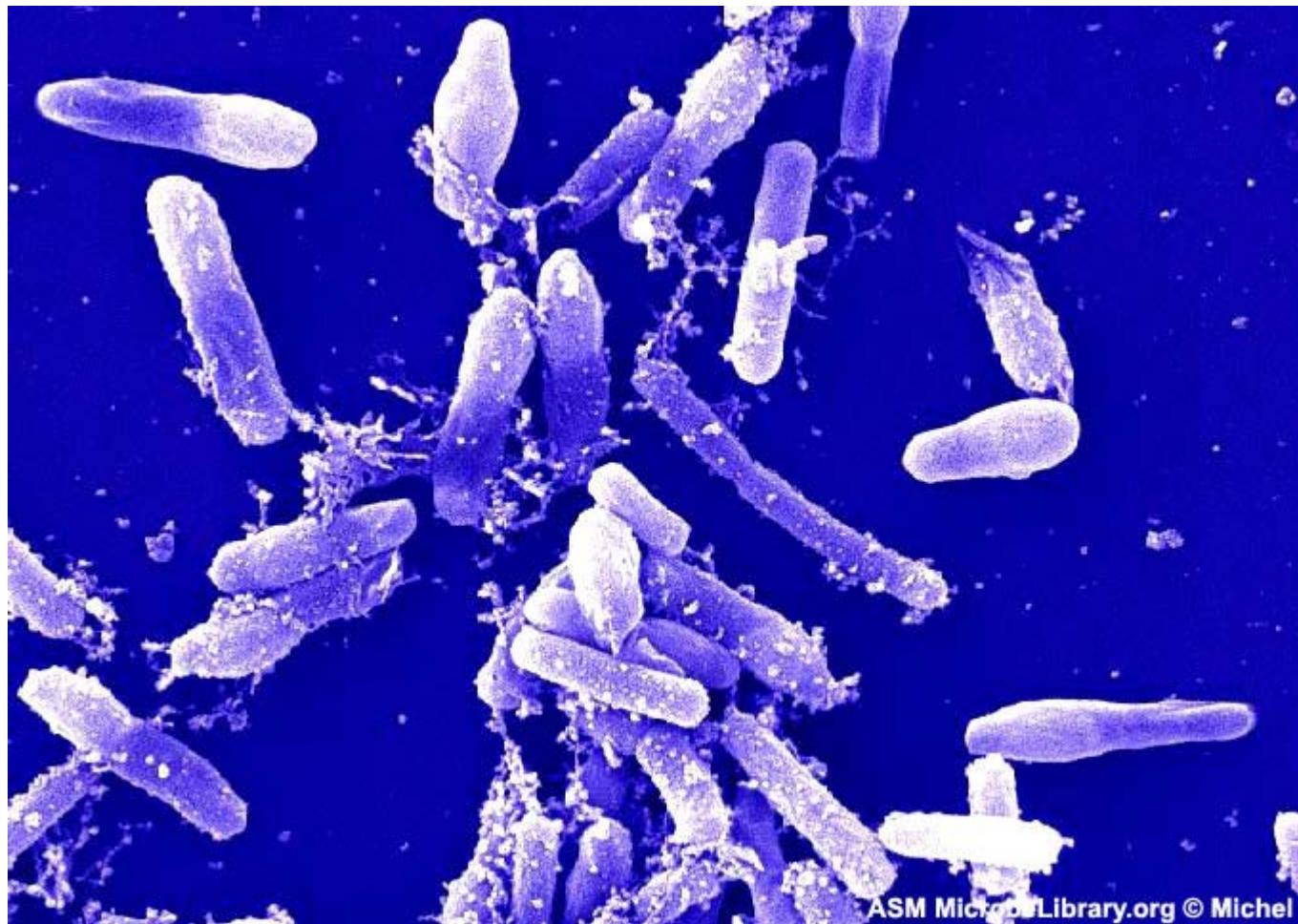


Figure 5 (above). SEM of a thermophilic *Bacillus* species isolated from a compost pile at 55° C. © Frederick C. Michel. The Ohio State University -OARDC, Wooster, Ohio. Licensed for use by ASM Microbe Library <http://www.microbelibrary.org/>. The rods are 3-5 microns in length and 0.5 to 1 micron in width with terminal endospores in a slightly-swollen sporangium.

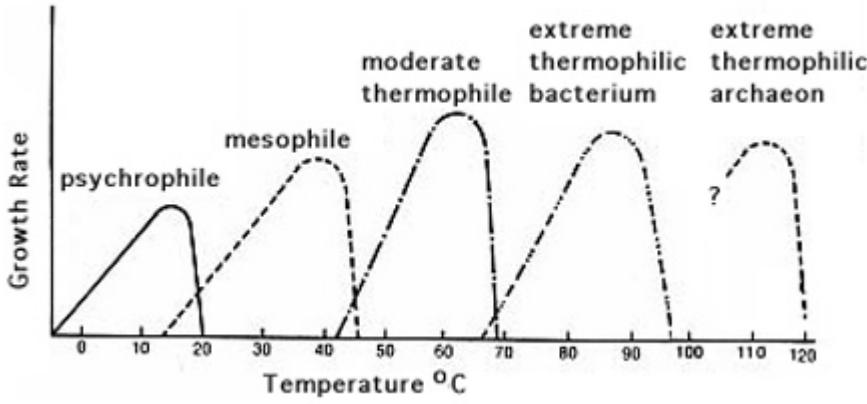


Figure 6 (below). Growth rate vs temperature for five environmental classes of prokaryotes. Most prokaryotes will grow over a temperature range of about 30 degrees. The curves exhibit three cardinal points: minimum, optimum and maximum temperatures for growth. There is a steady increase in growth rate 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

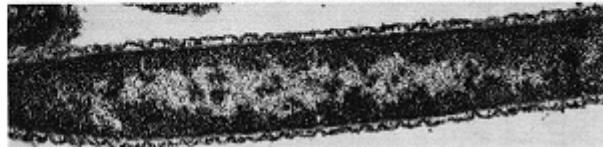
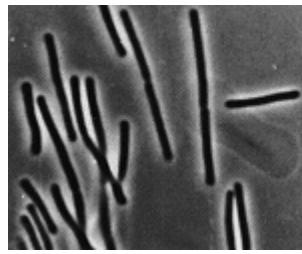


Figure 7. *Thermus aquaticus*, the thermophilic bacterium that is the source of taq polymerase.
L wet mount; R electron micrograph. T.D. Brock. [Life at High Temperatures](#).

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

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

Table 10b. Optimum growth temperature of some prokaryotes.

Genus and species	Optimal growth temp (degrees C)
<i>Vibrio cholerae</i>	18-37
<i>Photobacterium phosphoreum</i>	20
<i>Rhizobium leguminosarum</i>	20
<i>Streptomyces griseus</i>	25
<i>Rhodobacter sphaeroides</i>	25-30
<i>Pseudomonas fluorescens</i>	25-30
<i>Erwinia amylovora</i>	27-30

<i>Staphylococcus aureus</i>	30-37
<i>Escherichia coli</i>	37
<i>Mycobacterium tuberculosis</i>	37
<i>Pseudomonas aeruginosa</i>	37
<i>Streptococcus pyogenes</i>	37
<i>Treponema pallidum</i>	37
<i>Thermoplasma acidophilum</i>	59
<i>Thermus aquaticus</i>	70
<i>Bacillus caldolyticus</i>	72
<i>Pyrococcus furiosus</i>	100

Table 10c. Hyperthermophilic Archaea.**Temperature for growth(degrees C)**

Genus	Minimum	Optimum	Maximum	Optimum	pH
<i>Sulfolobus</i>	55	75-85	87	2-3	
<i>Desulfurococcus</i>	60	85	93	6	
<i>Methanothermus</i>	60	83	88	6-7	
<i>Pyrodictium</i>	82	105	113	6	
<i>Methanopyrus</i>	85	100	110	7	

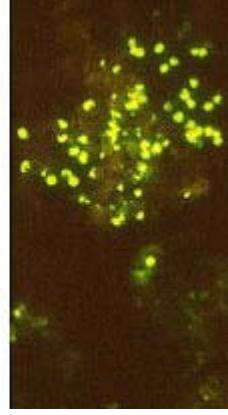


Figure 8. *Sulfolobus acidocaldarius* is an extreme thermophile and an acidophile found in geothermally-heated acid springs, mud pots and surface soils with temperatures from 60 to 95 degrees C, and a pH of 1 to 5. Left: Electron micrograph of a thin section (85,000X). Under the electron microscope the organism appears as irregular spheres which are often lobed. Right: Fluorescent photomicrograph of cells attached to a sulfur crystal. Fimbrial-like appendages have been observed on the cells attached to solid surfaces such as sulfur crystals. T.D. Brock. [Life at High Temperatures](#).

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.

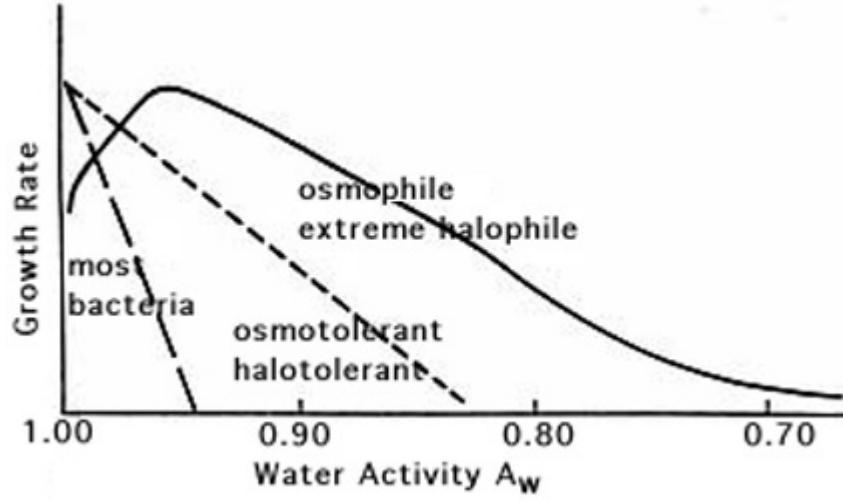
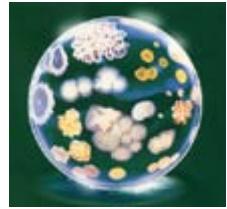


Figure 9. Growth rate vs osmolarity for different classes of prokaryotes. Osmolarity is determined by solute concentration in the environment. Osmolarity is inversely related to water activity (A_w), which is more like a measure of the concentration of water (H_2O) in a solution. Increased solute concentration means increased osmolarity and decreased A_w . From 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.

Table 11. Limiting water activities (A_w) for growth of certain prokaryotes.

Organism	Minimum A_w for growth
<i>Caulobacter</i>	1.00
<i>Spirillum</i>	1.00
<i>Pseudomonas</i>	.91
<i>Salmonella/E. coli</i>	.91
<i>Lactobacillus</i>	.90
<i>Bacillus</i>	.90
<i>Staphylococcus</i>	.85
<i>Halococcus</i>	.75

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GROWTH OF BACTERIAL POPULATIONS

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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**.

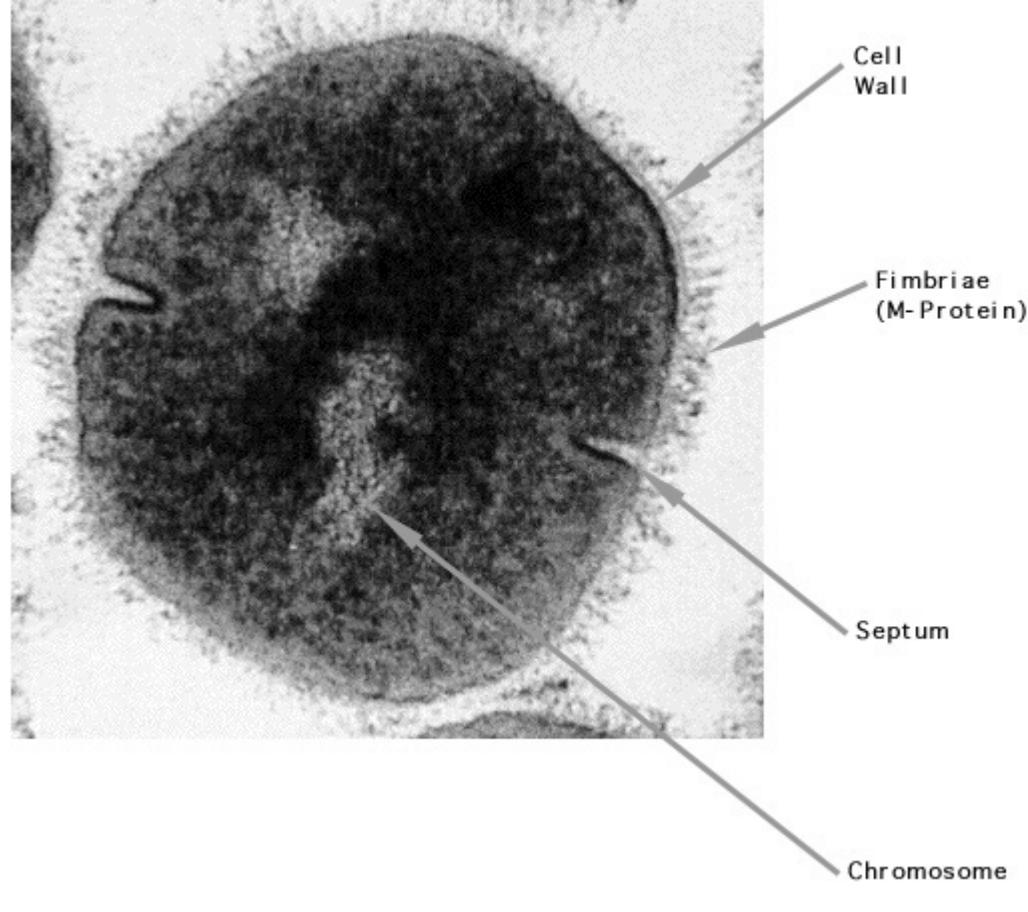


Figure 1. Bacterial growth by binary fission. Most bacteria reproduce by a relatively simple asexual process called **binary fission**: each cell increases in size and divides into two cells. During this process there is an orderly increase in cellular structures and components, replication and segregation 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* by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of](#)

Bacterial Pathogenesis and Immunology, Rockefeller University.

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 O₂ production or consumption, CO₂ 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 10⁷ 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 (>10⁷ 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 suspending medium must be very clean. Such electronic 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 prokaryotes in such an environment, but many species identified by genetic techniques have so far proven uncultivable.

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. O ₂ uptake CO ₂ 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



Figure 2. Bacterial colonies growing on a plate of nutrient agar.

Hans Knoll Institute, Jena, Germany.

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 $2^0, 2^1, 2^2, 2^3 \dots \dots 2^n$ (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 3 below).

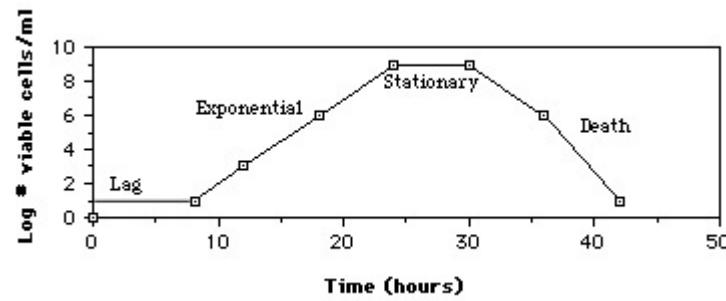


Figure 3. The typical bacterial growth curve. When bacteria are grown in a closed system (also called a batch culture), like a test tube, the population of cells almost always exhibits these growth dynamics: cells initially adjust to the new medium (lag phase) until they can start dividing regularly 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 physical 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 ($n = \text{number of generations}$). 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 is 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 microscopic 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 shown in Table 2.

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

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

Calculation of Generation Time

When growing exponentially by binary fission, the increase in a bacterial population is by geometric progression. If we start with one cell, when it divides, there are 2 cells in the first generation, 4 cells in the second generation, 8 cells in the third generation, and so on. The **generation time** is the time interval required for the cells (or population) to divide.

$$G \text{ (generation time)} = t \text{ (time, in minutes or hours)} / n \text{ (number of generations)}$$

$$G = t/n$$

G = generation time (time for the cells to divide)

t = time interval in hours or minutes

B = number of bacteria at the beginning of a time interval

b = number of bacteria at the end of the time interval

n = number of generations (number of times the cell population doubles during the time interval)

b = B \times 2ⁿ (This equation is an expression of growth by binary fission)

Solve for n:

$$\log b = \log B + n \log 2$$

$$n = \frac{\log b - \log B}{\log 2}$$

$$n = \frac{\log b - \log B}{.301}$$

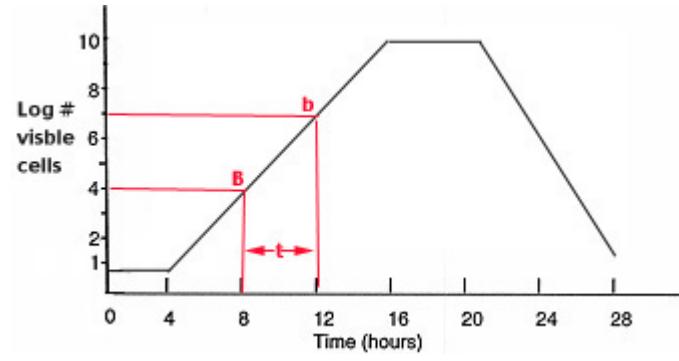
$$n = 3.3 \log b / B$$

$$G = t/n$$

Solve for G

$$G = \frac{t}{3.3 \log b/B}$$

Example: What is the generation time of a bacterial population that increases from 10,000 cells to 10,000,000 cells in four hours of growth?



$$G = \frac{t}{3.3 \log b/B}$$

$$G = \frac{240 \text{ minutes}}{3.3 \log 10^7/10^4}$$

$$G = \frac{240 \text{ minutes}}{3.3 \times 3}$$

$$G = 24 \text{ minutes}$$

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 4), 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.

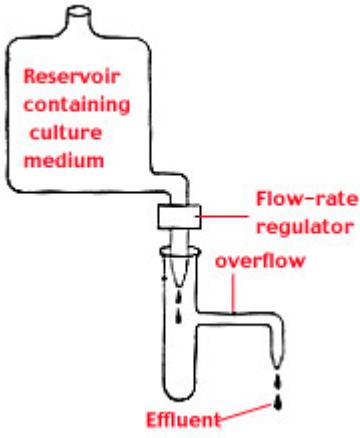


Figure 4. Schematic diagram of a chemostat, a device for the continuous culture of bacteria.
The chemostat relieves the environmental conditions that restrict growth by continuously supplying nutrients to cells and removing waste substances and spent cells from the culture medium.

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.

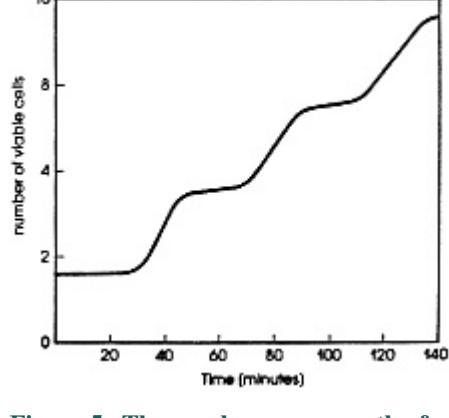
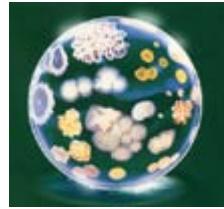


Figure 5. The synchronous growth of a bacterial population. By careful selection of cells that have just divided, a bacterial population can be synchronized in the bacterial cell division cycle. Synchrony can be maintained for only a few generations.

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THE CONTROL OF MICROBIAL GROWTH

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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 effected 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 thermophilic 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° for 30 minutes. Kills everything except some endospores (Actually, for the purposes of purifying drinking water 100° 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° 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): $160^{\circ}/2$ hours or $170^{\circ}/1$ hour. 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	100°	Vaporizes organic material on nonflammable surfaces but may destroy

	>500	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: involves 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 pasteurization of milk: batch method: 63°/30minutes; flash method: 71°/15 seconds.

Low temperature (refrigeration and freezing): Most organisms grow very little or not at all at 0°. Store perishable foods at low temperatures 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 H₂O): Most microorganisms cannot grow at reduced water activity ($A_w < 0.90$). 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 of 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 internally. 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% I ₂ 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. carbolic acid, lysol, hexylresorcinol, hexachlorophene)	Denature proteins and disrupt cell membranes	Antiseptics at low concentrations; disinfectants at high concentrations Disinfectant used to sterilize heat-sensitive objects such as rubber and plastics
Ethylene oxide gas	Alkylating agent	

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 source	Spectrum (effective against)	Mode of action
Beta-lactams (penicillins and cephalosporins)	Penicillin G, Cephalothin	<i>Penicillium notatum</i> and <i>Cephalosporium</i> 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	<i>Streptomyces clavuligerus</i>	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
Carboxyphenems	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. <i>Staphylococcus aureus</i>	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Lincomycins	Clindamycin	Streptomyces lincolnensis	Gram-positive and Gram-negative bacteria esp. anaerobic <i>Bacteroides</i>	Inhibits translation (protein synthesis)
Macrolides	Erythromycin	Streptomyces erythreus	Gram-positive bacteria, Gram-negative bacteria not enterics, <i>Neisseria</i> , <i>Legionella</i> , <i>Mycoplasma</i>	Inhibits translation (protein synthesis)
Polypeptides	Polymyxin	<i>Bacillus polymyxa</i>	Gram-negative bacteria	Damages cytoplasmic membranes
	Bacitracin	<i>Bacillus subtilis</i>	Gram-positive bacteria	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Polyenes	Amphotericin	Streptomyces nodosus	Fungi	Inactivate membranes containing sterols
	Nystatin	Streptomyces noursei	Fungi (<i>Candida</i>)	Inactivate membranes containing sterols
Rifamycins	Rifampicin	Streptomyces mediterranei	Gram-positive and Gram-negative bacteria, <i>Mycobacterium tuberculosis</i>	Inhibits transcription (eubacterial RNA polymerase)
Tetracyclines	Tetracycline	Streptomyces species	Gram-positive and Gram-negative bacteria, <i>Rickettsias</i>	Inhibit translation (protein synthesis)
Semisynthetic tetracycline	Doxycycline		Gram-positive and Gram-negative bacteria, <i>Rickettsias</i> <i>Ehrlichia</i> , <i>Borellia</i>	Inhibit translation (protein synthesis)
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 is expressed as its **spectrum of action**. Antibiotics effective against prokaryotes 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 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 of the molecule (6-aminopenicillanic acid) which can be modified chemically by the addition of side chains. 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.

Cephalosporins are beta lactam antibiotics with a similar mode of action to penicillins that are produced by species of *Cephalosporium*. They 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 polymyxa*. 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 prokaryotic cells and have 70S ribosomes, they are subject to inhibition by some of the protein synthesis inhibitors including chloramphenicol. 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 prokaryotes 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 tetrahydrofolic 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 sulfonilamides. 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 for most bacterial genes is approximately 10^{-8} . This means that if a bacterial population doubles from 10^8 cells to 2×10^8 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 genes to 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!

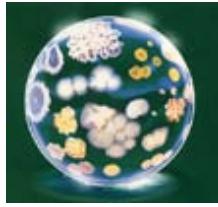
The medical problem of bacterial drug resistance

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.

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THE DIVERSITY OF METABOLISM IN PROCARYOTES

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Introduction

A lot of hoopla is made about **microbial diversity**. Based on superficial inspection, the bacteria and archaea hardly seem diversified. There are but a few basic morphologies, the possibilities of motility and resting cells (spores), and a major differential stain (the Gram stain) to distinguish the prokaryotes microscopically. In the eukaryotes, there may be more structural diversity within a single genus of organisms. So what is all the hoopla about? It is about biochemical or **metabolic diversity**, especially as it relates to energy-generating metabolism and biosynthesis of secondary metabolites. The prokaryotes, as a group, conduct all the same types of basic metabolism as eukaryotes, but, in addition, there are several types of energy-generating metabolism among the prokaryotes that are non-existent in eukaryotic cells or organisms. The diversity of prokaryotes is expressed by their great variation in modes of energy production and metabolism.

Even within a prokaryotic species, there may be great versatility in metabolism. Consider *Escherichia coli*. The bacterium can produce energy for growth by fermentation or respiration. It can respire aerobically using O₂ as a final electron acceptor, or it can respire under anaerobic conditions, using NO₃ or fumarate as a terminal electron acceptor. *E. coli* can use glucose or lactose as a sole carbon source for growth, with the metabolic ability to transform the sugar into all the necessary amino acids, vitamins and nucleotides that make up cells. A relative of *E. coli*, *Rhodospirillum rubrum*, has all the heterotrophic capabilities as *E. coli*, plus the ability to grow by photoautotrophic, photoheterotrophic or lithotrophic means. It does require one growth factor, however; biotin must be added to its growth media.

Fundamentally, most eukaryotes produce energy (ATP) through alcohol fermentation (e.g. yeast), lactic acid fermentation (e.g. muscle cells, neutrophils), aerobic respiration (e.g. molds, protozoa, animals) or oxygenic photosynthesis (e.g. algae, plants). These modes of energy-generating metabolism exist among prokaryotes, in addition to all following types of energy production which are virtually non-existent in eukaryotes.

Unique fermentations proceeding through the Embden-Meyerhof pathway

Other fermentation pathways such as the phosphoketolase (heterolactic) and Entner-Doudoroff pathways

Anaerobic respiration: respiration that uses substances other than O₂ as a final electron acceptor

Lithotrophy: use of inorganic substances as sources of energy

Photoheterotrophy: use of organic compounds as a carbon source during bacterial photosynthesis

Anoxygenic photosynthesis: photophosphorylation in the absence of O₂

Methanogenesis: an ancient type of archaeon metabolism that uses H₂ as an energy source and produces methane

Light-driven nonphotosynthetic photophosphorylation: unique archaeon metabolism that converts light energy into chemical energy

In addition, among autotrophic prokaryotes, there are three ways to fix CO₂, two of which are unknown among eukaryotes, the **CODH (acetyl CoA pathway)** and the **reverse TCA cycle**.

Energy-Generating Metabolism

The term **metabolism** refers to the sum of the biochemical reactions required for energy generation **and** the use of energy to synthesize cell material from small molecules in the environment. Hence, metabolism has an **energy-generating component**, called **catabolism**, and an **energy-consuming, biosynthetic component**, called **anabolism**. Catabolic reactions or sequences produce energy as **ATP**, which can be utilized in anabolic reactions to build cell material from nutrients in the environment. The relationship between catabolism and anabolism is illustrated in Figure 1 below.

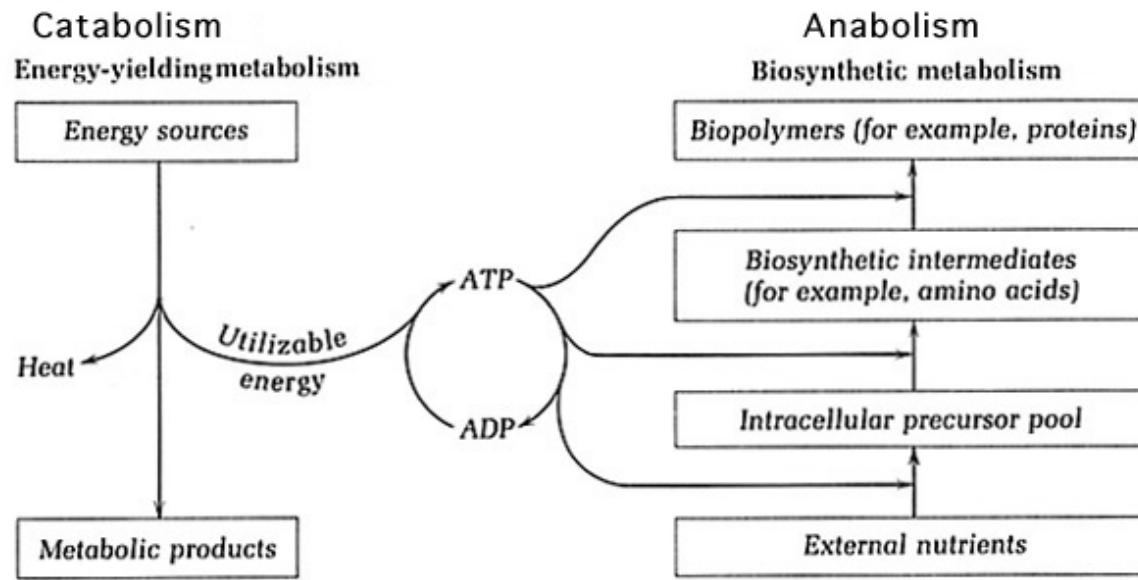


Figure 1. The relationship between catabolism and anabolism in a cell. During catabolism, energy is changed from one form to another, and keeping with the laws of thermodynamics, such energy transformations are never completely efficient, i.e., some energy is lost in the form of heat. The efficiency of a catabolic sequence of reactions is the amount of energy made available to the cell (for anabolism) divided by the total amount of energy released during the reactions.

ATP

During catabolism, useful energy is temporarily conserved in the "high energy bond" of **ATP - adenosine triphosphate**. No matter what form of energy a cell uses as its primary source, the energy is ultimately transformed and conserved as ATP - the universal currency of energy exchange in biological systems. When energy is required during anabolism, it may be spent as the high energy bond of ATP which has a value of about 8 kcal per mole. Hence, the conversion of ADP to ATP requires 8 kcal of energy, and the hydrolysis of ATP to ADP releases 8 kcal.

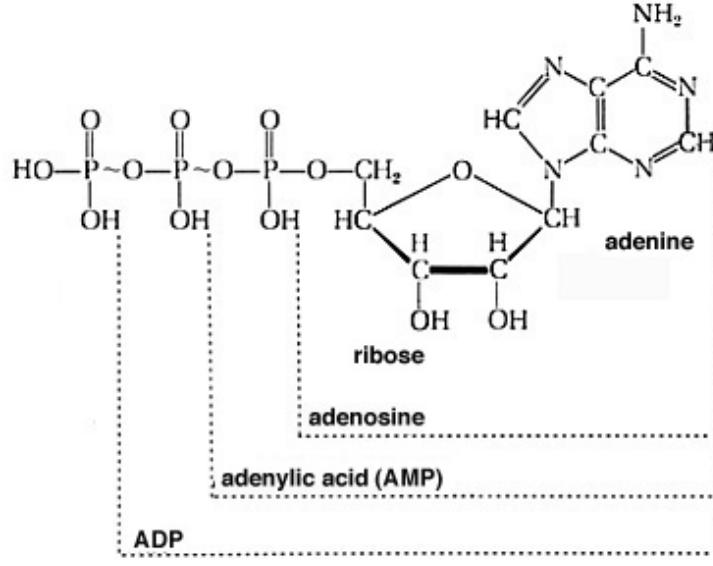


Figure 2. The structure of ATP. ATP is derived from the nucleotide adenosine monophosphate (AMP) or adenylic acid, to which two

additional phosphate groups are attached through pyrophosphate bonds ($\sim\text{P}$). These two bonds are energy rich in the sense that their hydrolysis yields a great deal more energy than a corresponding covalent bond. ATP acts as a coenzyme in energetic coupling reactions wherein one or both of the terminal phosphate groups is removed from the ATP molecule with the bond energy being used to transfer part of the ATP molecule to another molecule to activate its role in metabolism. For example, $\text{Glucose} + \text{ATP} \rightleftharpoons \text{Glucose-P} + \text{ADP}$ or $\text{Amino Acid} + \text{ATP} \rightleftharpoons \text{AMP-Amino Acid} + \text{PPi}$.

Because of the central role of ATP in energy-generating metabolism, expect to see its involvement as a coenzyme in most energy-producing processes in cells.

NAD

Another coenzyme commonly involved in energy-producing metabolism, derived from the vitamin niacin, is the pyridine nucleotide, **NAD (Nicotinamide Adenine Dinucleotide)**. The basis for chemical transformations of energy usually involves oxidation/reduction reactions. For a biochemical to become oxidized, electrons must be removed by an oxidizing agent. The oxidizing agent is an electron acceptor that becomes reduced in the reaction. During the reaction, the oxidizing agent is converted to a reducing agent that can add its electrons to another chemical, thereby reducing it, and reoxidizing itself. The molecule that usually functions as the electron carrier in these types of **coupled oxidation-reduction reactions** in biological systems is NAD and its phosphorylated derivative, **NADP**. NAD or NADP can become alternately oxidized or reduced by the loss or gain of two electrons. The oxidized form of NAD is symbolized NAD; the reduced form is symbolized as NADH, NADH_2 or $\text{NADH} + \text{H}^+$. The structure of NAD is drawn below.

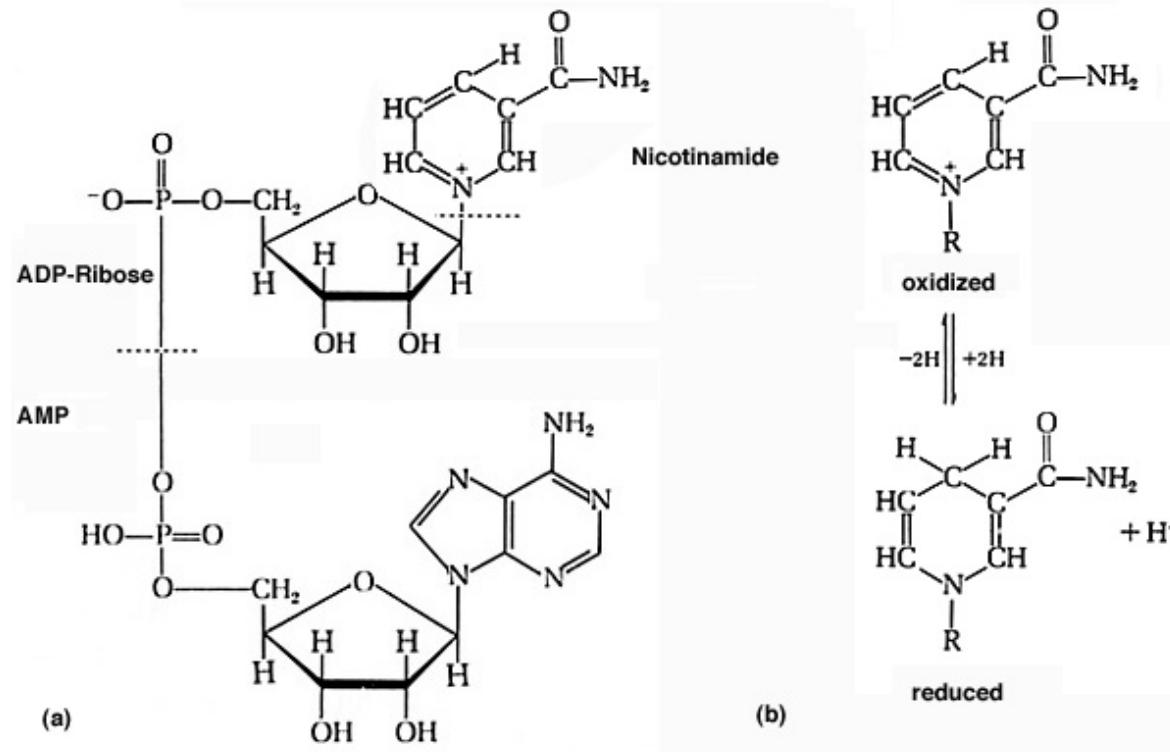
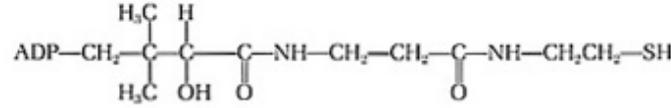


Figure 3. The Structure of NAD. (a) Nicotinamide Adenine Dinucleotide is composed of two nucleotide molecules: Adenosine monophosphate (adenine plus ribose-phosphate) and nicotinamide ribotide (nicotinamide plus ribose-phosphate). NADP has an identical structure except that it contains an additional phosphate group attached to one of the ribose residues. (b) The oxidized and reduced forms of the nicotinamide moiety of NAD. Nicotinamide is the active part of the molecule where the reversible oxidation and reduction takes place. The oxidized form of NAD has one hydrogen atom less than the reduced form and, in addition, has a positive charge on the nitrogen atom which allows it to accept a second electron upon reduction. Thus the correct way to symbolize the reaction is $\text{NAD}^+ + 2\text{H}^- \rightarrow \text{NADH} + \text{H}^+$. However, for convenience we will hereafter use the symbols NAD and NADH₂.

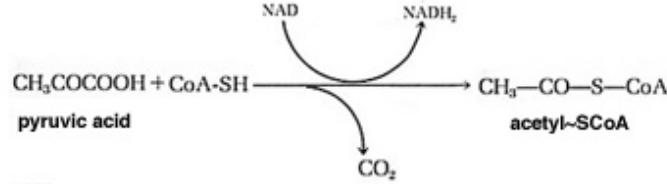
OFF THE WALL. Many bacterial protein toxins including the cholera toxin, pertussis toxin and diphtheria toxin, exert their enzymatic activity using NAD as a co-substrate. The toxins are referred to as ADP-ribosylation toxins, because they cleave NAD into nicotinamide plus ADP-ribose (ADPR) and then transfer the ADPR to some host molecule. For example, the diphtheria toxin transfers ADPR to elongation factor 2, irreversibly inactivating its role in chain elongation during protein synthesis. Thus, the biological activity of the diphtheria toxin is to inhibit protein synthesis in eukaryotic cells.

Coenzyme A

Coenzyme A is another coenzyme frequently involved in energy-generating metabolism of prokaryotes. Coenzyme A is involved in a type of ATP-generating reaction seen in some fermentative bacteria and in all respiratory organisms. The reaction occurs in association with the oxidation of keto acids such as pyruvic acid and alpha ketoglutaric acid. These substrates are central to glycolysis and the TCA cycle, respectively, and they are direct or indirect precursors of several essential macromolecules in a cell. The oxidations of pyruvate and alpha ketoglutarate, involving Coenzyme A, NAD, a dehydrogenation reaction and a decarboxylation reaction, are two of the most important, and complex, reactions in metabolism.



(a)



(b)

Figure 4. (a) The Structure of Coenzyme A. CoA-SH is a derivative of ADP. The molecule shown here attached to ADP is pantothenic acid, which carries a terminal thiol (-S) group. (b) the oxidation of the keto acid, pyruvic acid, to acetyl~SCoA. This is the reaction that enters two carbons from pyruvate into the TCA cycle.

In the oxidation of keto acids, coenzyme A (CoA or CoASH) becomes attached through a thioester linkage (~S) to the carboxyl group of the oxidized product. Part of the energy released in the oxidation is conserved in the thioester bond. This bond energy can be subsequently used to synthesize ATP, as in the case of the clostridia that convert $\text{acetyl-SCoA} + \text{ADP} + \text{Pi} \longrightarrow \text{acetic acid} + \text{CoASH} + \text{ATP}$. Or in the case of respiratory organisms, the thioester bond energy is expended when acetyl~SCoA condenses with oxalacetate in order to drive the TCA cycle into its oxidative branch.

ATP Synthesis in Prokaryotes

The objective of a catabolic pathway is to make ATP: to transform either chemical energy or electromagnetic (light) energy into the chemical energy contained within the high-energy bonds of ATP. Cells fundamentally can produce ATP in two ways: **substrate level phosphorylation** and **electron transport phosphorylation**.

Substrate level phosphorylation (SLP) is the simplest, oldest and least-evolved way to make ATP. In a substrate level phosphorylation, ATP is made during the conversion of an organic molecule from one form to another. Energy released during the conversion is partially conserved during the synthesis of the high energy bond of ATP. SLP occurs during fermentations and respiration (the TCA cycle), and even during some lithotrophic transformations of inorganic substrates.

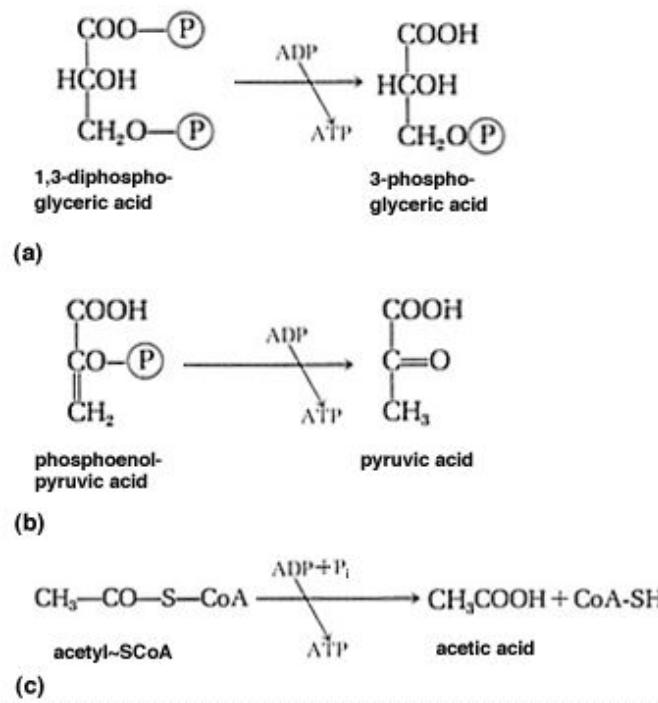


Figure 5. Three examples of substrate level phosphorylation. (a) and (b) are the two substrate level phosphorylations that occur during the Embden Meyerhof pathway, but they occur in all other fermentation pathways which have an Embden-Meyerhof component. (c) is a substrate level phosphorylation found in *Clostridium* and *Bifidobacterium*. These are two anaerobic (fermentative) bacteria who learned how to make one more ATP from glycolysis beyond the formation of pyruvate.

Electron Transport Phosphorylation (ETP) is a much more complicated affair that evolved long after SLP. Electron Transport Phosphorylation takes place during respiration, photosynthesis, lithotrophy and possibly other types of bacterial metabolism. ETP requires that electrons removed from substrates be dumped into an electron transport system (ETS) contained within a membrane. The electrons are transferred through the ETS to some final electron acceptor in the membrane (like O_2 in aerobic respiration), while their traverse through the ETS results in the extrusion of protons and the establishment of a **proton motive force (pmf)** across the membrane. An essential component of the membrane for synthesis of ATP is a **membrane-bound ATPase** (ATP synthetase) enzyme. The ATPase enzyme transports protons, thereby utilizing the pmf (protons) during the synthesis of ATP. The idea in electron transport phosphorylation is to drive electrons through an ETS in the membrane, establish a pmf, and use the pmf to synthesize ATP. Obviously, ETP take a lot more "gear" than SLP, in the form of membranes, electron transport systems, ATPase enzymes, etc.

A familiar example of energy-producing and energy-consuming functions of the bacterial membrane, related to the establishment and use of pmf and the production of ATP, is given in the following drawing of the plasma membrane of *Escherichia coli*.

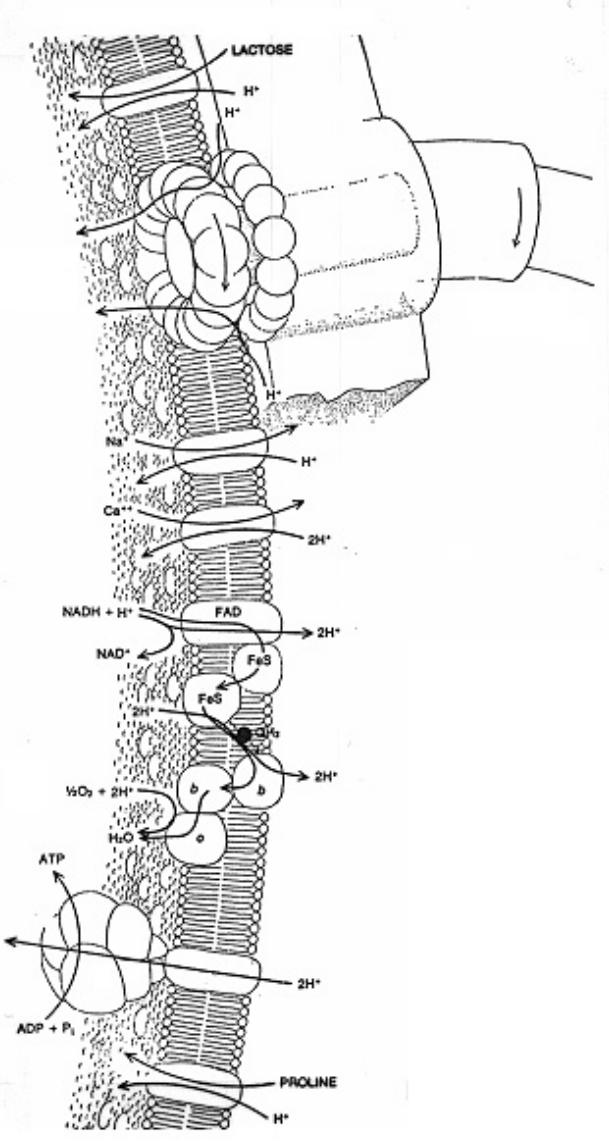


Figure 6. The plasma membrane of *Escherichia coli*. The membrane in cross-section reveals various transport systems, the flagellar motor apparatus (S and M rings), the respiratory electron transport system, and the membrane-bound ATPase enzyme. Reduced $NADH + H^+$ feeds pairs of electrons into the ETS. The ETS is the sequence of electron carriers in the membrane [$FAD \rightarrow FeS \rightarrow QH_2$ (Quinone) \rightarrow (cytochromes) b \rightarrow b \rightarrow o] that ultimately reduces O_2 to H_2O during respiration. At certain points in the electron transport process, the electrons pass "coupling sites" and this results in the translocation of protons from the inside to the outside of the membrane, thus establishing the proton motive force (pmf) on the membrane. The pmf is used in three ways by the bacterium to do work or conserve energy: active transport (e.g. lactose and proline symport; calcium and sodium antiport); motility (rotation of the bacterial flagellum); and ATP synthesis (via the ATPase enzyme during the process of oxidative phosphorylation or electron transport phosphorylation).

Heterotrophic Types of Metabolism

Heterotrophy (i.e. chemoheterotrophy) is the use of an organic compound as a source of carbon and energy. It is the complete metabolism package. The cell oxidizes organic molecules in order to produce energy (catabolism) and then uses the energy to synthesize cellular material from these the organic molecules (anabolism). We animals are familiar with heterotrophic metabolism. Many **Bacteria** (but just a few **Archaea**) are heterotrophs, particularly those that live in associations with animals. Heterotrophic bacteria are the masters of decomposition and biodegradation in the environment. Heterotrophic metabolism is driven mainly by two metabolic processes: fermentations and respirations.

Fermentation

Fermentation is an ancient mode of metabolism, and it must have evolved with the appearance of organic material on the planet. Fermentation is metabolism in which energy is derived from the **partial oxidation of an organic compound** using **organic intermediates as electron donors and electron acceptors**. No outside electron acceptors are involved; no membrane or electron transport system is required; **all ATP is produced by substrate level**

phosphorylation.

By definition, fermentation may be as simple as two steps illustrated in the following model. Indeed, some amino acid fermentations by the clostridia are this simple. But the **pathways of fermentation** are a bit more complex, usually involving several preliminary steps to prime the energy source for oxidation and substrate level phosphorylations.

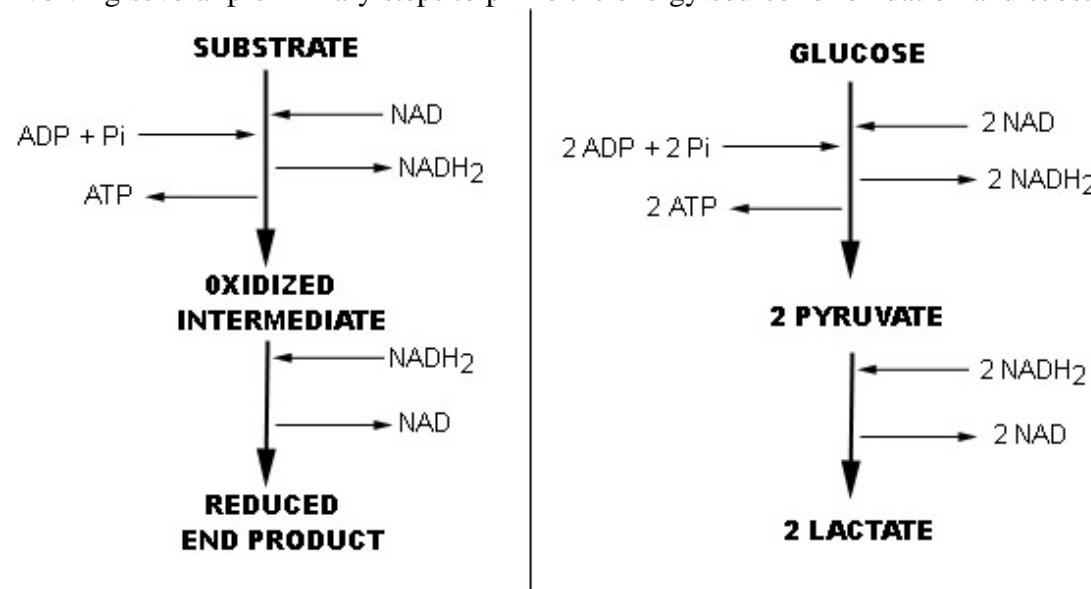


Figure 7. Model fermentation. L. The substrate is oxidized to an organic intermediate; the usual oxidizing agent is NAD. Some of the energy released by the oxidation is conserved during the synthesis of ATP by the process of substrate level phosphorylation. Finally, the oxidized intermediate is reduced to end products. Note that NADH₂ is the reducing agent, thereby balancing its redox ability to drive the energy-producing reactions. **R.** In lactic fermentation by *Lactobacillus*, the substrate (glucose) is oxidized to pyruvate, and pyruvate becomes reduced to lactic acid. Redox balance is maintained by coupling oxidations to reductions within the pathway. For example, in lactic acid fermentation via the Embden-Meyerhof pathway, the oxidation of glyceraldehyde phosphate to phosphoglyceric acid is coupled to the reduction of pyruvic acid to lactic acid.

In biochemistry, for the sake of convenience, fermentation pathways start with glucose. This is because it is the simplest molecule, requiring the fewest catalytic steps, to enter into a pathway of glycolysis and central metabolism. In prokaryotes there exist three major pathways of glycolysis (the dissimilation of sugars): the classic **Embden-Meyerhof pathway**, which is also used by most eukaryotes, including yeast (*Saccharomyces*); the **phosphoketolase or heterolactic pathway** related to the hexose-pentose shunt; and the **Entner-Doudoroff pathway**. Whether or not a bacterium is a fermenter, it will likely dissimilate sugars through one or more of these pathways (See Table 1 below).

The Embden-Meyerhof Pathway

This is the pathway of glycolysis most familiar to biochemists and eukaryotic biologists, as well as to brewers, breadmakers and cheeseheads. The pathway is operated by *Saccharomyces* to produce ethanol and CO₂. The pathway is used by the (homo)lactic acid bacteria to produce lactic acid, and it is used by many other bacteria to produce a variety of fatty acids, alcohols and gases. Some end products of Embden-Meyerhof fermentations are essential components of foods and beverages, and some are useful fuels and industrial solvents. Diagnostic microbiologists use bacterial fermentation profiles (e.g. testing an organism's ability to ferment certain sugars, or examining an organism's array of end products) in order to identify them, down to the genus level.

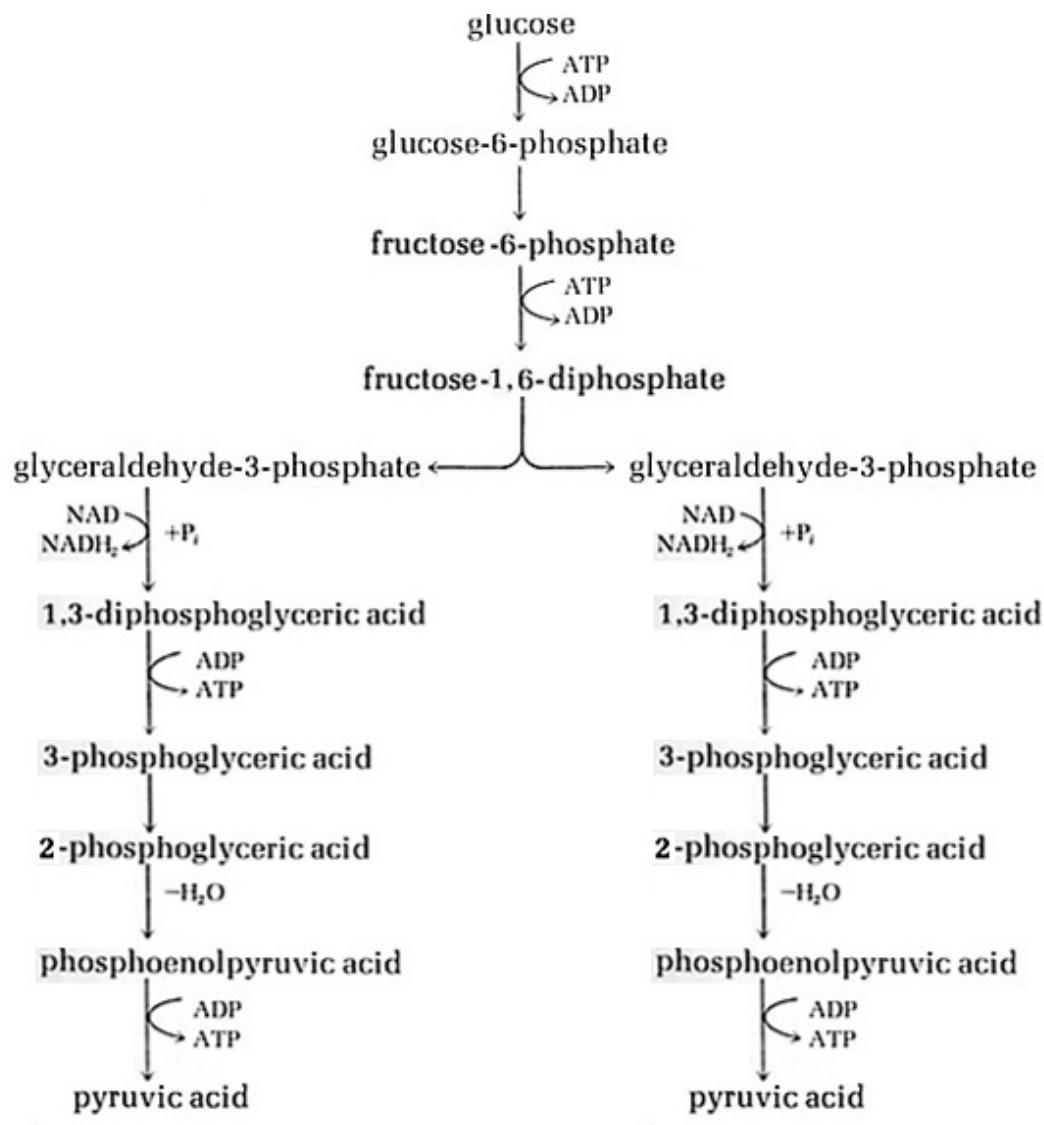


Figure 8. The Embden Meyerhof pathway for glucose dissimilation. The overall reaction is the oxidation of glucose to 2 pyruvic acid. The two branches of the pathway after the cleavage are identical, drawn in this manner for comparison with other bacterial pathways of glycolysis.

The first three steps of the pathway prime (phosphorylate) and rearrange the hexose for cleavage into 2 trioses (glyceraldehyde-phosphate). **Fructose 1,6-diphosphate aldolase** is the key (cleavage) enzyme in the E-M pathway. Each triose molecule is oxidized and phosphorylated followed by two substrate level phosphorylations that yield 4 ATP during the drive to pyruvate.

Lactic acid bacteria reduce the pyruvate to lactic acid; yeast reduce the pyruvate to alcohol (ethanol) and CO₂ as shown in Figure 9 below.

The oxidation of glucose to lactate yields a total of 56 kcal per mole of glucose. Since the cells harvest 2 ATP (16 kcal) as useful energy, the efficiency of the lactate fermentation is about 29 percent (16/56). Ethanol fermentations have a similar efficiency.

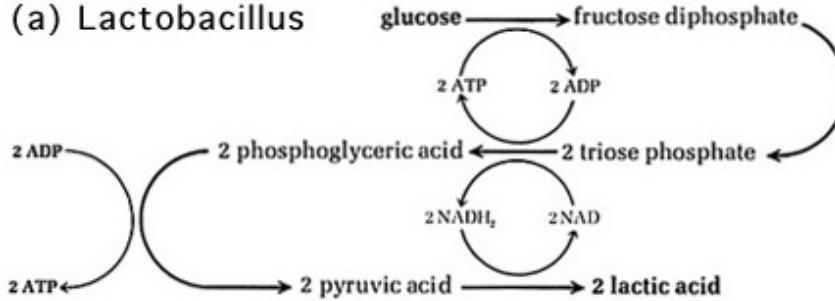
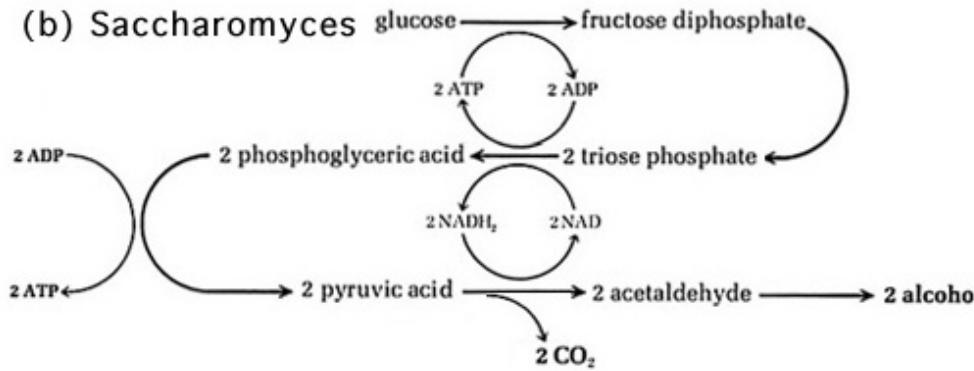
(a) *Lactobacillus*(b) *Saccharomyces*

Figure 9. (a) The Embden Meyerhof pathway of lactic acid fermentation in lactic acid bacteria (*Lactobacillus*) and (b) the Embden Meyerhof pathway of alcohol fermentation in yeast (*Saccharomyces*). The pathways yield two moles of end products and two moles of ATP per mole of glucose fermented. The steps in the breakdown of glucose to pyruvate are identical. The difference between the pathways is the manner of reducing pyruvic acid, thereby giving rise to different end products.

Besides lactic acid, Embden-Meyerhof fermentations in bacteria can lead to a wide array of end products depending on the pathways taken in the reductive steps after the formation of pyruvic acid. Figure 10 below shows some of the pathways proceeding from pyruvic acid in certain bacteria. Usually, these bacterial fermentations are distinguished by their end products into the following groups.

1. Homolactic Fermentation. **Lactic acid** is the sole end product. Pathway of the homolactic acid bacteria (*Lactobacillus* and most streptococci). The bacteria are used to ferment milk and milk products in the manufacture of yogurt, buttermilk, sour cream, cottage cheese, cheddar cheese, and most fermented dairy products.

2. Mixed Acid Fermentations. Mainly the pathway of the *Enterobacteriaceae*. End products are a mixture of **lactic acid**, **acetic acid**, **formic acid**, **succinate** and **ethanol**, with the possibility of gas formation (**CO₂** and **H₂**) if the bacterium possesses the enzyme formate dehydrogenase, which cleaves formate to the gases.

2a. Butanediol Fermentation. Forms mixed acids and gases as above, but, in addition, **2,3 butanediol** from the condensation of 2 pyruvate. The use of the pathway decreases acid formation (butanediol is neutral) and causes the formation of a distinctive intermediate, **acetoin**. Water microbiologists have specific tests to detect low acid and acetoin in order to distinguish non fecal enteric bacteria (butanediol formers, such as *Klebsiella* and *Enterobacter*) from fecal enterics (mixed acid fermenters, such as *E. coli*, *Salmonella* and *Shigella*).

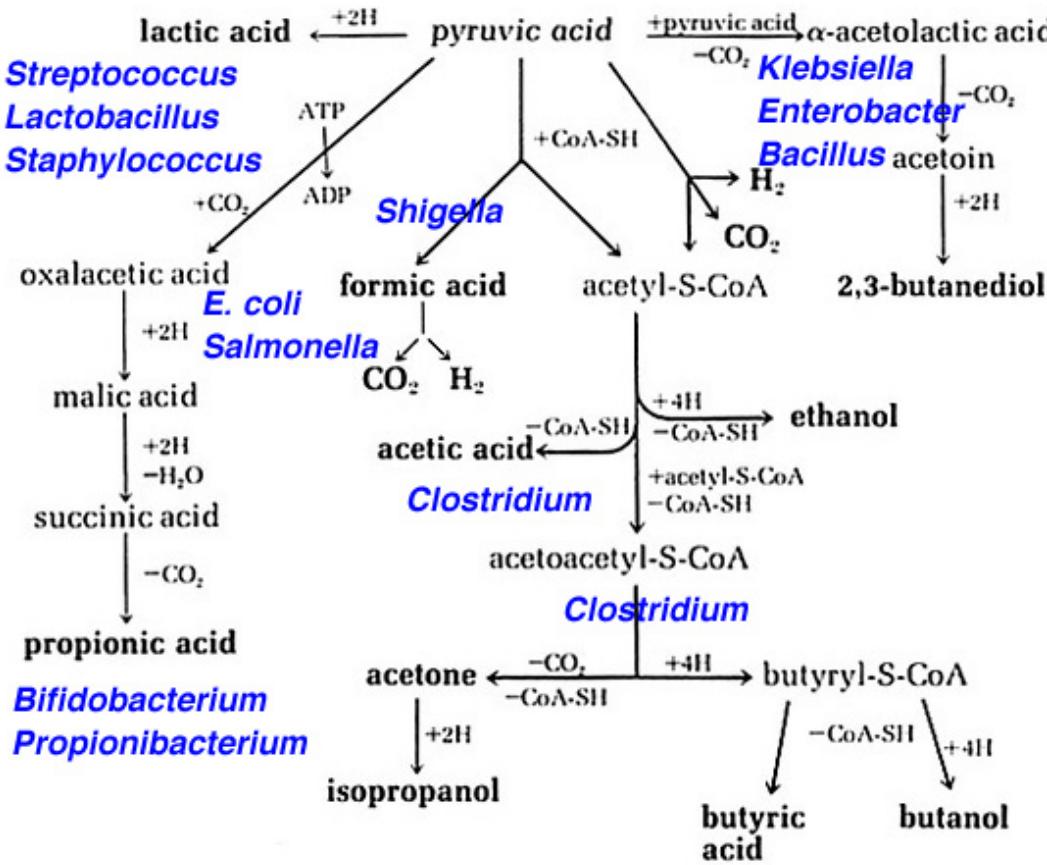
3. Butyric acid fermentations, as well as the butanol-acetone fermentation (below), are run by the clostridia, the masters of fermentation. In addition to butyric acid, the clostridia form acetic acid, CO₂ and H₂ from the fermentation of sugars. Small amounts of ethanol and isopropanol may also be formed.

3a. Butanol-acetone fermentation. Butanol and acetone were discovered as the main end products of fermentation by *Clostridium acetobutylicum* during the World War I. This discovery solved a critical problem of explosives manufacture (acetone is required in the manufacture gunpowder) and is said to have affected the outcome of the War. Acetone was distilled from the fermentation liquor of *Clostridium acetobutylicum*, which worked out pretty good if you were on our side, because organic chemists hadn't figured out how to synthesize it chemically. You can't run a war without gunpowder, at least you couldn't in those days.

4. Propionic acid fermentation. This is an unusual fermentation carried out by the propionic acid bacteria which

include corynebacteria, *Propionibacterium* and *Bifidobacterium*. Although sugars can be fermented straight through to propionate, propionic acid bacteria will ferment lactate (the end product of lactic acid fermentation) to acetic acid, CO₂ and propionic acid. The formation of propionate is a complex and indirect process involving 5 or 6 reactions. Overall, 3 moles of lactate are converted to 2 moles of propionate + 1 mole of acetate + 1 mole of CO₂, and 1 mole of ATP is squeezed out in the process. The propionic acid bacteria are used in the manufacture of Swiss cheese, which is distinguished by the distinct flavor of propionate and acetate, and holes caused by entrapment of CO₂.

Figure 10. Fermentations in bacteria that proceed through the Embden-Meyerhof pathway.



The Embden-Meyerhof pathway for glucose dissimilation (Figure 8), as well as the TCA cycle discussed below (Figure 14), are two pathways that are at the center of metabolism in nearly all organisms. Not only do these pathways dissipilate organic compounds and provide energy, they also provide the precursors for biosynthesis of macromolecules that make up living systems (see Figure 25 below). These are rightfully-called **amphibolic pathways** since they have both an anabolic and a catabolic function.

The Heterolactic (Phosphoketolase) Pathway

The phosphoketolase pathway (Figure 11) is distinguished by the key cleavage enzyme, **phosphoketolase**, which cleaves pentose phosphate into glyceraldehyde-3-phosphate and acetyl phosphate. As a fermentation pathway, it is employed mainly by the **heterolactic acid bacteria**, which include some species of *Lactobacillus* and *Leuconostoc*. In this pathway, glucose-phosphate is oxidized to 6-phosphogluconic acid, which becomes oxidized and decarboxylated to form pentose phosphate. Unlike the Embden-Meyerhof pathway, NAD-mediated oxidations take place before the cleavage of the substrate being utilized. Pentose phosphate is subsequently cleaved to glyceraldehyde-3-phosphate (GAP) and acetyl phosphate. GAP is converted to lactic acid by the same enzymes as the E-M pathway. This branch of the pathway contains an oxidation coupled to a reduction while 2 ATP are produced by substrate level phosphorylation. Acetyl phosphate is reduced in two steps to ethanol, which balances the two oxidations before the cleavage but does not yield ATP. The overall reaction is Glucose -----> 1 lactic acid + 1 ethanol + 1 CO₂ with a net gain of 1 ATP. The efficiency is about half that of the E-M pathway.

Heterolactic species of bacteria are occasionally used in the fermentation industry. For example, one type of fermented milk called kefir, analogous to yogurt which is produced by homolactic acid bacteria, is produced using a heterolactic *Lactobacillus* species. Likewise, sauerkraut fermentations use *Leuconostoc* species of bacteria to

complete the fermentation.

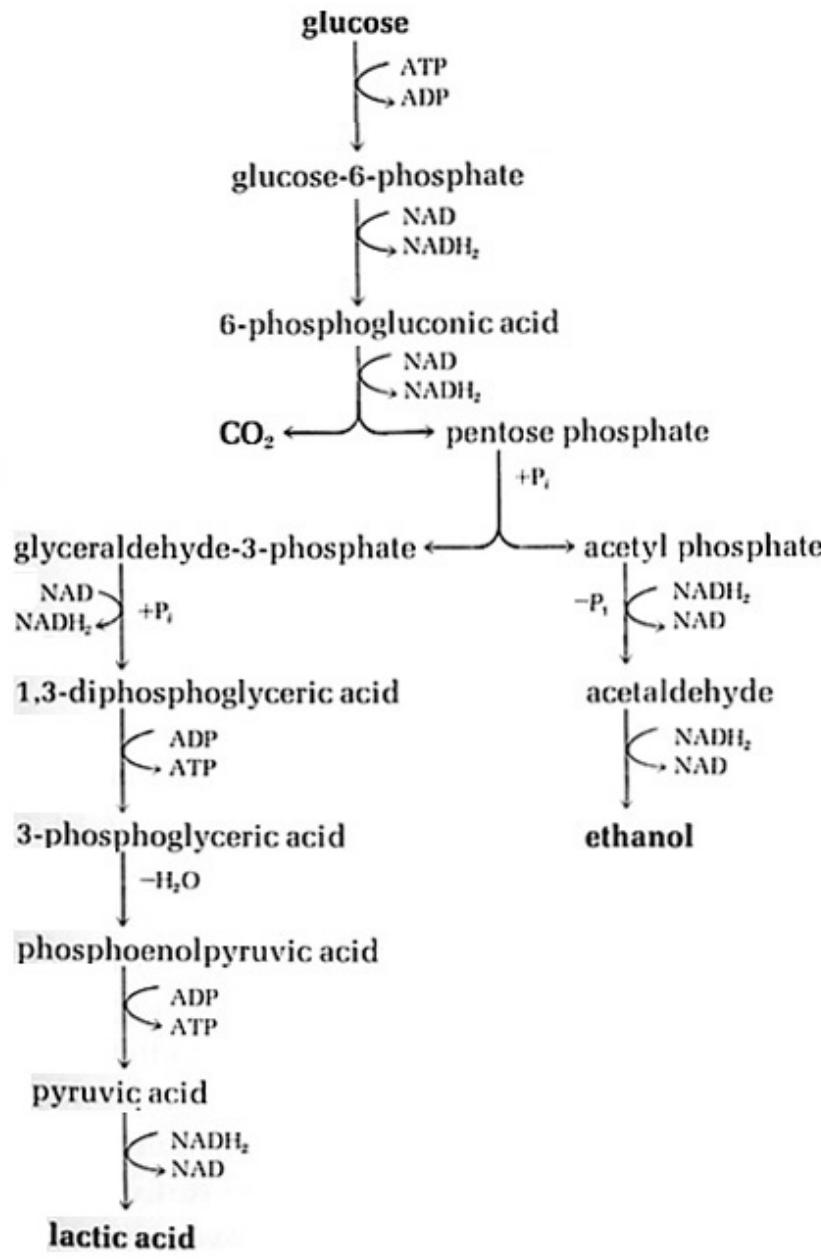


Figure 11. The heterolactic (phosphoketolase) pathway of fermentation. Compare with the Embden-Meyerhof pathway in Figure 9. This pathway differs in the early steps before the cleavage of the molecule. The overall reaction in the fermentation of glucose is Glucose -----> Lactic acid + ethanol + CO₂ + 1 ATP (net).

The Entner-Doudoroff Pathway

Only a few bacteria, most notably *Zymomonas*, employ the Entner-Doudoroff pathway as a fermentation path. However, many bacteria, especially those grouped around the pseudomonads, use the pathway as a way to degrade carbohydrates for respiratory metabolism (see Table 1 below). The E-D pathway yields 2 pyruvic acid from glucose (same as the E-M pathway) but like the phosphoketolase pathway, oxidation occurs before the cleavage, and the net energy yield per mole of glucose utilized is one mole of ATP.

In the E-D pathway, glucose phosphate is oxidized to 2-keto-3-deoxy-6-phosphogluconic acid (KDPG) which is cleaved by **KDPG aldolase** to pyruvate and GAP. The latter is oxidized to pyruvate by E-M enzymes wherein 2 ATP are produced by substrate level phosphorylations. Pyruvic acid from either branch of the pathway is reduced to ethanol and CO₂, in the same manner as yeast, by the "yeast-like bacterium", *Zymomonas* (Figure 12 below). Thus, the overall reaction is Glucose -----> 2 ethanol + 2 CO₂, and a net gain of 1 ATP.

Zymomonas is a bacterium that lives on the surfaces of plants, including the succulent Maguey cactus which is

indigenous to Mexico. Just as grapes are crushed and fermented by resident yeast to wine, so may the Maguey flesh be crushed and allowed to ferment with *Zymomonas*, which gives rise to "cactus beer" or "pulque", as it is known in Mexico. Distilled pulque yields tequila in the state of Jalisco or mescal in the state of Oaxaca. Many cultures around the world prepare their native fermented brews with *Zymomonas* in deference to the yeast, *Saccharomyces*, although they may not have a choice in the matter. *Zymomonas* has potential advantages over yeast for the industrial production of alcohol, but the industry is geared to do what it can do, and no change in organisms is forthcoming.

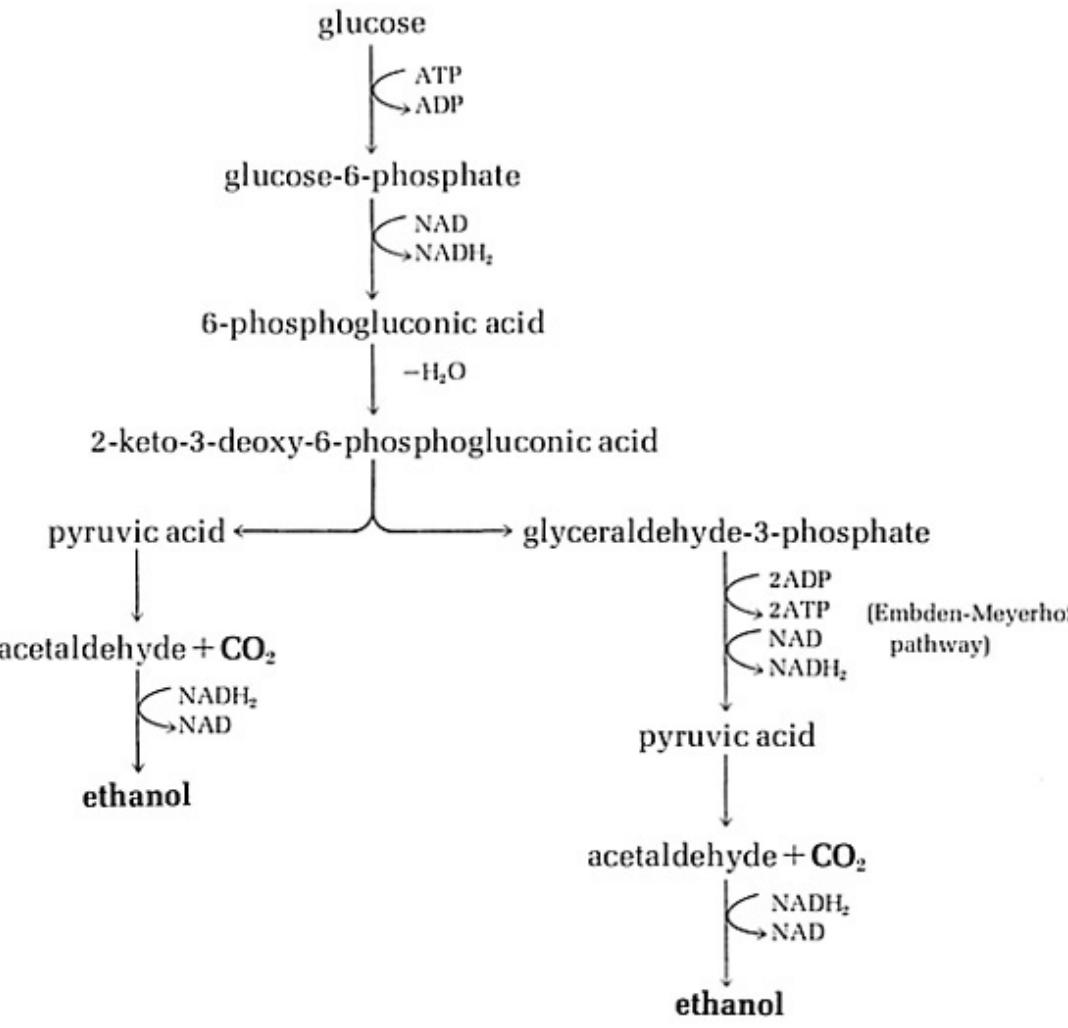


Figure 12. The Entner-Doudoroff Pathway of Fermentation. The overall reaction is Glucose -----> 2 ethanol + 2 CO₂ + 1 ATP (net).

Table 1. Oxidative pathways of glycolysis employed by various bacteria.

Bacterium	Embden-Meyerhof pathway	Phosphoketolase (heterolactic) pathway	Entner-Doudoroff pathway
<i>Acetobacter aceti</i>	-	+	-
<i>Agrobacterium tumefaciens</i>	-	-	+
<i>Azotobacter vinelandii</i>	-	-	+
<i>Bacillus subtilis</i>	major	minor	-
<i>Escherichia coli</i>	+	-	-
<i>Lactobacillus acidophilus</i>	+	-	-
<i>Leuconostoc mesenteroides</i>	-	+	-
<i>Pseudomonas aeruginosa</i>	-	-	+

<i>Vibrio cholerae</i>	minor	-	major
<i>Zymomonas mobilis</i>	-	-	+

Table 2. End product yields in microbial fermentations.

Pathway	Key enzyme	Ethanol	Lactic Acid	CO ₂	ATP
Embden-Meyerhof <i>Saccharomyces</i>	fructose 1,6-diP aldolase	2	0	2	2
Embden-Meyerhof <i>Lactobacillus</i>	fructose 1,6-diP aldolase	0	2	0	2
Heterolactic <i>Streptococcus</i>	phosphoketolase	1	1	1	1
Entner-Doudoroff <i>Zymomonas</i>	KDPG aldolase	2	0	2	1

Respiration

Compared to fermentation as a means of oxidizing organic compounds, respiration is a lot more complicated. Respirations result in the **complete oxidation of the substrate** by an **outside electron acceptor**. In addition to a pathway of glycolysis, four essential structural or metabolic components are needed:

1. The **tricarboxylic acid (TCA) cycle** (also known as the citric acid cycle or the Kreb's cycle): when an organic compound is utilized as a substrate, the TCA cycle is used for the complete oxidation of the substrate. The end product that always results from the complete oxidation of an organic compound is CO₂.
2. A **membrane and an associated electron transport system (ETS)**. The ETS is a **sequence of electron carriers in the plasma membrane** that transports electrons taken from the substrate through the chain of carriers to a final electron acceptor. The electrons enter the ETS at a very low redox potential (E'₀) and exit at a relatively high redox potential. This drop in potential releases energy that can be harvested by the cells in the process of ATP synthesis by the mechanisms of **electron transport phosphorylation**. The operation of the ETS establishes a proton motive force (pmf) due to the formation of a proton gradient across the membrane.
3. An **outside electron acceptor** ("outside", meaning it is not internal to the pathway, as is pyruvate in a fermentation). For **aerobic respiration** the electron acceptor is O₂, of course. Molecular oxygen is reduced to H₂O in the last step of the electron transport system. But in the bacterial processes of **anaerobic respiration**, the final electron acceptors may be SO₄ or S or NO₃ or NO₂ or certain other inorganic compounds, or even an organic compound, such as fumarate.
4. A transmembranous **ATPase enzyme** (ATP synthetase). This enzyme utilizes the proton motive force established on the membrane (by the operation of the ETS) to synthesize ATP in the process of **electron transport phosphorylation**. It is believed that the transmembranous Fo subunit is a proton transport system that transports 2H⁺ to the F1 subunit (the actual ATPase) on the inside of the membrane. The 2 protons are required and consumed during the synthesis of ATP from ADP plus Pi. See Figure 6 -the membrane of *E. coli*. The reaction catalyzed by the ATPase enzyme is ADP + Pi + 2 H⁺ <-----> ATP. (It is important to appreciate the reversibility of this reaction in order to account for how a fermentative bacterium, without an ETS, could establish a necessary pmf on the membrane for transport or flagellar rotation. If such an organism has a transmembranous ATPase, it could produce ATP by SLP, and subsequently the ATPase could hydrolyze the ATP, thereby releasing protons to the outside of the membrane.)

The diagram below of aerobic respiration (Figure 13) integrates these metabolic processes into a scheme that

represents the overall process of respiratory metabolism. A substrate such as glucose is completely oxidized to CO_2 by the combined pathways of glycolysis and the TCA cycle. Electrons removed from the glucose by NAD are fed into the ETS in the membrane. As the electrons traverse the ETS, a pmf becomes established across the membrane. The electrons eventually reduce an outside electron acceptor, O_2 , and reduce it to H_2O . The pmf on the membrane is used by the ATPase enzyme to synthesize ATP by a process referred to as "oxidative phosphorylation".

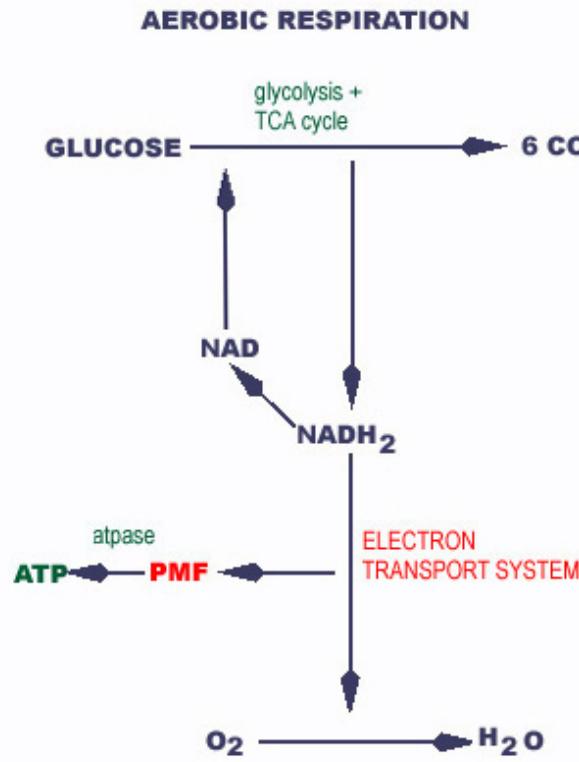


Figure 13. Model of Aerobic respiration.

Paramount to appreciation of respiration, is an understanding of the role of the TCA cycle. The TCA cycle (including the steps leading into it) accounts for the complete oxidation of the substrate and provides 10 pairs of electrons (from glucose) for transit through the ETS. For every pair of electrons put into the ETS, 2 or 3 ATP may be produced, so a huge amount of ATP is produced in a respiration, compared to a fermentation.

Glucose is dissimilated in a pathway of glycolysis to the intermediate, pyruvate, and it is the pyruvate that is moved into the TCA cycle, eventually becoming oxidized to 3 CO_2 . Since 2 pyruvate are formed from one glucose, the cycle must turn twice for every molecule of glucose oxidized to 6 CO_2 .

Initially, pyruvate is oxidized and decarboxylated in a complex reaction involving NAD, Coenzyme A, and pyruvate dehydrogenase (pyruvate decarboxylase), forming the most central molecule in metabolism, Acetyl CoA. (See Figure 4). Acetyl CoA condenses with the 4C-compound, oxaloacetic acid, to form the first stable intermediate of the TCA cycle, 6C-citric acid (citrate), a tricarboxylic acid. Citrate is isomerized to isocitrate, which is oxidized and decarboxylated forming alpha-ketoglutarate (akg). Alpha ketoglutarate dehydrogenase uses CoA and NAD to oxidize akg to succinyl CoA in a reaction analogous to the pyruvate dehydrogenase reaction above. Succinyl CoA is converted to succinate during a substrate level phosphorylation yielding high energy GTP (equivalent to ATP). This completes the decarboxylation of pyruvate forming 3 CO_2 . The remaining three steps in the cycle complete the oxidation of succinate and regenerate the oxalacetate necessary to drive the cycle. During the oxidation of pyruvic acid to 3 CO_2 by one turn of the TCA cycle, 4 NADH₂, 1 FADH₂ and one ATP (actually GTP) are produced. Since the TCA cycle is an important amphibolic pathway, several intermediates of the cycle may be withdrawn for anabolic (biosynthetic) pathways (See Figure 25).

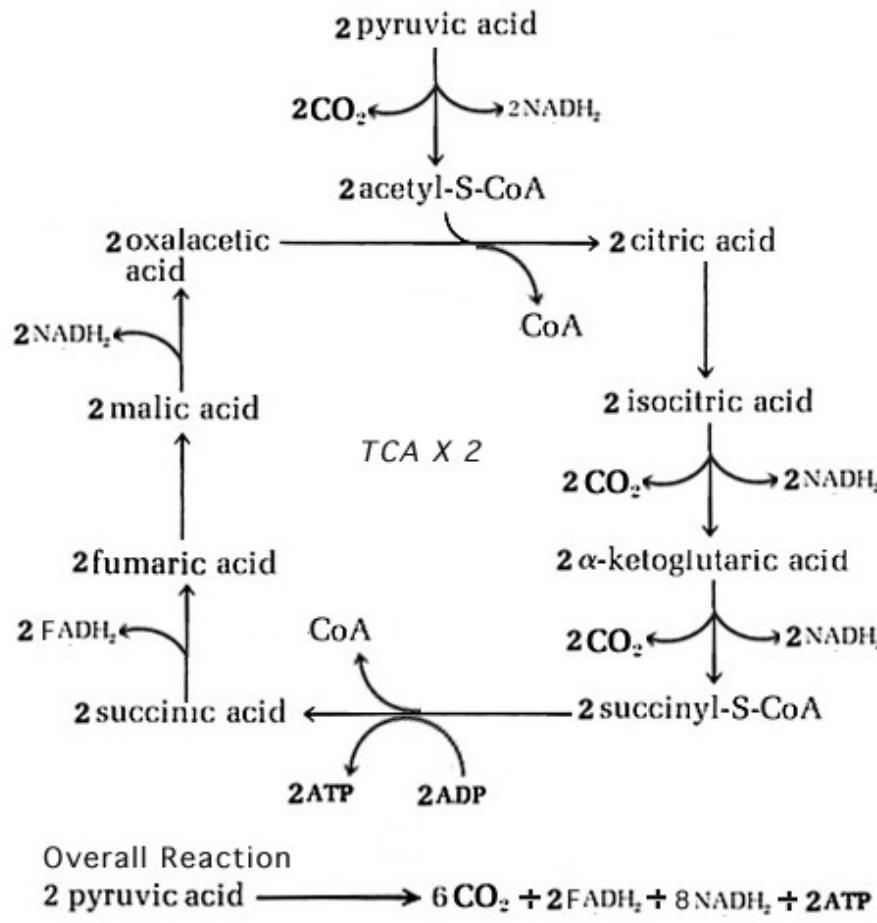
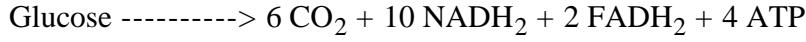


Figure 14. The tricarboxylic acid (TCA) or Kreb's cycle

The overall reaction for the aerobic respiration of glucose is

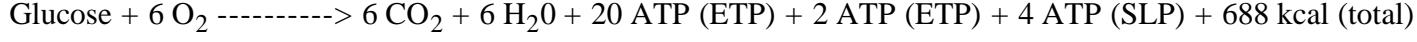


which can be written

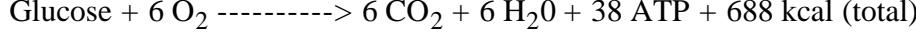


(2NADH₂ from glycolysis, 8NADH₂ from two turns of TCA, 2 FADH₂ from two turns of TCA; 2ATP (net) from glycolysis, 2 ATP (GTP) from two turns of TCA)

In *E. coli*, 2 ATP are produced for each pair of electrons that are introduced into the ETS by NADH₂. One ATP is produced from a pair of electrons introduced by FADH₂. Hence, the equation can be rewritten



Since a total of 26 ATP is formed during the release of 688 kcal of energy, the efficiency of this respiration is 26x8/688 or about 30 percent. In *Pseudomonas* (or mitochondria), due to the exact nature of the ETS, 3 ATP are produced for each pair of electrons that are introduced into the ETS by NADH₂ and 2 ATP are produced from a pair of electrons introduced by FADH₂. Hence, the overall reaction in *Pseudomonas*, using the same dissimilatory pathways as *E. coli*, is



and the corresponding efficiency is about 45 percent.

Respiration in some prokaryotes is possible using electron acceptors other than oxygen (O₂). This type of respiration

in the absence of oxygen is referred to as **anaerobic respiration**. Although anaerobic respiration is more complicated than the foregoing statement, in its simplest form it represents the substitution or **use of some compound other than O₂ as a final electron acceptor in the electron transport chain**. Electron acceptors used by prokaryotes for respiration or methanogenesis (an analogous type of energy generation in archaea) are described in the table below.

Table 3. Electron acceptors for respiration and methanogenesis in prokaryotes

electron acceptor	reduced end product	name of process	organism
O ₂	H ₂ O	aerobic respiration	<i>Escherichia, Streptomyces</i>
NO ₃	NO ₂ , NH ₃ or N ₂	anaerobic respiration: denitrification	<i>Bacillus, Pseudomonas</i>
SO ₄	S or H ₂ S	anaerobic respiration: sulfate reduction	<i>Desulfovibrio</i>
fumarate	succinate	anaerobic respiration: using an organic e- acceptor	<i>Escherichia</i>
CO ₂	CH ₄	methanogenesis	<i>Methanococcus</i>

Biological **methanogenesis** is the source of methane (natural gas) on the planet. Methane is preserved as a fossil fuel (until we use it all up) because it is produced and stored under anaerobic conditions, and oxygen is needed to oxidize the CH₄ molecule. Methanogenesis is not really a form of anaerobic respiration, but it is a type of energy-generating metabolism that requires an outside electron acceptor in the form of CO₂.

Denitrification is an important process in agriculture because it removes NO₃ from the soil. NO₃ is a major source of nitrogen fertilizer in agriculture. Almost one-third the cost of some types of agriculture is in nitrate fertilizers. The use of nitrate as a respiratory electron acceptor is usually an alternative to the use of oxygen. Therefore, soil bacteria such as *Pseudomonas* and *Bacillus* will use O₂ as an electron acceptor if it is available, and disregard NO₃. This is the rationale in maintaining well-aerated soils by the agricultural practices of plowing and tilling. *E. coli* will utilize NO₃ (as well as fumarate) as a respiratory electron acceptor and so it may be able to continue to respire in the anaerobic intestinal habitat.

Sulfate reduction is not an alternative to the use of O₂ as an electron acceptor. It is an obligatory process that occurs only under anaerobic conditions. Methanogens and sulfate reducers may share habitat, especially in the anaerobic sediments of eutrophic lakes such as Lake Mendota, where they crank out methane and hydrogen sulfide at a surprising rate.

Anaerobic respiring bacteria and methanogens play an essential role in the biological cycles of carbon, nitrogen and sulfur. In general, they convert oxidized forms of the elements to a more reduced state. The lithotrophic prokaryotes metabolize the reduced forms of nitrogen and sulfur to a more oxidized state in order to produce energy. The methanotrophic bacteria, which uniquely possess the enzyme methane monooxygenase, can oxidize methane as a source of energy. Among all these groups of prokaryotes there is a minicycle of the elements in a model ecosystem.

Lithotrophic Types of Metabolism

Lithotrophy is the use of an inorganic compound as a source of energy. Most lithotrophic bacteria are aerobic respirers that produce energy in the same manner as all aerobic respiring organisms: they remove electrons from a substrate and put them through an electron transport system that will produce ATP by electron transport phosphorylation. Lithotrophs just happen to get those electrons from an inorganic, rather than an organic compound.

Some lithotrophs are **facultative lithotrophs**, meaning they are able to use organic compounds, as well, as sources of energy. Other lithotrophs do not use organic compounds as sources of energy; in fact, they won't transport organic compounds. CO₂ is the sole source of carbon for the methanogens and the nitrifying bacteria and a few other species scattered about in other groups. These **lithoautotrophs** are often referred to as "chemoautotrophs", but the term **lithoautotroph** is a more accurate description of their metabolism. The lithotrophs are a very diverse group of prokaryotes, united only by their ability to oxidize an inorganic compound as an energy source.

Lithotrophy runs through the **Bacteria** and the **Archaea**. If one considers methanogen oxidation of H₂ a form of lithotrophy, then probably most of the **Archaea** are lithotrophs. Lithotrophs are usually organized into "physiological groups" based on their inorganic substrate for energy production and growth (see Table 5 below).

Table 5. Physiological groups of lithotrophs

physiological group	energy source	oxidized end product	organism
hydrogen bacteria	H ₂	H ₂ O	<i>Alcaligenes, Pseudomonas</i>
methanogens	H ₂	H ₂ O	<i>Methanobacterium</i>
carboxydobacteria	CO	CO ₂	<i>Rhodospirillum, Azotobacter</i>
nitrifying bacteria*	NH ₃	NO ₂	<i>Nitrosomonas</i>
nitrifying bacteria*	NO ₂	NO ₃	<i>Nitrobacter</i>
sulfur oxidizers	H ₂ S or S	SO ₄	<i>Thiobacillus, Sulfolobus</i>
iron bacteria	Fe ⁺⁺	Fe ⁺⁺⁺	<i>Gallionella, Thiobacillus</i>

* The overall process of **nitrification**, conversion of NH₃ to NO₃, requires a consortium of microorganisms.

The **hydrogen bacteria** oxidize H₂ (hydrogen gas) as an energy source. The hydrogen bacteria are **facultative lithotrophs** as evidenced by the pseudomonads that fortuitously possess a hydrogenase enzyme that will oxidize H₂ and put the electrons into their respiratory ETS. They will use H₂ if they find it in their environment even though they are typically heterotrophic. Indeed, most hydrogen bacteria are nutritionally-versatile in their ability to use a wide range of carbon and energy sources. Some hydrogen bacteria possess an NAD-linked **hydrogenase** that transfers electrons from H₂ to NAD in a one-step process. NAD then delivers the electrons to the ETS. Others have hydrogenase enzymes that pass electrons to different carriers in the bacterial electron transport system.

The **methanogens** used to be considered a major group of hydrogen bacteria - until it was discovered that they are **Archaea**. The methanogens are able to oxidize H₂ as a sole source of energy while transferring the electrons from H₂ to CO₂ in its reduction to methane. Apparently, H₂ has more energy available than CH₄, for all you physical chemists out there. Metabolism of the methanogens is absolutely unique, yet methanogens represent the most prevalent and diverse group of **Archaea**. Methanogens use H₂ and CO₂ to produce cell material and methane. They have unique coenzymes and electron transport processes. Their type of energy generating metabolism is never seen in the **Bacteria**, and their mechanism of autotrophic CO₂ fixation is very rare, except in methanogens.

The **carboxydobacteria** are able to oxidize CO (carbon monoxide) to CO₂, using an enzyme **CODH (carbon monoxide dehydrogenase)**. The carboxydobacteria are not obligate CO users, i.e., some are also hydrogen bacteria, and some are phototrophic bacteria. Interestingly, the enzyme CODH used by the carboxydobacteria to oxidize CO to CO₂, is used by the methanogens for the reverse reaction - the reduction of CO₂ to CO - during CO₂ fixation by the CODH pathway (Figure 23).

The **nitrifying bacteria** are represented by two genera, *Nitrosomonas* and *Nitrobacter*. Together these bacteria can accomplish the oxidation of NH₃ to NO₃, known as the process of **nitrification**. No single organism can carry out the whole oxidative process. *Nitrosomonas* oxidizes ammonia to NO₂ and *Nitrobacter* oxidizes NO₂ to NO₃. Most of the nitrifying bacteria are **obligate lithoautotrophs**, the exception being a few strains of *Nitrobacter* that will utilize acetate. CO₂ fixation utilizes RUBP carboxylase and the Calvin Cycle. Nitrifying bacteria grow in environments rich in ammonia, where extensive protein decomposition is taking place. Nitrification in soil and aquatic habitats is an essential part of the nitrogen cycle.

Lithotrophic **sulfur oxidizers** include both **Bacteria** (e.g. *Thiobacillus*) and **Archaea** (e.g. *Sulfolobus*). Sulfur oxidizers oxidize H₂S (sulfide) or S (elemental sulfur) as a source of energy. Similarly, the purple and green sulfur bacteria oxidize H₂S or S as an electron donor for photosynthesis, and use the electrons for CO₂ fixation (the dark reaction of photosynthesis). Obligate autotrophy, which is nearly universal among the nitrifiers, is variable among the sulfur oxidizers. Lithoautotrophic sulfur oxidizers are found in environments rich in H₂S, such as volcanic hot springs

and fumaroles, and deep-sea thermal vents. Some are found as symbionts and endosymbionts of higher organisms. Since they can generate energy from an inorganic compound and fix CO₂ as autotrophs, they may play a fundamental role in **primary production** in environments that lack sunlight. As a result of their lithotrophic oxidations, these organisms produce sulfuric acid (SO₄), and therefore tend to acidify their own environments. Some of the sulfur oxidizers are **acidophiles** that will grow at a pH of 1 or less. Some are **hyperthermophiles** that grow at temperatures of 115 degrees C.

Iron bacteria oxidize Fe⁺⁺ (ferrous iron) to Fe⁺⁺⁺ (ferric iron). At least two bacteria probably oxidize Fe⁺⁺ as a source of energy and/or electrons and are capable of lithoautotrophic growth: the stalked bacterium *Gallionella*, which forms flocculant rust-colored colonies attached to objects in nature, and *Thiobacillus ferrooxidans*, which is also a sulfur-oxidizing lithotroph.

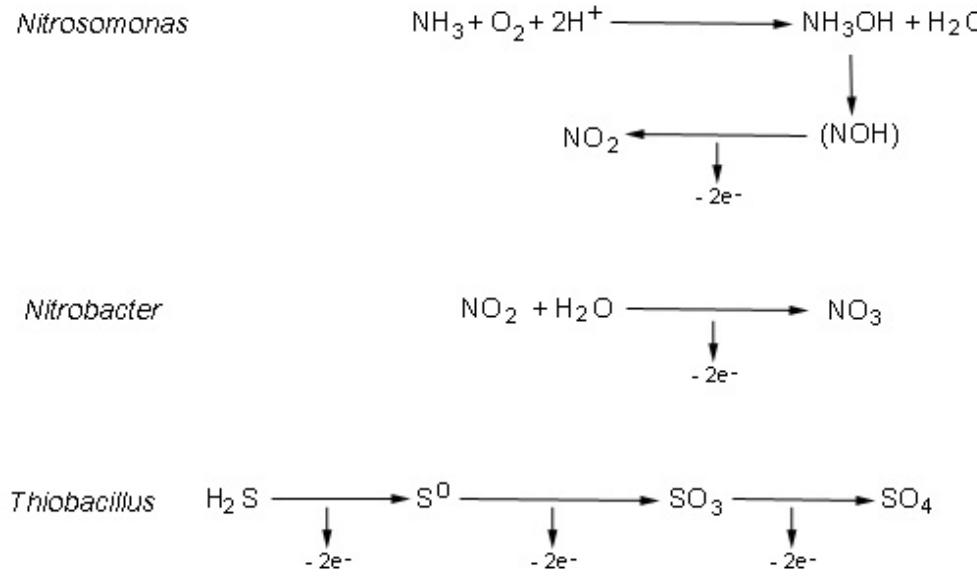


Figure 15. Lithotrophic oxidations

Phototrophic Metabolism

Phototrophy is the use of light as a source of energy for growth, more specifically the conversion of light energy into chemical energy in the form of ATP. Prokaryotes that can convert light energy into chemical energy include the photosynthetic cyanobacteria, the purple and green bacteria and the "halobacteria" (actually archaea). The cyanobacteria conduct plant photosynthesis, called **oxygenic photosynthesis**; the purple and green bacteria conduct bacterial photosynthesis or **anoxygenic photosynthesis**; the extreme halophilic archaea use a type of **nonphotosynthetic photophosphorylation** mediated by bacteriorhodopsin to transform light energy into ATP.

Photosynthesis is the conversion of light energy into chemical energy that can be used in the formation of cellular material from CO₂. Photosynthesis is a type of metabolism separable into a catabolic and anabolic component. The catabolic component of photosynthesis is the **light reaction**, wherein light energy is transformed into electrical energy, then chemical energy. The anabolic component involves the fixation of CO₂ and its use as a carbon source for growth, usually called the **dark reaction**. In photosynthetic prokaryotes there are two types of photosynthesis and two types of CO₂ fixation.

The Light Reactions depend upon the presence of chlorophyll, the **primary light-harvesting pigment** in the membrane of photosynthetic organisms. Absorption of a quantum of light by a chlorophyll molecule causes the displacement of an electron at the reaction center. The displaced electron is an energy source that is moved through a membrane photosynthetic electron transport system, being successively passed from an iron-sulfur protein (X) to a quinone to a cytochrome and back to chlorophyll (Figure 16 below). As the electron is transported, a proton motive force is established on the membrane, and ATP is synthesized by an ATPase enzyme. This manner of converting light energy into chemical energy is called **cyclic photophosphorylation**.

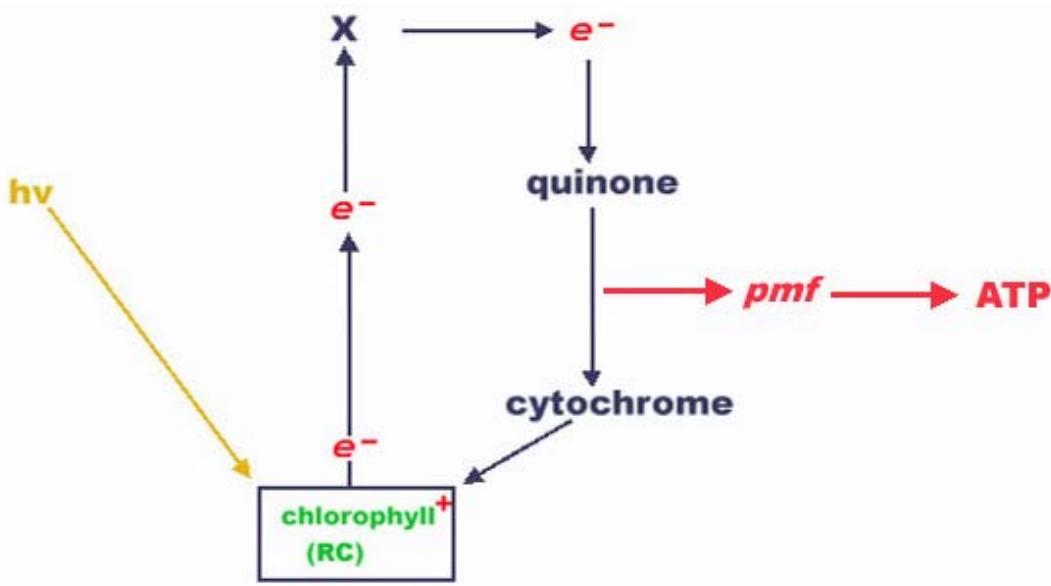


Figure 16. Photosystem I: cyclical electron flow coupled to photophosphorylation

The functional components of the photochemical system are **light harvesting pigments**, a membrane **electron transport system**, and an **ATPase** enzyme. The photosynthetic electron transport system is fundamentally similar to a respiratory ETS, except that there is a low redox electron acceptor (e.g. **ferredoxin**) at the top (low redox end) of the electron transport chain, that is first reduced by the electron displaced from chlorophyll.

There are several types of pigments distributed among various phototrophic organisms. **Chlorophyll** is the primary light-harvesting pigment in all photosynthetic organisms. Chlorophyll is a tetrapyrrole which contains magnesium at the center of the porphyrin ring. It contains a long hydrophobic side chain that associates with the photosynthetic membrane. Cyanobacteria have **chlorophyll a**, the same as plants and algae. The chlorophylls of the purple and green bacteria, called **bacteriochlorophylls** are chemically different than chlorophyll a in their substituent side chains. This is reflected in their light absorption spectra. Chlorophyll a absorbs light in two regions of the spectrum, one around 450nm and the other between 650 - 750nm; bacterial chlorophylls absorb from 800-1000nm in the far red region of the spectrum.

The chlorophylls are partially responsible for light harvesting at the photochemical reaction center. The energy of a photon of light is absorbed by a special chlorophyll molecule at the reaction center, which becomes instantaneously oxidized by a nearby electron acceptor of low redox potential. The energy present in a photon of light is conserved as a separation of electrical charge which can be used to generate a proton gradient for ATP synthesis.

Carotenoids are always associated with the photosynthetic apparatus. They function as **secondary light-harvesting pigments**, absorbing light in the blue-green spectral region between 400-550 nm. Carotenoids transfer energy to chlorophyll, at near 100 percent efficiency, from wave lengths of light that are missed by chlorophyll. In addition, carotenoids have an indispensable function to protect the photosynthetic apparatus from photooxidative damage. Carotenoids have long hydrocarbon side chains in a conjugated double bond system. Carotenoids "quench" the powerful oxygen radical, singlet oxygen, which is invariably produced in reactions between chlorophyll and O_2 (molecular oxygen). Some nonphotosynthetic bacterial pathogens, i.e., *Staphylococcus aureus*, produce carotenoids that protect the cells from lethal oxidations by singlet oxygen in phagocytes.

Phycobiliproteins are the major light harvesting pigments of the cyanobacteria. They also occur in some groups of algae. They may be red or blue, absorbing light in the middle of the spectrum between 550 and 650nm. Phycobiliproteins consist of proteins that contain covalently-bound linear tetrapyrroles (**phycobilins**). They are contained in granules called **phycobilisomes** that are closely associated with the photosynthetic apparatus. Being closely linked to chlorophyll they can efficiently transfer light energy to chlorophyll at the reaction center.

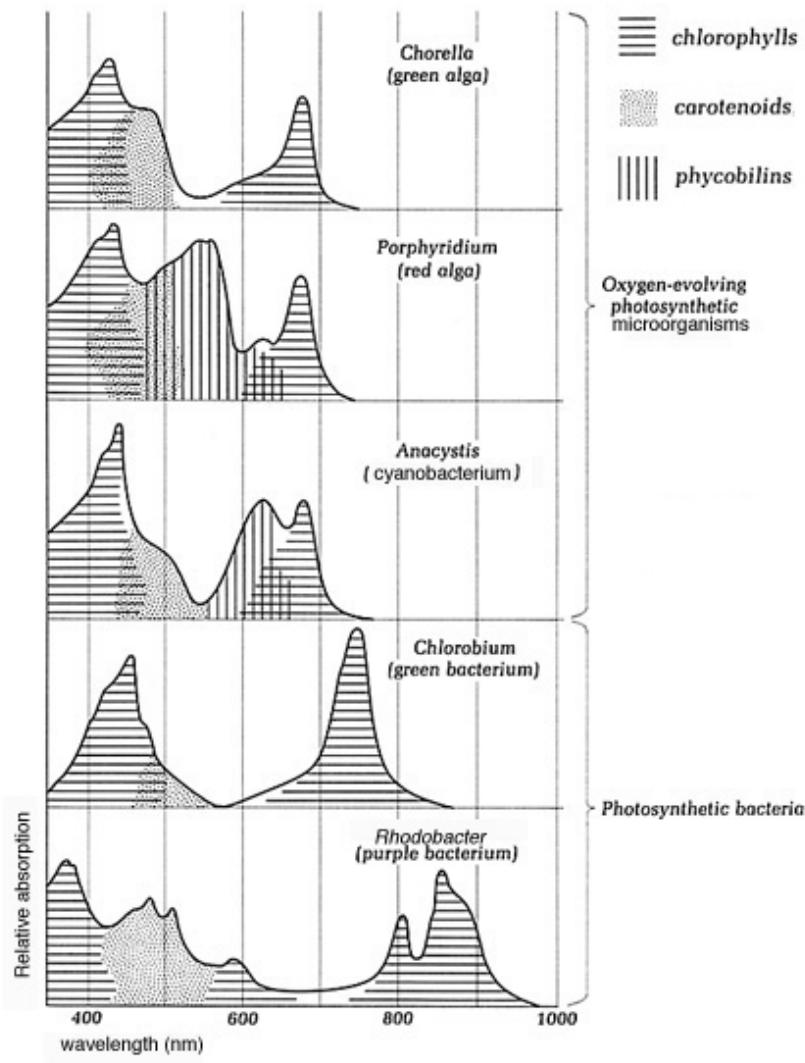


Figure 17. The distribution of photosynthetic pigments among photosynthetic microorganisms.

All phototrophic bacteria are capable of performing cyclic photophosphorylation as described above and in Figure 16 and below in Figure 18. This universal mechanism of cyclic photophosphorylation is referred to as **Photosystem I**. Bacterial photosynthesis uses only Photosystem I (PSI), but the more evolved cyanobacteria, as well as algae and plants, have an additional light-harvesting system called Photosystem II (PSII). **Photosystem II** is used to reduce Photosystem I when electrons are withdrawn from PSI for CO_2 fixation. PSII transfers electrons from H_2O and produces O_2 , as shown in Figure 20.

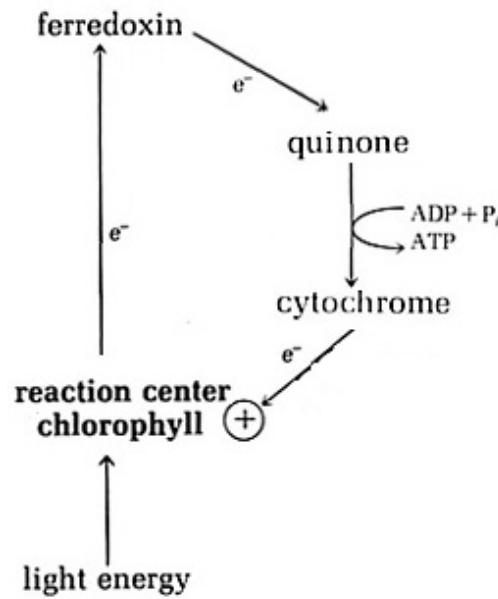


Figure 18. The cyclical flow of electrons during bacterial (anoxygenic) photosynthesis. A cluster of carotenoid and chlorophyll molecules at the Reaction Center harvests a quantum of light. A bacterial chlorophyll molecule becomes instantaneously oxidized by the loss of an electron. The light energy is used to boost the electron to a low redox intermediate, ferredoxin, (or some other iron sulfur protein) which can enter electrons into the photosynthetic electron transport system in the membrane. As the electrons traverse the ETS a proton motive force is established that is used to make ATP in the process of photophosphorylation. The last cytochrome in the ETS returns the electron to chlorophyll. Since light energy causes the electrons to turn in a cycle while ATP is synthesized, the process is called cyclic photophosphorylation. Compare bacterial photosynthesis with the scheme that operates in Photosystem I in Figure 16 above. Bacterial photosynthesis uses only Photosystem I for the conversion of light energy into chemical energy.

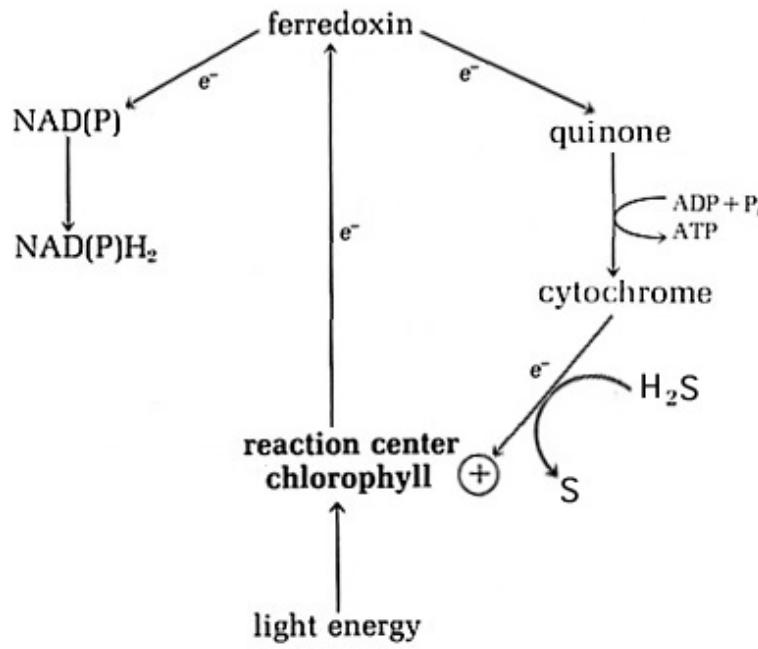


Figure 19. The normally cyclical flow of electrons during bacterial photosynthesis must be opened up in order to obtain electrons for CO_2 fixation. In the case of the purple sulfur bacteria, they use H_2S as a source of electrons. The oxidation of H_2S is coupled to PSI. Light energy boosts an electron, derived from H_2S , to the level of ferredoxin, which reduces NADP to provide electrons for autotrophic CO_2 fixation.

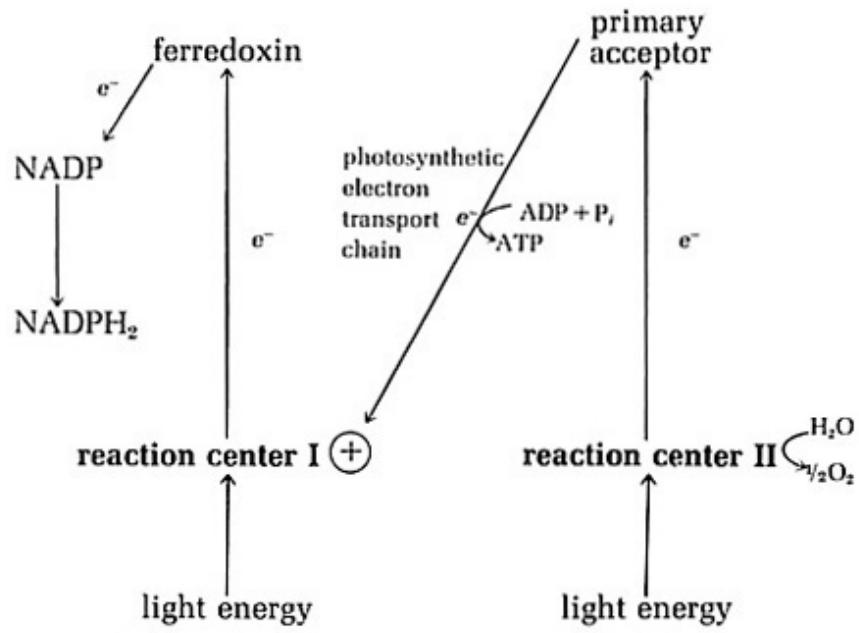


Figure 20. Electron flow in plant (oxygenic) photosynthesis. Photosystem I and the mechanisms of cyclic photophosphorylation operate in plants, algae and cyanobacteria, as they do in bacterial photosynthesis. In plant photosynthesis, chlorophyll a is the major chlorophyll species at the reaction center and the exact nature of the primary electron acceptors (X or ferredoxin) and the components of the ETS are different than bacterial photosynthesis. But the fundamental mechanism of cyclic photophosphorylation is the same. However, when electrons must be withdrawn from photosystem I (ferredoxin-- e^- -->NADP in upper left), those electrons are replenished by the operation of Photosystem II. In the Reaction Center of PSII, a reaction between light, chlorophyll and H_2O removes electrons from H_2O (leading to the formation of O_2) and transfers them to a component of the photosynthetic ETS (primary electron acceptor). The electrons are then transferred through a chain of electron carriers consisting of cytochromes and quinones until they reach shlorophyll in PSI. The resulting drop in redox potential allows for the synthesis of ATP in a process called noncyclic photophosphorylation. The operation of photosystem II is what fundamentally differentiates plant photosynthesis from bacterial photosynthesis. Photosystem II accounts for the source of reductant for CO_2 fixation (provided by H_2O), the production of O_2 , and ATP synthesis by noncyclic photophosphorylation

Most of the phototrophic prokaryotes are obligate or facultative autotrophs, which means that they are able to fix CO_2 as a sole source of carbon for growth. Just as the oxidation of organic material yields energy, electrons and CO_2 , in order to build up CO_2 to the level of cell material (CH_2O), energy (ATP) and electrons (reducing power) are required. The overall reaction for the fixation of CO_2 in the Calvin cycle is $CO_2 + 3ATP + 2NADPH_2 \text{ ----->} CH_2O + 2ADP + 2Pi + 2NADP$. The light reactions operate to produce ATP to provide energy for the dark reactions of CO_2 fixation. The dark reactions also need reductant (electrons). Usually the provision of electrons is in some way connected to the light reactions. A model for coupling the light and dark reactions of photosynthesis is illustrated in Figure 21 below.

The general scheme for finding electrons for CO_2 fixation is to open up Photosystem I and remove the electrons, eventually getting them to NADP which can donate them to the dark reaction. In bacterial photosynthesis the process may be quite complex. The electrons are removed from Photosystem I at the level of a cytochrome, then moved through an energy-consuming **reverse electron transport system** to an iron-sulfur protein, **ferredoxin**, which reduces NADP to $NADPH_2$. The electrons that replenish Photosystem I come from the oxidation of an external **photosynthetic electron donor**, which may be H_2S , other sulfur compounds, H_2 , or certain organic compounds.

In plant photosynthesis, the photosynthetic electron donor is H_2O , which is lysed by photosystem II, resulting in the production of O_2 . Electrons removed from H_2O travel through Photosystem II to Photosystem I as described in Figure 20 above. Electrons removed from Photosystem I reduce ferredoxin directly. Ferredoxin, in turn, passes the electrons to NADP.

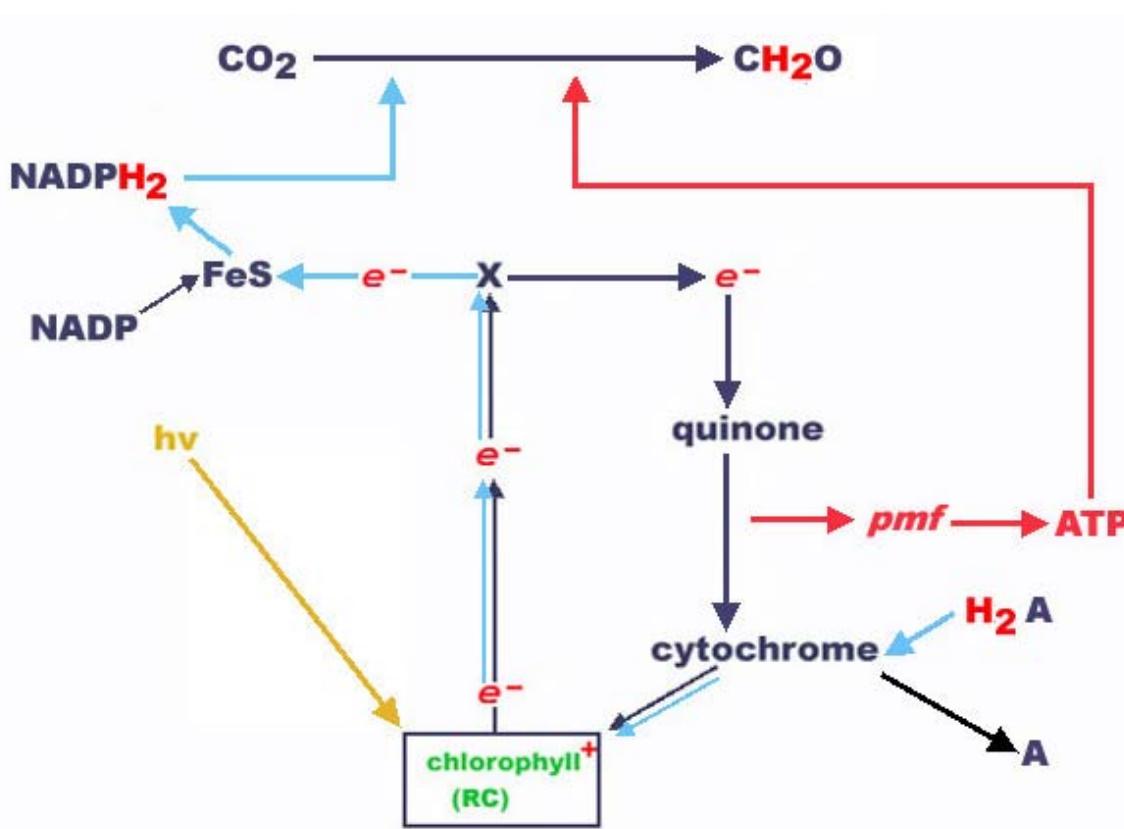


Figure 21. Model for coupling the light and dark reactions of photosynthesis.

The differences between plant and bacterial photosynthesis are summarized in Table 6 below. Bacterial photosynthesis is an anoxygenic process. The external electron donor for bacterial photosynthesis is never H_2O , and therefore, purple and green bacteria never produce O_2 during photosynthesis. Furthermore, bacterial photosynthesis is usually inhibited by O_2 and takes place in microaerophilic and anaerobic environments. Bacterial chlorophylls use light at longer wave lengths not utilized in plant photosynthesis, and therefore they do not have to compete with oxygenic phototrophs for light. Bacteria use only cyclic photophosphorylation (Photosystem I) for ATP synthesis and lack a second photosystem.

Table 6. Differences between plant and bacterial photosynthesis

	plant photosynthesis	bacterial photosynthesis
organisms	plants, algae, cyanobacteria	purple and green bacteria
type of chlorophyll	chlorophyll a absorbs 650-750nm	bacteriochlorophyll absorbs 800-1000nm
Photosystem I (cyclic photophosphorylation)	present	present
Photosystem II (noncyclic photophosphorylation)	present	absent
Produces O_2	yes	no
Photosynthetic electron donor	H_2O	H_2S , other sulfur compounds or certain organic compounds

While photosynthesis is highly-evolved in the prokaryotes, it apparently originated in the **Bacteria** and did not spread or evolve in **Archaea**. But the Archaea, in keeping with their unique ways, are not without representatives which can conduct a type of light-driven photophosphorylation. The **extreme halophiles**, archaea that live in natural

environments such as the Dead Sea and the Great Salt Lake at very high salt concentration (as high as 25 percent NaCl) adapt to the high-salt environment by the development of "purple membrane", actually patches of light-harvesting pigment in the plasma membrane. The pigment is a type of rhodopsin called **bacteriorhodopsin** which reacts with light in a way that forms a proton gradient on the membrane allowing the synthesis of ATP. This is the only example in nature of **non photosynthetic photophosphorylation**. These organisms are heterotrophs that normally respire by aerobic means. The high concentration of NaCl in their environment limits the availability of O₂ for respiration so they are able to supplement their ATP-producing capacity by converting light energy into ATP using bacteriorhodopsin.

Autotrophic CO₂ fixation

The use of **RUBP carboxylase and the Calvin cycle** is the most common mechanism for CO₂ fixation among autotrophs. Indeed, RUBP carboxylase is said to be the most abundant enzyme on the planet (nitrogenase, which fixes N₂ is second most abundant). This is the only mechanism of autotrophic CO₂ fixation among eukaryotes, and it is used, as well, by all cyanobacteria and purple bacteria. Lithoautotrophic bacteria also use this pathway. But the green bacteria and the methanogens, as well as a few isolated groups of prokaryotes, have alternative mechanisms of autotrophic CO₂ fixation and do not possess RUBP carboxylase.

RUBP carboxylase (**ribulose bisphosphate carboxylase**) uses ribulose bisphosphate (RUBP) and CO₂ as co-substrates. In a complicated reaction the CO₂ is "fixed" by addition to the RUBP, which is immediately cleaved into two molecules of 3-phosphoglyceric acid (PGA). The fixed CO₂ winds up in the -COO group of one of the PGA molecules. Actually, this is the reaction which initiates the Calvin cycle (Figure 22 below).

The Calvin cycle is concerned with the conversion of PGA to intermediates in glycolysis that can be used for biosynthesis, and with the regeneration of RUBP, the substrate that drives the cycle. After the initial fixation of CO₂, 2 PGA are reduced and combined to form hexose-phosphate by reactions which are essentially the reverse of the oxidative Embden-Meyerhof pathway. (Now is a good time to go back to Figure 8 and look at the E-M pathway for the location of PGA and glucose-phosphate). The hexose phosphate is converted to pentose-phosphate, which is phosphorylated to regenerate RUBP. An important function of the Calvin cycle is to provide the organic precursors for the biosynthesis of cell material. Intermediates must be constantly withdrawn from the Calvin cycle in order to make cell material. In this regard, the Calvin cycle is an anabolic pathway. The fixation of CO₂ to the level of glucose (C₆H₁₂O₆) requires 18 ATP and 12 NADPH₂.

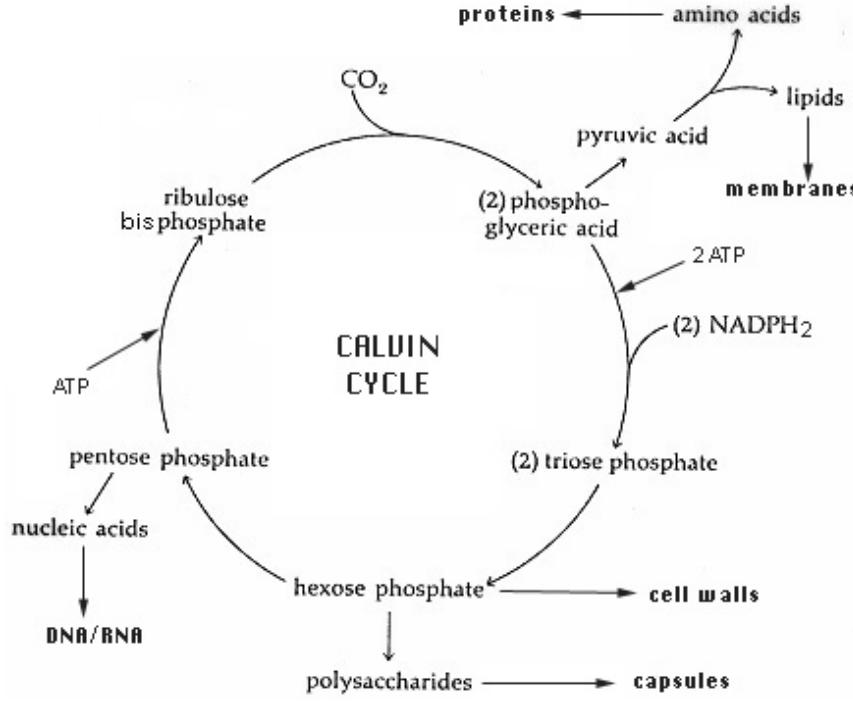


Figure 22. The Calvin cycle and its relationship to the synthesis of cell materials.

The methanogens, a very abundant group of prokaryotes, use CO_2 as a source of carbon for growth, and as a final electron acceptor in an energy-producing process that produces methane. If a methanogen is fed labeled CO_2 as a sole form of carbon, 95 percent of the label winds up in methane and 5 percent winds up in cell material. The methanogens fix CO_2 by means of the enzyme **CODH** (**carbon monoxide dehydrogenase**) and the **Acetyl CoA pathway** (Figure 23 below).

The pathway of methanogenesis steadily reduces CO_2 to the methyl (CH_3) level, mediated by the coenzyme methanopterin (MP), related to folic acid. MP- CH_3 may be reduced to methane (not shown) or the MP may be replaced by a vitamin B_{12} -like molecule to enter the pathway of CO_2 fixation. The " B_{12} "- CH_3 is substrate for CO fixation mediated by the CODH. CODH reduces CO_2 to CO and adds the CO to " B_{12} "- CH_3 to form acetyl-[CODH]. Coenzyme A (CoA) then replaces the CODH, resulting in the formation of Acetyl CoA, which is in the heart of biosynthetic metabolism. The net effect is the reduction of 2 CO_2 to Acetyl CoA.

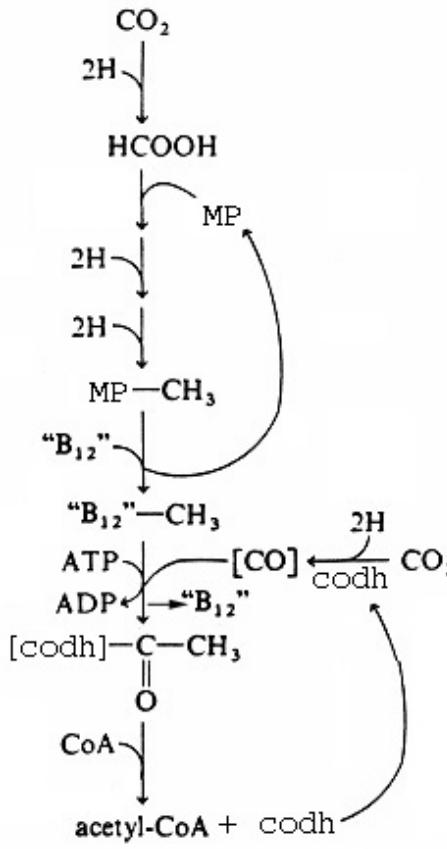


Figure 23. The CODH or acetyl CoA pathway of CO_2 fixation in the methanogens

Finally, in the photosynthetic Green Bacteria, the pathway of autotrophic CO_2 fixation involves the reversal of familiar decarboxylation reactions in and around the TCA cycle. The two primary reactions utilized by the Green Bacteria are two Ferredoxin (FD)-mediated reactions, the reduction of Acetyl CoA to pyruvate, and the reduction of succinyl CoA to alpha-ketoglutarate. This is referred to as the **reverse TCA cycle** for CO_2 uptake.

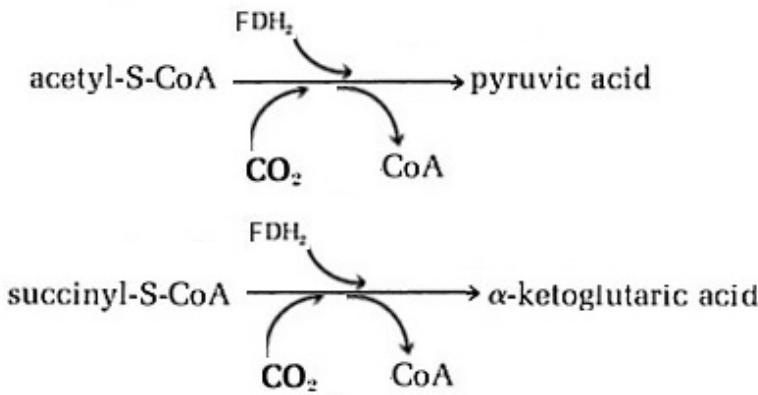


Figure 24. The two ferredoxin (FD)-mediated reactions used for CO₂ uptake in the green bacteria are a reversal of the oxidation of keto acids mediated by NAD and CoA (Figure 4).

Biosynthesis

The pathways of central metabolism (i.e., glycolysis and the TCA cycle), with a few modifications, always run in one direction or another in all organisms. The reason - these pathways provide the precursors for the biosynthesis of cell material. When a pathway, such as the Embden-Meyerhof pathway or the TCA cycle, functions to provide energy in addition to chemical intermediates for the synthesis of cell material, the pathway is referred to as an **amphibolic pathway**. Pathways of glycolysis and the TCA cycle are amphibolic pathways because they provide ATP and chemical intermediates to build new cell material. The main metabolic pathways, and their relationship to biosynthesis of cell material, are shown in Figure 25 below.

Biosynthesis or intermediary metabolism is a topic of biochemistry, more so than microbiology. It will not be dealt with in detail here. The fundamental metabolic pathways of biosynthesis are similar in all organisms, in the same way that protein synthesis or DNA structure are similar in all organisms. When biosynthesis proceeds from central metabolism as drawn below, some of the main precursors for synthesis of prokaryotic cell structures and components are as follows.

Polysaccharide capsules or inclusions are polymers of **glucose** and **other sugars**.

Cell wall peptidoglycan (NAG and NAM) is derived from **glucose phosphate**.

Amino acids for the manufacture of **proteins** have various sources, the most important of which are **pyruvic acid**, **alpha ketoglutaric acid** and **oxalacetic acid**.

Nucleotides (DNA and RNA) are synthesized from **ribose phosphate**. **ATP** and **NAD** are part of purine (nucleotide) metabolism.

Triose-phosphates are precursors of **glycerol**, and **acetyl CoA** is a main precursor of **lipids** for **membranes**

Vitamins and **coenzymes** are synthesized in various pathways that leave central metabolism. In the example given in Figure 25, **heme** synthesis proceeds from the serine pathway, as well as from succinate in the TCA cycle.

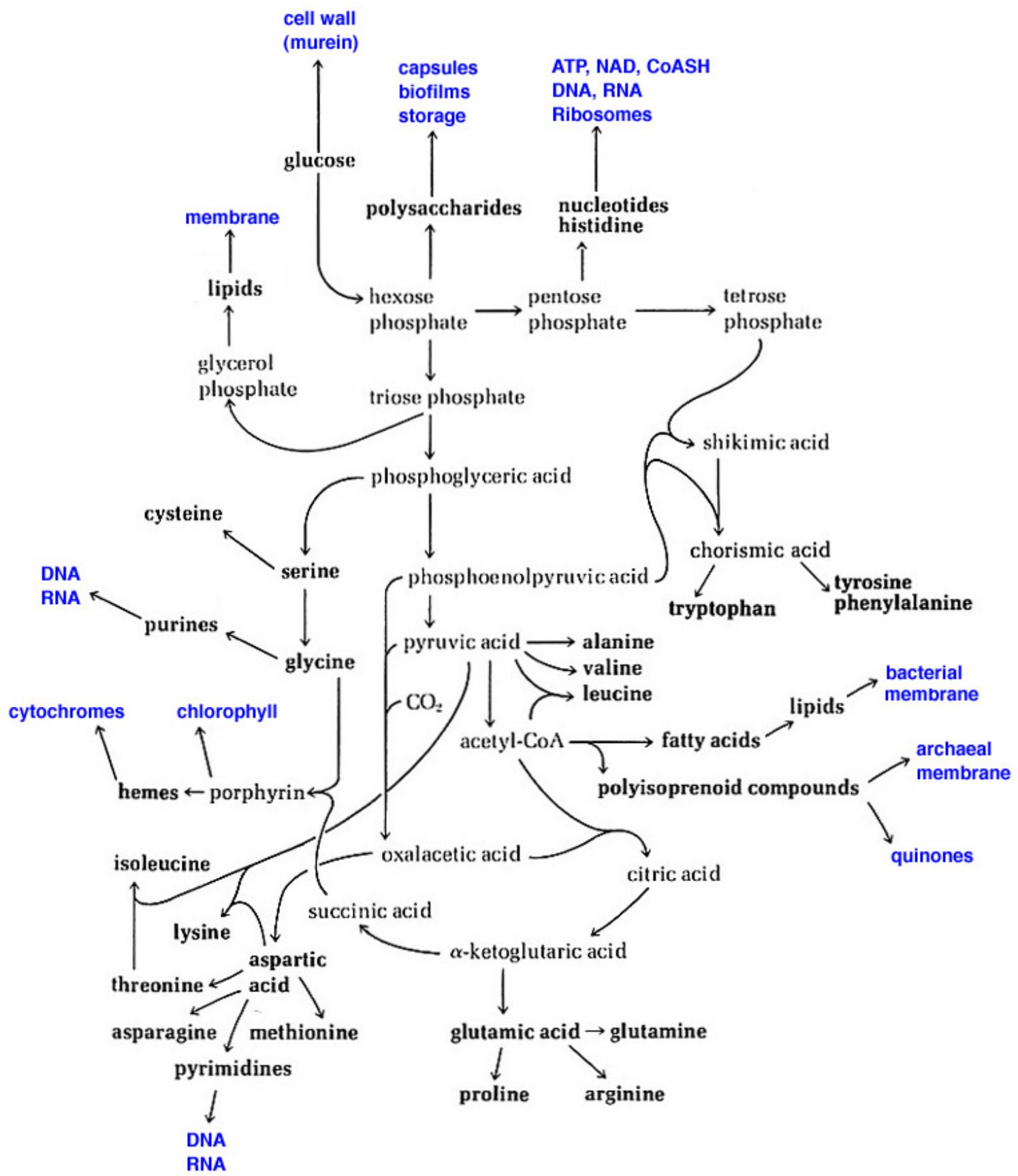
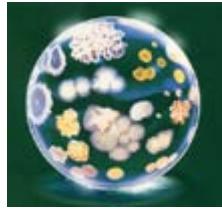


Figure 25. The main pathways of biosynthesis in prokaryotic cells

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REGULATION AND CONTROL OF METABOLIC ACTIVITY

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Adaptation to the Nutritional and Physical Environment

Unlike plant and animal cells, most bacteria are exposed to a constantly changing physical and chemical environment. Within limits, bacteria can react to changes in their environment through changes in patterns of structural proteins, transport proteins, toxins, enzymes, etc., which adapt them to a particular ecological situation. For example, *E. coli* does not produce fimbriae for colonization purposes when living in a planktonic (free-floating or swimming) environment. *Vibrio cholerae* does not produce the cholera toxin that causes diarrhea unless it is in the human intestinal tract. *Bacillus subtilis* does not make the enzymes for tryptophan biosynthesis if it can find preexisting tryptophan in its environment. If *E. coli* is fed glucose and lactose together, it will use the glucose first because it takes two less enzymes to use glucose than it does to use lactose. In *Neisseria gonorrhoeae*, the bacterium will develop a sophisticated iron gathering and transport system if it senses that iron is in short supply in its environment.

Bacteria have developed sophisticated mechanisms for the regulation of both catabolic and anabolic pathways. Generally, bacteria do not synthesize degradative (catabolic) enzymes unless the substrates for these enzymes are present in their environment. For example, synthesis of enzymes that degrade lactose would be wasteful unless the substrate for these enzymes (lactose) was available in the environment. Similarly, bacteria have developed diverse mechanisms for the control of biosynthetic (anabolic) pathways. Bacterial cells shut down biosynthetic pathways when the end products of the pathway are not needed or are readily obtained by transport from the environment. For example, if a bacterium could find a preformed amino acid like tryptophan in its environment, it would make sense to shut down its own pathway of tryptophan biosynthesis, and thereby conserve energy. However, in real bacterial life, the control mechanisms for all these metabolic pathways must be reversible, since the environment can change quickly and drastically.

Some of the common mechanisms by which bacterial cells can regulate and control their metabolic activities are discussed in this chapter. It is important for the reader to realize that most of these mechanisms have been observed or described in the bacterium, *Escherichia coli*, and they are mostly untested and unproved to exist in many other bacteria or prokaryotes (although, whenever they are looked for, they are often found). The perceptive reader will appreciate that the origins of the modern science of molecular biology are found in the experiments that explained these regulatory processes in *E. coli*.

Conditions Affecting Enzyme Formation in Bacteria

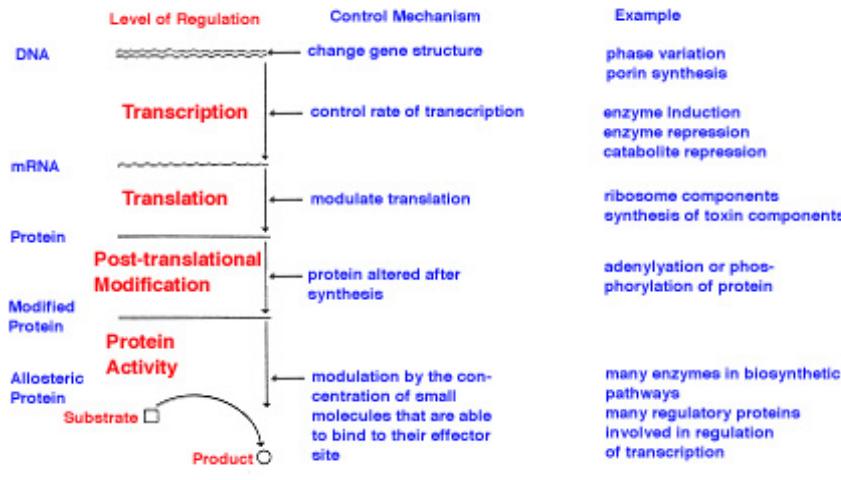
As stated above, bacterial cells can change patterns of enzymes, in order to adapt them to their specific environment. Often the concentration of an enzyme in a bacterial cell depends on the presence of the substrate for the enzyme. **Constitutive enzymes** are always produced by cells independently of the composition of the medium in which the cells are grown. The enzymes that operate during glycolysis and the TCA cycle are generally constitutive: they are present at more or less the same concentration in cells at all times. **Inducible enzymes** are produced ("turned on") in cells in response to a particular substrate; they are produced only when needed. In the process of induction the substrate, or a compound structurally similar to the substrate, evokes formation of the enzyme and is sometimes called an **inducer**. A **repressible enzyme** is one whose synthesis is downregulated or "turned off" by the presence of (for example) the end product of a pathway that the enzyme normally participates in. In this case, the end product is called a **corepressor** of the enzyme.

Regulation of Enzyme Reactions

Not all enzymatic reactions occur in a cell to the same extent. Some compounds are needed in large amounts and the reactions involved in their synthesis must therefore occur in large amounts. Other compounds are needed in small amounts and the corresponding reactions involved in their synthesis need only occur in small amounts.

In bacterial cells, enzymatic reactions may be regulated by two unrelated modes: (1) control or **regulation of enzyme activity** (**feedback inhibition** or end product inhibition), which mainly operates to regulate biosynthetic pathways; and (2) control or **regulation of enzyme synthesis** including, **end-product repression**, which functions in the regulation of biosynthetic pathways, and **enzyme induction** and **catabolite repression**, which regulate mainly degradative pathways. The process of feedback inhibition regulates the activity of preexisting enzymes in the cells. The processes of end-product repression, enzyme induction and catabolite repression are involved in the control of synthesis of enzymes. These latter processes which regulate the synthesis of enzymes may be either a form of positive control or negative control. End-product repression and enzyme induction are mechanisms of **negative control** which lead to a **decrease in the transcription** of proteins. Catabolite repression is considered a form of **positive control** because it affects an **increase in transcription** of proteins.

Table 1. Points for regulation of various metabolic processes. Bacteria exert control over their metabolism at every possible stage starting at the level of the gene that encodes for a protein and ending with alteration or modifications in the protein after it is produced. For example, variation in gene structure can vary the activity or production of a protein, just as modifications of a protein after it is produced can alter or change its activity. One of the most important sites for control of metabolism at the genetic level is regulation of transcription. At this level, in positive control mechanisms (e.g. catabolite repression), a regulatory protein has an effect to increase the rate of transcription of a gene, while in negative control mechanisms (e.g. enzyme induction or end product repression) a regulatory protein has the effect to decrease the rate of transcription of a gene. Sometimes this nomenclature may seem counter-intuitive, but molecular biologists have stuck us with it.



Allosteric Proteins

Although there are examples of regulatory processes that occur at all stages in molecular biology of bacterial cells (see Table 1 above), the most common points of regulation are at the level of transcription (e.g. enzyme induction and enzyme repression) and changing the activity of preexisting proteins. In turn, these levels of control are usually modulated by proteins with the property of **allostery**.

An **allosteric protein** is one which has an **active (catalytic) site** and an **allosteric (effector) site**. In an allosteric enzyme, the active site binds to the substrate of the enzyme and converts it to a product. The allosteric site is occupied by some small molecule which is not a substrate. However, when the allosteric site is occupied by the effector molecule, the configuration of the active site is changed so that it is now unable to recognize and bind to its substrate (Figure 1). If the protein is an enzyme, when the allosteric site is occupied, the enzyme is inactive, i.e., the effector molecule decreases the activity of the enzyme. There is an alternative situation, however. The effector molecule of certain allosteric enzymes binds to its allosteric site and consequently transforms the enzyme from an inactive to an active state (Figure 2). Some multicomponent allosteric enzymes have several sites occupied by various effector molecules that modulate enzyme activity over a range of conditions.

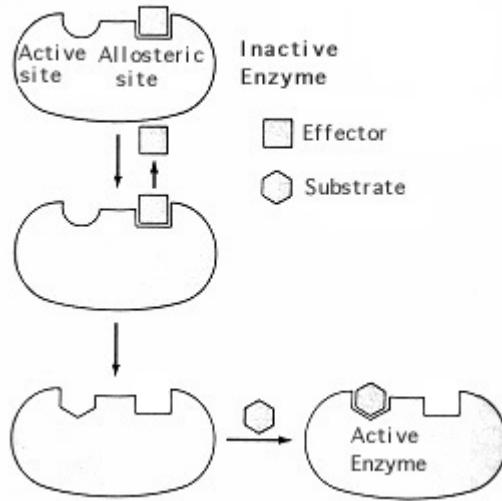


Figure 1. Example of an allosteric enzyme with a negative effector site. When the effector molecule binds to the allosteric site, substrate binding and catalytic activity of the enzyme are inactivated. When the effector is detached from the allosteric site the enzyme is active.

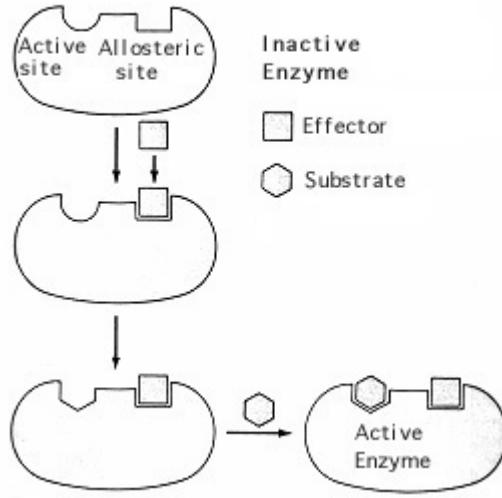


Figure 2. Example of an allosteric enzyme with a positive effector site. The effector molecule binds to the allosteric site resulting in alteration of the active site that stimulates substrate binding and catalytic activity.

Some allosteric proteins are not enzymes, but nonetheless have an active site and an allosteric site. The regulatory proteins that control metabolic pathways involving end product repression, enzyme induction and catabolite repression are allosteric proteins. In their case, the **active site is a DNA binding site**, which, when active, binds to a specific sequence of DNA, and which, when inactive, does not bind to DNA. The allosteric or effector molecule is a small molecule which can occupy the allosteric site and affect the active site. In the case of enzyme repression, a positive effector molecule (called a **corepressor**) binds to the allosteric regulatory protein and activates its ability to bind to DNA. In the case of enzyme induction a negative effector molecule (called an **inducer**) binds to the allosteric site, causing the active site to change conformation thereby detaching the protein from its DNA binding site.

Feedback Inhibition

Feedback inhibition (or end product inhibition) is a mechanism for the **inhibition of preformed enzymes** that is seen primarily in the regulation of whole biosynthetic pathways, e.g. pathways involved in the synthesis of the amino acids. Such pathways usually involve many enzymatic steps, and the final (end) product is many steps removed from the starting substrate. By this mechanism, the final product is able to feed back to the first step in the pathway and to regulate its own biosynthesis.

In feedback inhibition, the end product of a biosynthetic pathway inhibits the activity of the first enzyme that is

unique to the pathway, thus controlling production of the end product. The first enzyme in the pathway is an allosteric enzyme. Its allosteric site will bind to the end product (e.g. amino acid) of the pathway which alters its active site so that it cannot mediate the enzymatic reaction which initiates the pathway. Other enzymes in the pathway remain active, but they do not see their substrates. The pathway is shut down as long as adequate amounts of the end product are present. If the end product is used up or disappears, the inhibition is relieved, the enzyme regains its activity, and the organism can resume synthesis of the end product. Thus, if a *E. coli* bacterium swims out of a glucose minimal medium into milk or some other medium rich in growth factors, the bacterium can stop synthesizing any of the essential metabolites that are made available directly from the new environment.

One of the most intensely studied bacterial pathways is the pathway of tryptophan biosynthesis (Figure 3). The pathway of tryptophan biosynthesis is regulated by feed back inhibition. Tryptophan is the effector molecule for allosteric enzyme a. When the end product of the pathway (tryptophan) attaches to enzyme a, the enzyme is inactive and can no longer join glutamine and chorismic acid into anthranilate. If tryptophan is disjoined from the enzyme the pathway is resumed, and tryptophan synthesis will continue. Tryptophan biosynthesis is also regulated at a genetic level by the processes of enzyme repression (below) and attenuation.

Note: In the case of feedback inhibition (above), the signal molecule, tryptophan, is a negative effector of Enzyme a in the pathway of tryptophan biosynthesis, because when it binds to Enzyme a, it inactivates the enzyme. In enzyme repression (below) tryptophan is a signal molecule that acts as a positive effector of the trp repressor protein because when it binds to the repressor it activates the protein, so that it binds to the trp DNA.

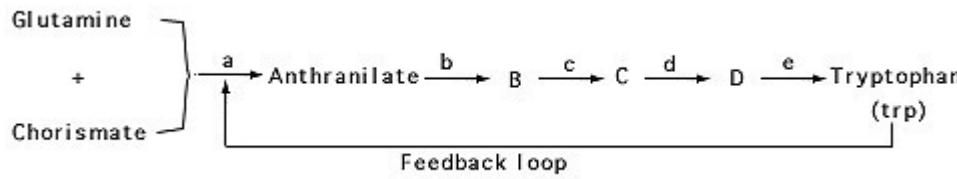


Figure 3. The pathway of tryptophan biosynthesis in *E. coli*. The pathway is regulated by the process of feedback inhibition. Tryptophan (trp), the end product of the pathway, is the effector molecule that binds to the allosteric site of Enzyme a, the first enzyme in the pathway. When trp is bound to the enzyme the catalytic (active) site of Enzyme a is altered so that it is unable to react with its substrates and the synthesis of anthranilate is inhibited.

If a metabolic pathway branches, leading to the synthesis of two amino acids, each end product (amino acid) can control its own synthesis without affecting the other (Figure 4). For example, the amino acids proline and arginine are both synthesized from glutamic acid. Each amino acid can regulate the first enzyme unique to its own synthesis without affecting the other, so that a surplus of arginine, for example, will not shut off the synthesis of proline.

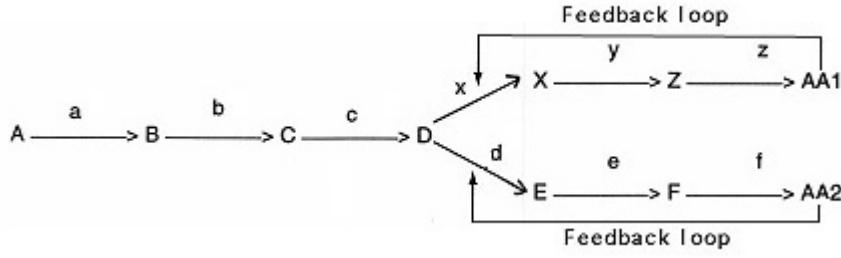


Figure 4. Generalized scheme for regulation of a branched metabolic pathway by the process of feedback inhibition.

Enzyme Repression

Enzyme repression is a form of **negative control** (down-regulation) of bacterial transcription. This process, along with that of enzyme induction, is called **negative control** because a regulatory protein brings about **inhibition** of mRNA synthesis which leads to **decreased** synthesis of enzymes.

Although feedback inhibition shuts off synthesis of the end product of a pathway, it still allows some waste of energy and carbon if the cell continued to manufacture enzymes for which it has no use. It is the process of enzyme repression that **prevents the synthesis of the enzymes concerned with the synthesis of that particular end product**. In the case of the pathway of tryptophan biosynthesis (Figure 3), the end product of the pathway, tryptophan, serves as an effector molecule that can shutdown the synthesis of the Enzymes a, b, c, d, and e that are

concerned with tryptophan biosynthesis. This results in saving of many molecules of ATP which must be expended during protein synthesis, and it conserves amino acid precursors for synthesis of other proteins. The process is slower to act than is feedback inhibition (which acts immediately) because pre-existing enzymes have to be diluted out as a result of cell division before its effects are seen.

The genes for tryptophan biosynthesis in *Escherichia coli* are organized on the bacterial chromosome in the **tryptophan operon (trp operon)**. An operon is a cluster of genes that are controlled by the same elements and which are coordinately transcribed and translated. The trp operon consists of a Promoter (P) region, an Operator (O) region, an Attenuator (A) region, and the five structural genes for the enzymes involved in tryptophan biosynthesis (Trp A-E). The components of the trp operon and its control elements are described in Figure 5 and Table 2 below.

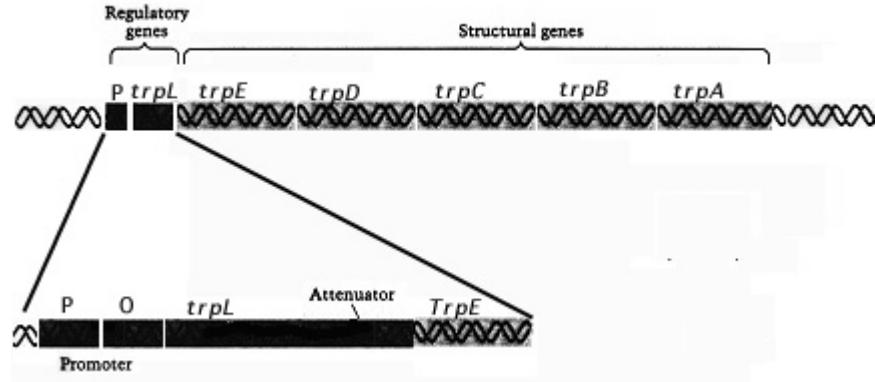


Figure 5. Genetic organization of the Trp operon and its control elements

Table 2. The Trp operon and its control elements

R = Regulatory gene that encodes for the trp Repressor protein that is concerned with regulating the synthesis of the 5 gene products. An active repressor binds to a specific nucleotide sequence in the operator region and thereby blocks binding of RNAP to the promoter to initiate transcription.

O = Operator specific nucleotide sequence on DNA to which an active Repressor binds.

P = Promoter specific nucleotide sequence on DNA to which RNA polymerase binds to initiate transcription. If the repressor protein binds to the operator, RNAP is prevented from binding with the promoter and initiating transcription. Therefore, none of the enzymes concerned with tryptophan biosynthesis are synthesized.

A = Attenuator DNA sequence which lies between the operator and the structural genes for trp biosynthesis. The attenuator is a barrier that RNA polymerase must traverse if it is to transcribe the genes for tryptophan biosynthesis. In the presence of trp, most RNAP molecules fall off the DNA before transcribing the trp genes. In the absence of trp, RNAP is able to traverse the attenuator region to successfully transcribe the trp genes.

Trp A, B, C, D, E = Trp Genes structural genes for enzymes involved in tryptophan biosynthesis.

Trp = tryptophan end product of the tryptophan biosynthetic pathway. When combined with the repressor protein the Repressor is active. Trp is called a corepressor.

The trp operon is regulated by a regulatory gene (Trp L) upstream of the trp promoter. The product of the R (L) gene, the trp Repressor, is an allosteric protein which is regulated by tryptophan. The Repressor is produced constitutively in small amounts in an inactive form. When the Repressor combines with tryptophan it becomes activated and binds to the DNA of the trp operon in such a way that it blocks the transcription of the structural genes for tryptophan. Thus, in the presence of tryptophan, transcription of the genes for tryptophan biosynthesis is repressed (tryptophan is not produced), while in the absence of tryptophan, the genes for tryptophan biosynthesis can be transcribed (tryptophan is produced); See Figure 6 below.

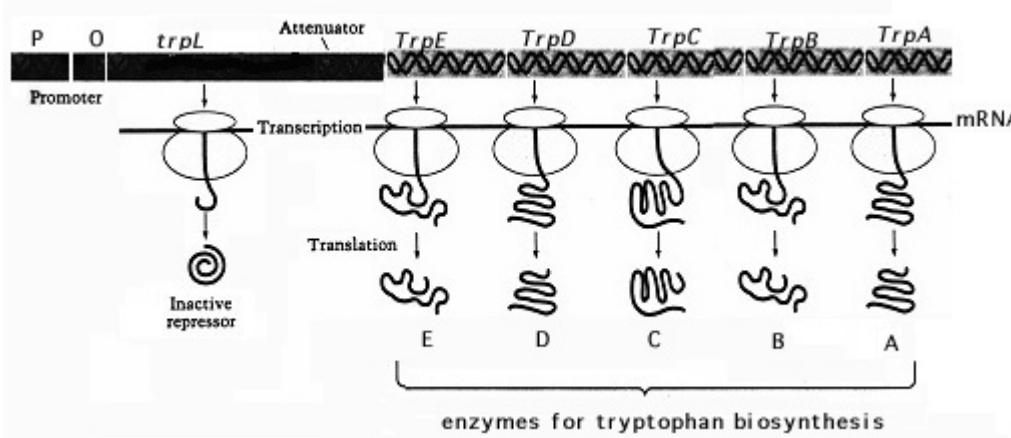


Figure 6a. Derepression of the trp operon. In the absence of trp the inactive repressor cannot bind to the operator to block transcription. The cell must synthesize the amino acid.

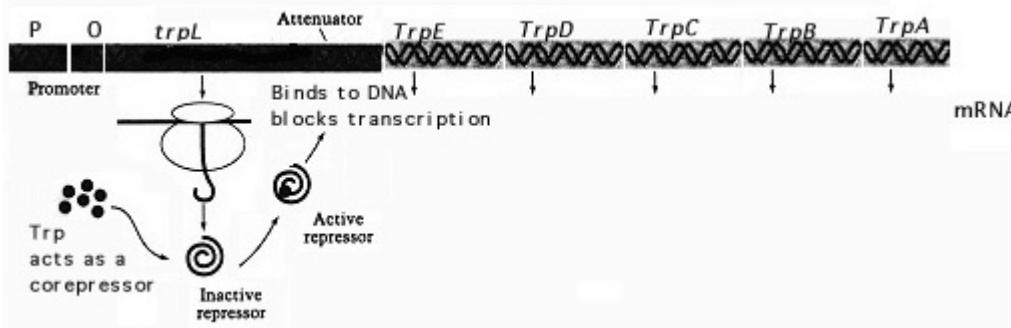


Figure 6b. Repression of the trp operon. In the presence of tryptophan the trp operon is repressed because trp activates the repressor. Transcription of is blocked because the active repressor binds to the DNA and prevents binding of RNA polymerase.

Enzyme Induction

In some cases, metabolites or substrates can turn on inactive genes so that they are transcribed. In the process of **enzyme induction**, the substrate or a compound structurally similar to the substrate, evokes the formation of enzyme(s) which are usually involved in the degradation of the substrate. Enzymes that are synthesized as a result of genes being turned on are called **inducible enzymes** and the substance that activates gene transcription is called the **inducer**. Inducible enzymes are produced only in response to the presence of a their substrate and, in a sense, are produced only when needed. In this way the cell does not waste energy synthesizing unneeded enzymes.

The best known and best studied case of enzyme induction involves the enzymes of lactose degradation in *E. coli*. Only in the presence of lactose does the bacterium synthesize the enzymes that are necessary to utilize lactose as a carbon and energy source for growth. Two enzymes are required for the initial breakdown of lactose: **lactose permease**, which actively transports the sugar into the cell, and **beta galactosidase**, which splits lactose into glucose plus galactose. The genes for these enzymes are contained within the **lac operon** (lac operon) in the bacterial chromosome (Figure 7).

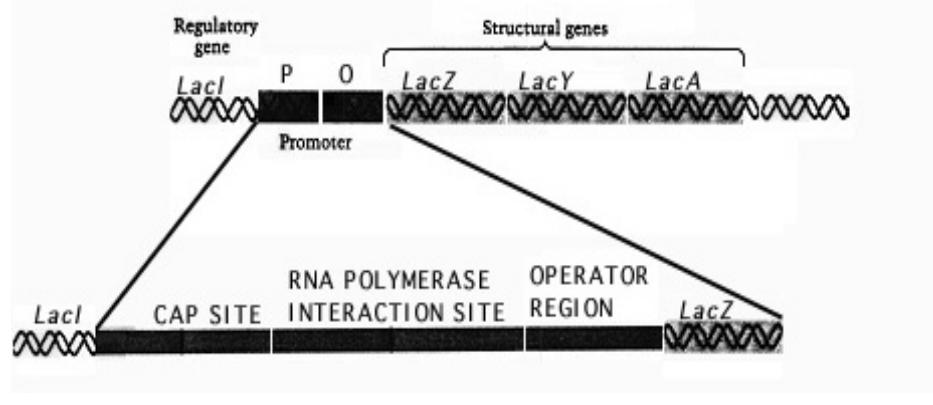


Figure 7. The Lac operon and its control elements

The mechanism of enzyme induction is similar to end product repression in that a regulatory gene, a promoter, and an operator are involved, but a major difference is that the **lac Repressor is active only in the absence of the inducer molecule (lactose)**. In the presence of lactose, the Repressor cannot bind to the operator region, so that the genes for lactose transport and cleavage are transcribed. In the absence of lactose, the Repressor is active and will bind to the operator with the result that the genes for lactose metabolism are not transcribed. The induction (presence of lactose) and the repression (absence of lactose) of the lac operon is represented in Figure 8 . The function of the components and control elements are shown in Table 3.

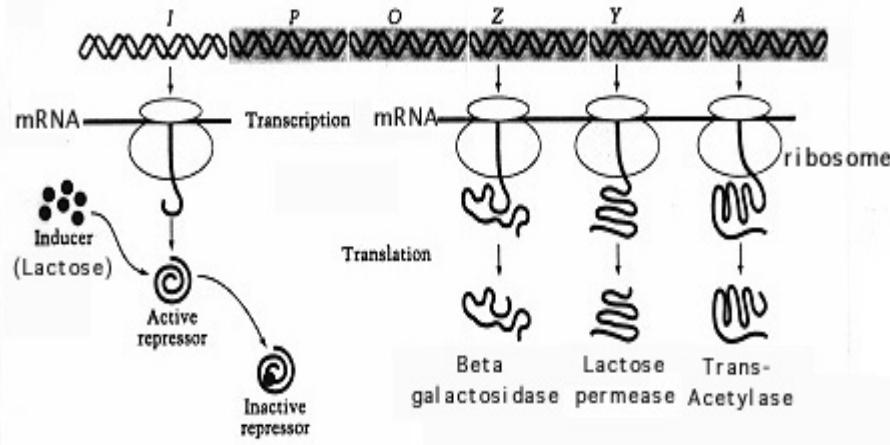


Figure 8. Enzyme Induction. Induction (or derepression) of the lac operon.

Table 3. Functional and regulatory components of the lac operon

Lac R = Regulatory gene (also known as Lac I) gene that encodes for the lac Repressor protein that is concerned with regulating the synthesis of the structural genes in the operon. **Lac I** is adjacent to the Promoter site of the operon. An active repressor binds to a specific nucleotide sequence in the operator region and thereby blocks binding of RNAP to the promoter to initiate transcription. The lac repressor is inactivated by lactose, and is active in the absence of lactose.

O = Operator specific nucleotide sequence on DNA to which an active Repressor binds.

P = Promoter specific nucleotide sequence on DNA to which RNA polymerase binds to initiate transcription. (The promoter site of the lac operon is further divided into two regions, an upstream region called the CAP site, and a downstream region consisting of the RNAP interaction site. The CAP site is involved in catabolite repression of the lac operon.). If the Repressor protein binds to the operator, RNAP is prevented from binding with the promoter and initiating transcription. Under these conditions the enzymes concerned with lactose utilization are not synthesized.

Lac Z, Y and A = Structural Genes in the lac operon. Lac Z encodes for Beta-galactosidase; Lac Y encodes the lactose permease; Lac A encodes a transacetylase whose function is not known.

lac = lactose the inducer molecule. When lactose binds to the Repressor protein, the Repressor is inactivated; the operon is derepressed; the transcription of the genes for lactose utilization occurs.

Catabolite Repression

Enzyme Induction is still considered a form of negative control because the effect of the regulatory molecule (the active repressor) is to decrease or downregulate transcription. **Catabolite repression** is a type of **positive control of transcription**, since a regulatory protein affects an **increase** (upregulation) in the rate of transcription of an operon. The process was discovered in *E. coli* and was originally referred to as the **glucose effect** because it was found that **glucose repressed the synthesis of certain inducible enzymes**, even though the inducer of the pathway was present in the environment. The discovery was made during study of the regulation of lac operon in *E. coli*. Since glucose is degraded by constitutive enzymes and lactose is initially degraded by inducible enzymes, what would happen if the bacterium was grown in limiting amounts of glucose and lactose? A plot of the bacterial growth rate resulted in a **diauxic growth curve** which showed two distinct phases of active growth (Figure 9). During the first phase of exponential growth, the bacteria utilize glucose as a source of energy until all the glucose

is exhausted. Then, after a secondary lag phase, the lactose is utilized during a second stage of exponential growth.

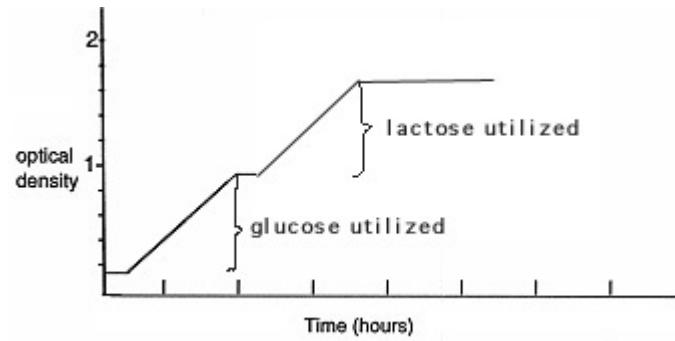


Figure 9. The Diauxic Growth Curve of *E. coli* grown in limiting concentrations of a mixture of glucose and lactose

During the period of glucose utilization, lactose is not utilized because the cells are unable to transport and cleave the disaccharide lactose. Glucose is always metabolized first in preference to other sugars. Only after glucose is completely utilized is lactose degraded. The lac operon is repressed even though lactose (the inducer) is present. The ecological rationale is that glucose is a better source of energy than lactose since its utilization requires two less enzymes.

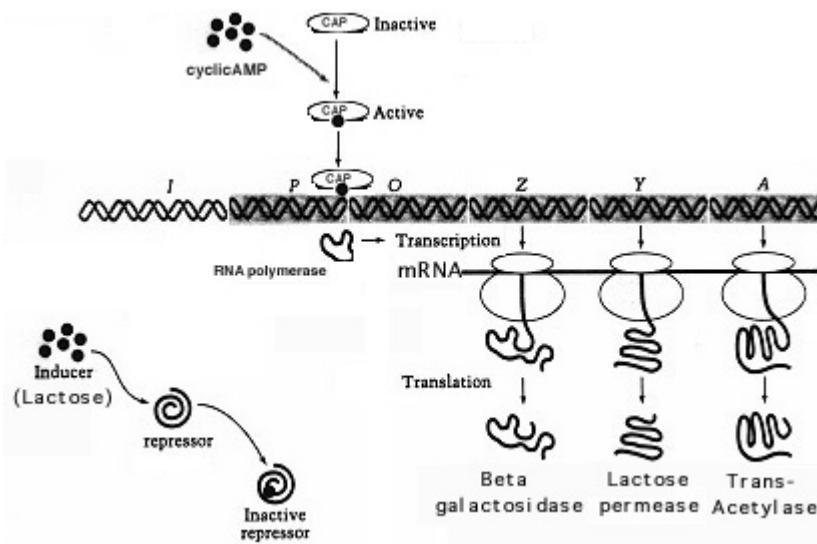
Only after glucose is exhausted are the enzymes for lactose utilization synthesized. The secondary lag during diauxic growth represents the time required for the complete induction of the lac operon and synthesis of the enzymes necessary for lactose utilization (lactose permease and beta-galactosidase). Only then does bacterial growth occur at the expense of lactose. Since the availability of **glucose represses the enzymes for lactose utilization**, this type of repression became known as **catabolite repression** or the **glucose effect**.

Glucose is known to repress a large number of inducible enzymes in many different bacteria. Glucose represses the induction of inducible operons by inhibiting the synthesis of **cyclic AMP (cAMP)**, a nucleotide that is required for the initiation of transcription of a large number of inducible enzyme systems including the lac operon.

The role of cyclic cAMP is complicated. cAMP is required to activate an allosteric protein called **CAP (catabolite activator protein)** which binds to the promoter CAP site and stimulates the binding of RNAP polymerase to the promoter for the initiation of transcription. Thus to efficiently promote gene transcription of the lac operon, not only must lactose be present to inactivate the lac repressor, but cAMP must be available to bind to CAP which binds to DNA to facilitate transcription. **In the presence of glucose**, adenylate cyclase (AC) activity is blocked. AC is required to synthesize cAMP from ATP. Therefore, if cAMP levels are low, CAP is inactive and **transcription does not occur**. **In the absence of glucose**, cAMP levels are high, CAP is activated by cAMP, and **transcription occurs** (in the presence of lactose).

Many positively controlled promoters, such as the lac promoter, are not fully functional in the presence of RNAP alone and require activation by CAP. CAP is encoded by a separate Regulatory gene, and is present in constitutive levels. CAP is active only in the presence of cAMP. The binding of cAMP to CAP causes a conformational change in the protein allowing it to bind to the promoter near the RNAP binding site. CAP can apparently interact with RNAP to increase operon transcription about 50-fold. Positive control of the lac operon is illustrated in Figure 10.

Figure 10. Catabolite repression is positive control of the lac operon. The effect is an increase in the rate of transcription. In this case, the CAP protein is activated by cAMP to bind to the lac operon and facilitate the binding of RNA polymerase to the promoter to transcribe the genes for lactose utilization.



As a form of catabolite repression, the glucose effect serves a useful function in bacteria: it requires the cells to use the best available source of energy. For many bacteria, glucose is the most common and readily utilizable substrate for growth. Thus, it inhibits indirectly the synthesis of enzymes that metabolize poorer sources of energy.

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PROCARYOTES IN THE ENVIRONMENT

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The most significant effect that prokaryotes have on their environment is their underlying ability to recycle the essential elements that make up cells. The earth is a closed system with limited amounts of carbon, oxygen and nitrogen available to all forms of life. These essential elements must be converted from one form to another and shared among all living organisms. The total biomass of microbial cells in the biosphere, their metabolic diversity and their persistence in all habitats that support life, guarantee that microbes will play crucial roles in the transformations and recycling of these elements.

The table below lists the major elements that make up a typical prokaryotic cell (in this case, *E. coli*). As expected, over 90 percent of the elemental analysis consists of carbon, hydrogen, oxygen, nitrogen, phosphorus and sulfur. These are the elements that become combined to form all the biochemicals that comprise living systems. C, H, O, N, P and S are the constituents of organic material (An organic compound is a chemical that contains a carbon to hydrogen bond. Organic compounds on earth are evidence of life. Organic compounds may be symbolized as CH₂O, which is the empirical formula for a sugar such as glucose.) H and O are the constituents of water (H₂O), that makes up over 95 percent of the cell composition. Calcium (Ca⁺⁺), iron (Fe⁺⁺), magnesium (Mg⁺⁺) and potassium (K⁺) are present as inorganic salts in the cytoplasm of cells.

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

Element	% of dry weight	Source	Function
Carbon	50	organic compounds or CO ₂	Main constituent of cellular material
Oxygen	20	H ₂ O, 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 ₂ O, organic compounds, H ₂	Main constituent of organic compounds and cell water
Phosphorus	3	inorganic phosphates (PO ₄)	Constituent of nucleic acids, nucleotides, phospholipids, LPS, teichoic acids
Sulfur	1	SO ₄ , H ₂ S, S, 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

The table ignores the occurrence of "trace elements" in cells. **Trace elements** are metal ions required in cellular nutrition in such small amounts that it is difficult to determine their presence in cells. The usual metals that qualify as trace elements are Mn⁺⁺, Co⁺⁺, Zn⁺⁺, Cu⁺⁺ and Mo⁺⁺. Trace elements are usually built into vitamins and enzymes. For example, vitamin B₁₂ contains cobalt (Co⁺⁺) and the bacterial nitrogenase enzyme contains molybdenum (Mo⁺⁺).

The structure and metabolism of any organism adapts it to its environment. Thus the various groups of microbes are adapted to certain environmental niches based on their predominant type of metabolism relevant to the elemental nutrients available.

The **fungi** (molds and yeasts). The molds are aerobic organisms that utilize organic compounds for growth. They play an important role in decomposition or biodegradation of organic matter, particularly in soil. Yeasts can grow anaerobically (without oxygen) through the process of fermentation. They play a role in fermentations in environments high in sugar. The prominent role of fungi in the environment is in the carbon cycle, during the process of decomposition, especially in the soil.

The **algae** are also an important part of the carbon cycle. They are the predominant photosynthetic organisms in many aquatic environments. The algae are **autotrophs**, which means they use carbon dioxide (CO₂) as a source of carbon for growth. Hence they convert atmospheric CO₂ into organic material (i.e., algal cells). Algae also play a role in the oxygen (O₂) cycle since their style of photosynthesis, similar to plants, produces O₂ in the atmosphere. The **cyanobacteria** are a group of prokaryotic microbes, as prevalent as algae, that have this type of metabolism. Photosynthetic algae and cyanobacteria can be found in most environments where there is moisture and light. They are a major component of marine plankton which is the basis of the food chain in the oceans.

Protozoans are heterotrophic organisms that have to catch or trap their own food. Therefore, they have developed elaborate mechanisms for movement and acquiring organic food which they can digest. Their food usually turns out to be bacterial cells, so one might argue that they are ecological predators that keep bacterial populations under control....in soil, aquatic environments, intestinal tracts of animals, and many other environments.

The prokaryotic **bacteria** and **archaea**, as a result of their diversity and unique types of metabolism, are involved in the cycles of virtually all essential elements. In two cases, methanogenesis (conversion of carbon dioxide into methane) and nitrogen fixation (conversion of nitrogen in the atmosphere into biological nitrogen) are unique to prokaryotes and earn them their "essential role" in the carbon and nitrogen cycles.

There are other metabolic processes that are unique, or nearly so, in the prokaryotes that bear significantly on the cycles of elements. For example, prokaryotes called **lithotrophs** use inorganic compounds like ammonia and hydrogen sulfide as a source of energy, and others called **anaerobic respirers** use nitrate (NO₃) or sulfate (SO₄) in the place of oxygen, so they can respire without air. Most of the archaea are lithotrophs that use hydrogen (H₂) or hydrogen sulfide (H₂S) as a source of energy, while many soil bacteria are anaerobic respirers that can use their efficient respiratory metabolism in the absence of O₂.

The basic processes of heterotrophy are spread throughout the bacteria. Most of the bacteria in the soil and water, and in associations with animals and plants, are heterotrophs. **Heterotrophy** means living off of dead organic matter, usually by some means of respiration (same as animals) or fermentation (same as yeast or lactic acid bacteria). Bacterial heterotrophs in the carbon chain are important in the processes of biodegradation and decomposition under aerobic and anaerobic conditions.

In bacteria, there is a unique type of photosynthesis that does not use H₂O or produce O₂ which impacts on the carbon and sulfur cycles.

Meanwhile, the cyanobacteria (mentioned above) fix CO₂ and produce O₂ during photosynthesis, and they make a very large contribution to the carbon and oxygen cycles.

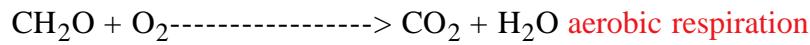
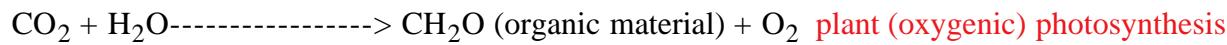
The list of examples of microbial involvement in the cycles of elements that make up living systems is endless, and

probably every microbe in the web is involved in an intimate and unique way.

The Oxygen Cycle

Basically, O₂ is derived from the photolysis of H₂O during **plant (oxygenic) photosynthesis**. Two major groups of microorganisms are involved in this process, the eukaryotic algae, and the prokaryotic cyanobacteria (formerly known as "blue-green algae"). The cyanobacteria and algae are the source of much of the O₂ in the earth's atmosphere. Of course, plants account for some O₂ production as well, but the microbes predominate in marine habitats which cover the majority of the planet.

Since most aerobic organisms need the O₂ that results from plant photosynthesis, this establishes a relationship between plant photosynthesis and aerobic respiration, two prominent types of metabolism on earth. Photosynthesis produces O₂ needed for aerobic respiration. Respiration produces CO₂ needed for autotrophic growth.

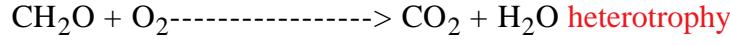


Since these photosynthetic microbes are also autotrophic (meaning they convert CO₂ to organic material during growth) they have a similar impact on the carbon cycle (below).

The Carbon Cycle

Carbon is the backbone of all organic molecules and is the most prevalent element in cellular (organic) material. In its most oxidized form, CO₂, it can be viewed as an "inorganic" molecule (no C - H bond). **Autotrophs**, which include plants, algae, photosynthetic bacteria, lithotrophs, and methanogens, use CO₂ as a sole source of carbon for growth, which reduces the molecule to organic cell material (CH₂O). **Heterotrophs** require organic carbon for growth, and ultimately convert it back to CO₂.

Thus, a relationship between autotrophs and heterotrophs is established wherein autotrophs fix carbon needed by heterotrophs, and heterotrophs produce CO₂ used by the autotrophs.



Since CO₂ is the most prevalent greenhouse gas in the atmosphere, it isn't good if these two equations get out of balance (i.e. heterotrophy predominating over autotrophy, as when rain forests are destroyed and replaced with cattle).

Autotrophs are referred to as **primary producers** at the "bottom of the food chain" because they convert carbon to a form required by heterotrophs. Among prokaryotes, the cyanobacteria, the lithotrophs and the methanogens are a formidable biomass of autotrophs that account for a corresponding amount of CO₂ fixation in the global carbon cycle.

The lithotrophic bacteria and archaea that oxidize reduced N and S compounds and play important roles in the natural cycles of N and S (discussed below), are virtually all autotrophs. The prevalence of these organisms in sulfur-rich environments (marine sediments, thermal vents, hot springs, endosymbionts, etc. may indicate an unappreciated role of these prokaryotes as primary producers of organic carbon on earth.

The **methanogens** play a dual role in the carbon cycle. These archaea are inhabitants of virtually all anaerobic environments in nature where CO₂ and H₂ (hydrogen gas) occur. They use CO₂ in their metabolism in two distinct ways. About 5 percent of CO₂ taken up is reduced to cell material during autotrophic growth; the remaining 95

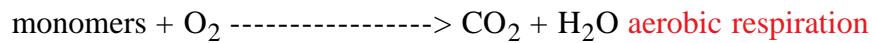
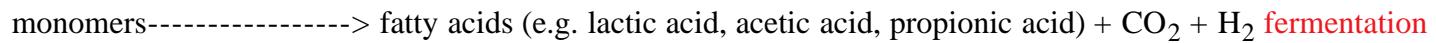
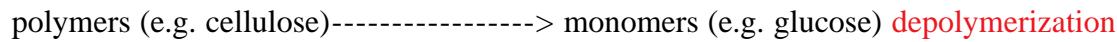
percent is reduced to CH_4 (methane gas) during a unique process of generating cellular energy. Hence, methane accumulates in rocks as fossil fuel ("natural gas"), in the rumen of cows and guts of termites, in sediments, swamps, landfills and sewage digestors. Since CH_4 is the second-most prevalent of the greenhouse gases, it is best to discourage processes that lead to its accumulation in the atmosphere.



Under aerobic conditions, methane and its derivatives (methanol, formaldehyde, etc.) can be oxidized as energy sources by bacteria called **methylotrophs**. Metabolically this is a version of decomposition or biodegradation during the carbon cycle which is discussed below.

Biodegradation is the process in the carbon cycle for which microbes get most credit (or blame). **Biodegradation** is the **decomposition** of organic material (CH_2O) back to $\text{CO}_2 + \text{H}_2\text{O}$ and H_2 . In soil habitats, the fungi play a significant role in biodegradation, but the prokaryotes are equally important. The typical decomposition scenario involves the initial degradation of biopolymers (cellulose, lignin, proteins, polysaccharides) by extracellular enzymes, followed by oxidation (fermentation or respiration) of the monomeric subunits. The ultimate end products are CO_2 , H_2O and H_2 , perhaps some NH_3 (ammonia) and sulfide (H_2S), depending on how one views the overall process. These products are scarfed up by lithotrophs and autotrophs for recycling. Prokaryotes which play an important role in biodegradation in nature include the actinomycetes, clostridia, bacilli, arthrobacters and pseudomonads.

Overall Process of Biodegradation (Decomposition)



The importance of microbes in biodegradation is embodied in the adage that "there is no known natural compound that cannot be degraded by some microorganism." The proof of the adage is that we aren't up to our ears in whatever it is that couldn't be degraded in the last 3.5 billion years. Actually, we are up to our ears in cellulose and lignin, which is better than concrete, and some places are getting up to their ears in teflon, plastic, styrofoam, insecticides, pesticides and poisons that are degraded slowly by microbes, or not at all.

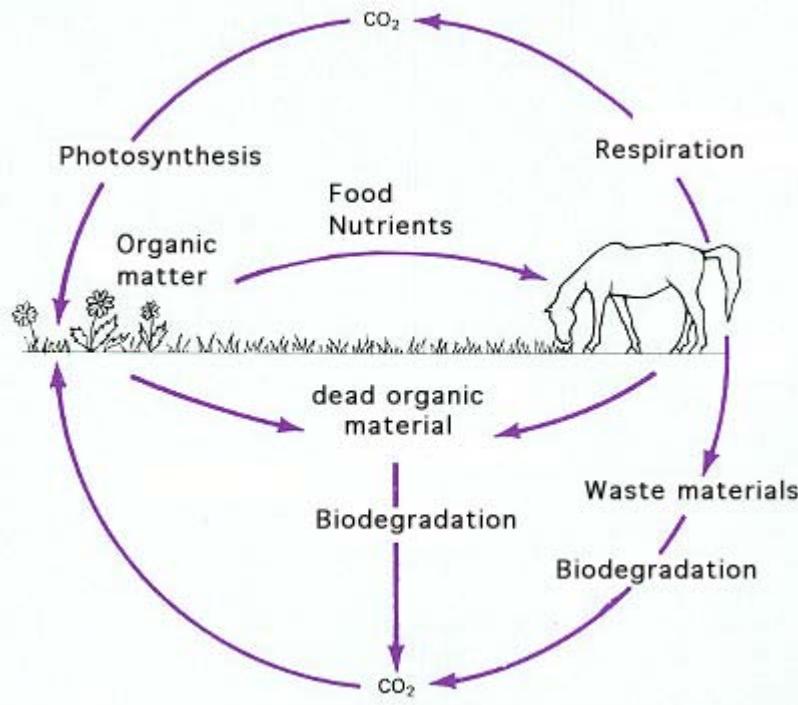


Figure 1. The Carbon Cycle. Organic matter (CH_2O) derived from photosynthesis (plants, algae and cyanobacteria) provides nutrition for heterotrophs (e.g. animals and associated bacteria), which convert it back to CO_2 . Organic wastes, as well as dead organic matter

in the soil and water, are ultimately broken down to CO₂ by microbial processes of biodegradation.

OFF THE WALL

The figure above mostly ignores the role of methanogenesis in the carbon cycle. Since methanogens have the potential to remove CO₂ from the atmosphere, converting it to cell material and CH₄, these prokaryotes not only influence the carbon cycle, but their metabolism also affects the concentration of major greenhouse gases in earth's atmosphere.

Recently, I asked a colleague, Professor Paul Weimer of the University of Wisconsin Department of Bacteriology, whether methanogenesis which utilizes CO₂ while producing CH₄ was better or worse on the greenhouse effect. This is his response.

"Worse. For methanogenesis by CO₂ reduction, the stoichiometry is 4H₂ + CO₂ → CH₄ + 2 H₂O, so one mole of a greenhouse gas is exchanged for another. But methane is about 15 times more potent than is CO₂ in terms of heat absorption capability on a per-molecule basis, so the net effect is a functional increase in heat absorption by the atmosphere. Remember also that in most natural environments, around two-thirds of the methane is produced by aceticlastic methanogenesis (CH₃COOH → CH₄ + CO₂) - an even less welcome situation, as BOTH products are greenhouse gases."

Even though methane concentrations in the atmosphere are two orders of magnitude below those of CO₂, methane is thought to account for about 15% of the anthropogenic climate forcing, compared to about 60% from CO₂. Most of the rest of the contribution is from nitrous oxide (N₂O, a respiratory denitrification product that has something like 300 times the heat absorbing capacity as CO₂) and the old chlorofluorocarbons (CFCs), even stronger heat absorbers yet, but more famous and dangerous as stratospheric ozone-depleters."

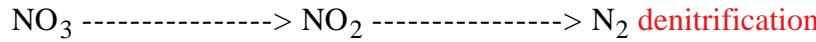
The Nitrogen Cycle

The nitrogen cycle is the most complex of the cycles of elements that make up biological systems. This is due to the importance and prevalence of N in cellular metabolism, the diversity of types of nitrogen metabolism, and the existence of the element in so many forms. Prokaryotes are essentially involved in the biological nitrogen cycle in three unique processes.

Nitrogen Fixation: this process converts N₂ in the atmosphere into NH₃ (ammonia), which is assimilated into amino acids and proteins. Nitrogen fixation occurs in many free-living bacteria such as clostridia, azotobacters and cyanobacteria, and in symbiotic bacteria such as *Rhizobium* and *Frankia*, which associate with plant roots to form characteristic nodules. Biological nitrogen fixation is the most important way that N₂ from the air enters into biological systems.



Anaerobic Respiration: this relates to the use of oxidized forms of nitrogen (NO₃ and NO₂) as final electron acceptors for respiration. Anaerobic respirers such as *Bacillus* and *Pseudomonas* are common soil inhabitants that will use nitrate (NO₃) as an electron acceptor. NO₃ is reduced to NO₂ (nitrite) and then to a gaseous form of nitrogen such as N₂ or N₂O (nitrous oxide) or ammonia (NH₃). The process is called **denitrification**. Denitrifying bacteria are typical aerobes that respire whenever oxygen is available by aerobic respiration. If O₂ is unavailable for respiration, they will turn to the alternative anaerobic respiration which uses NO₃. Since NO₃ is a common and expensive form of fertilizer in soils, denitrification may not be so good for agriculture, and one rationale for tilling the soil is to keep it aerobic, thereby preserving nitrate fertilizer in the soil.



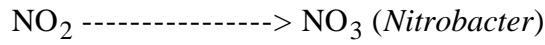
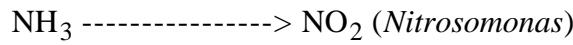
OFF THE WALL

The overall reactions of denitrification shown above proceed through the formation of nitrous oxide (N₂O).

A recent article by Wunsch and Zumft in *Journal of Bacteriology*, vol. 187 (2005), sheds new light on the process of denitrification. N_2O is a bacterial metabolite in the REVERSAL of Nitrogen fixation. The anthropogenic atmospheric increase of N_2O is a cause for concern, as noted above (as a greenhouse gas, N_2O has 300 times the heat absorbing capacity as CO_2). Denitrifying bacteria respire using N_2O as an electron acceptor yielding N_2 and thereby provide a sink for N_2O . This article provides new insight into this process by identifying a membrane-bound protein in denitrifying bacteria called NosR, that is necessary for the expression of N_2O reductase from the *nosZ* gene. The NosR protein has redox centers positioned on opposite sides of the cytoplasmic membrane, which allows it to sustain whole-cell N_2O respiration by acting on N_2O reductase.

Nitrification is a form of lithotrophic metabolism that is chemically the opposite of denitrification. Nitrifying bacteria such as *Nitrosomonas* utilize NH_3 as an energy source, oxidizing it to NO_2 , while *Nitrobacter* will oxidize NO_2 to NO_3 . Nitrifying bacteria generally occur in aquatic environments and their significance in soil fertility and the global nitrogen cycle is not well understood.

The Overall process of Nitrification



A final important aspect of the nitrogen cycle that involves prokaryotes, though not exclusively, is **decomposition** of nitrogen-containing compounds. Most organic nitrogen (in protein, for example) yields ammonia (NH_3) during the process of **deamination**. Fungi are involved in decomposition, as well.

Plants, animals and protista, as well as the prokaryotes, complete the nitrogen cycle during the uptake of the element for their own nutrition. Nitrogen **assimilation** is usually in the form of nitrate, an amino group, or ammonia.

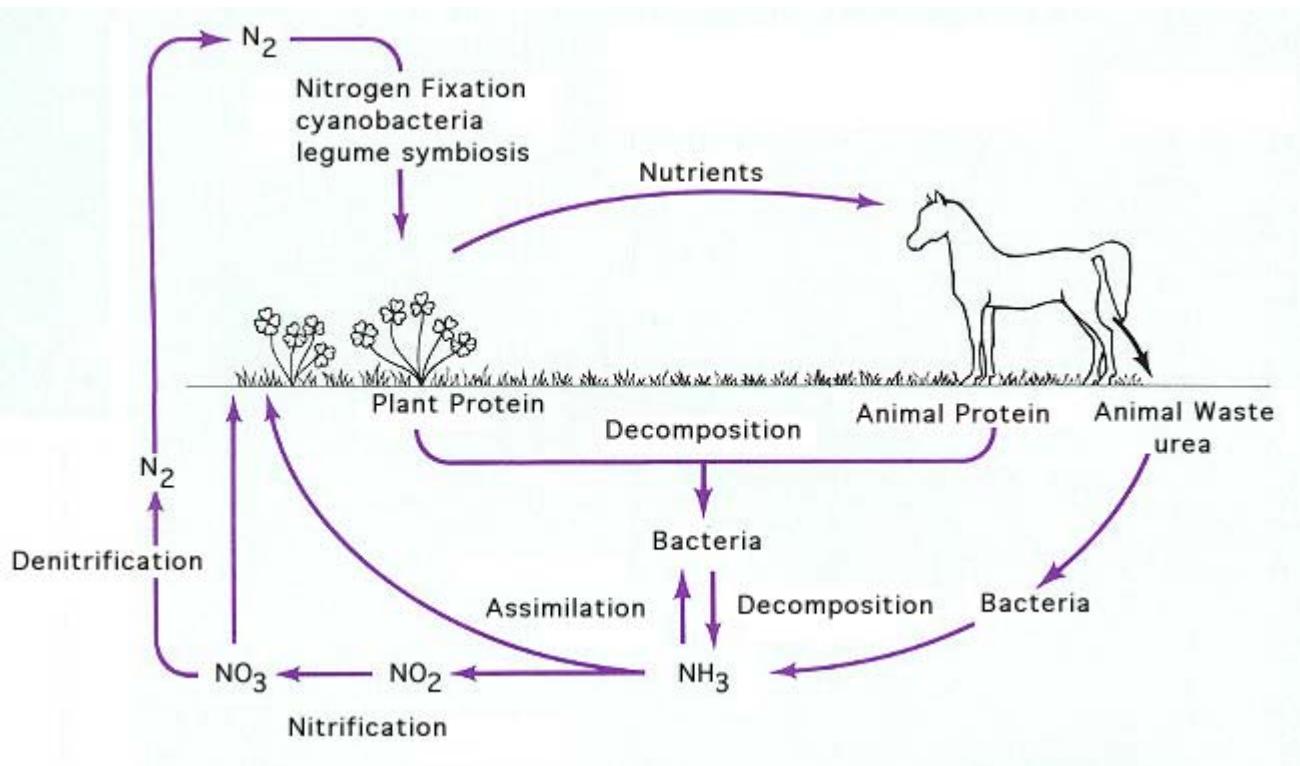


Figure 2. The Nitrogen Cycle

The Sulfur Cycle

Sulfur is a component of a couple of vitamins and essential metabolites and it occurs in two amino acids, cysteine and methionine. In spite of its paucity in cells, it is an absolutely essential element for living systems. Like nitrogen and carbon, the microbes can transform sulfur from its most oxidized form (sulfate or SO_4) to its most reduced state (sulfide or H_2S). The sulfur cycle, in particular, involves some unique groups of prokaryotes and prokaryotic processes. Two unrelated groups of prokaryotes oxidize H_2S to S and S to SO_4 . The first is the anoxygenic photosynthetic purple and green sulfur bacteria that oxidize H_2S as a source of electrons for cyclic photophosphorylation. The second is the "colorless sulfur bacteria" (now a misnomer because the group contains many Archaea) which oxidize H_2S and S as sources of energy. In either case, the organisms can usually mediate the complete oxidation of H_2S to SO_4 .



Sulfur-oxidizing prokaryotes are frequently thermophiles found in hot (volcanic) springs and near deep sea thermal vents that are rich in H_2S . They may be acidophiles, as well, since they acidify their own environment by the production of sulfuric acid.

Since SO_4 and S may be used as electron acceptors for respiration, sulfate reducing bacteria produce H_2S during a process of anaerobic respiration analogous to denitrification. The use of SO_4 as an electron acceptor is an obligatory process that takes place only in anaerobic environments. The process results in the distinctive odor of H_2S in anaerobic bogs, soils and sediments where it occurs.

Sulfur is assimilated by bacteria and plants as SO_4 for use and reduction to sulfide. Animals and bacteria can remove the sulfide group from proteins as a source of S during decomposition. These processes complete the sulfur cycle.

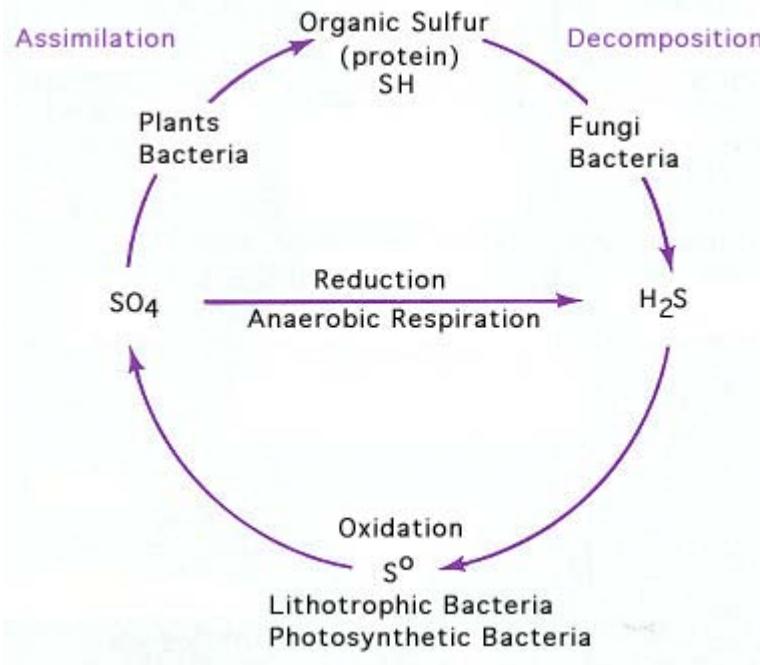


Figure 3. The Sulfur Cycle

The Phosphorus cycle

The phosphorus cycle is comparatively simple. Inorganic phosphate exists in only one form. It is interconverted from an inorganic to an organic form and back again, and there is no gaseous intermediate.

Phosphorus is an essential element in biological systems because it is a constituent of nucleic acids, (DNA and RNA) and it occurs in the phospholipids of cell membranes. Phosphate is also a constituent of ADP and ATP which are universally involved in energy exchange in biological systems.

Dissolved phosphate (PO_4) inevitably ends up in the oceans. It is returned to land by shore animals and birds that feed on phosphorus containing sea creatures and then deposit their feces on land. Dissolved PO_4 is also returned to land by a geological process, the uplift of ocean floors to form land masses, but the process is very slow. However, the figure below considers how PO_4 is recycled among land-based groups of organisms.

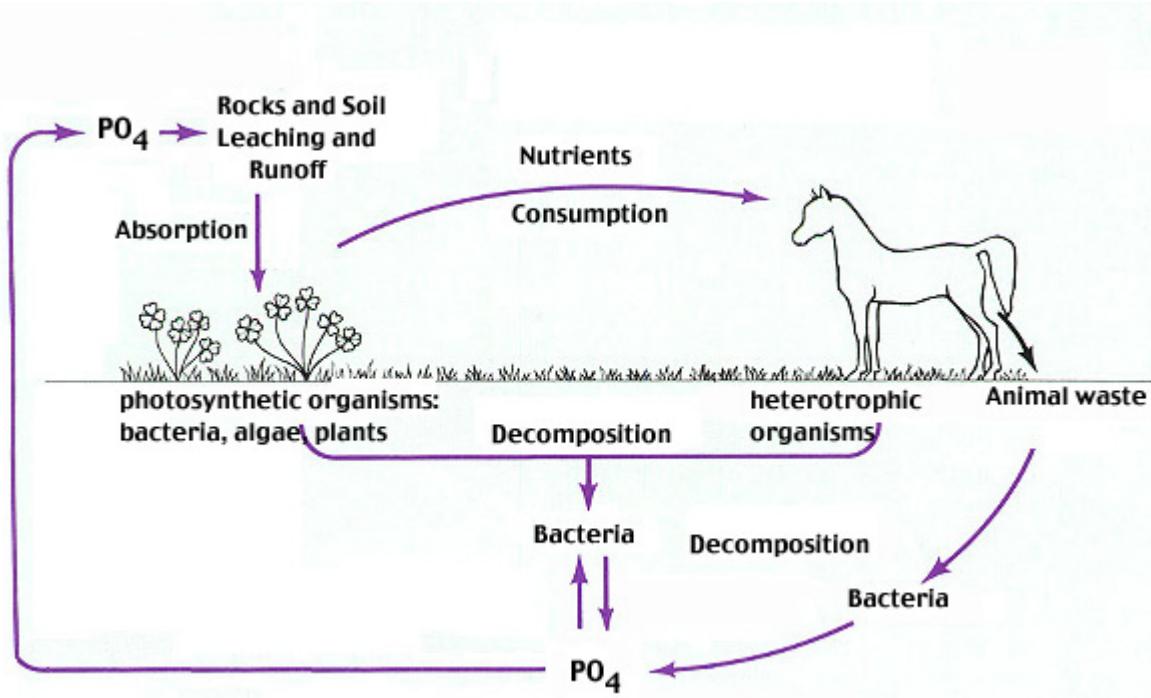


Figure 4. The Phosphorus Cycle. Plants, algae and photosynthetic bacteria can absorb phosphate (PO_4) dissolved in water, or if it washes out of rocks and soils. They incorporate the PO_4 into various organic forms, including such molecules as DNA, RNA, ATP, and phospholipid. The plants are consumed by animals wherein the organic phosphate in the plant becomes organic phosphate in the animal and in the bacteria that live with the animal. Animal waste returns inorganic PO_4 to the environment and also organic phosphate in the form of microbial cells. Dead plants and animals, as well as animal waste, are decomposed by microbes in the soil. The phosphate eventually is mineralized to the soluble PO_4 form in water and soil, to be taken up again by photosynthetic organisms.

Ecology of a Stratified Lake

The role of microbes in the global cycle of elements (described above) can be visited on a smaller scale, in a lake, for example, like Lake Mendota, which may become stratified as illustrated in Figure 5. The surface of the lake is well-lighted by the sun and is aerobic. The bottom of the lake and its sediments are dark and anaerobic. Generally there is less O_2 and less light as the water column is penetrated from the surface. Assuming that the nutrient supply is stable and there is no mixing between layers of lake water, we should, for the time being, have a stable ecosystem with recycling of essential elements among the living systems. Here is how it would work.

At the surface, light and O_2 are plentiful, CO_2 is fixed and O_2 is produced. Photosynthetic plants, algae and cyanobacteria produce O_2 , cyanobacteria can even fix N_2 ; aerobic bacteria, insects, animals and plants live here.

At the bottom of the lake and in the sediments, conditions are dark and anaerobic. Fermentative bacteria produce fatty acids, H_2 and CO_2 , which are used by methanogens to produce CH_4 . Anaerobic respiration bacteria use NO_3^- and SO_4^{2-} as electron acceptors, producing NH_3 and H_2S . Several soluble gases are in the water: H_2 , CO_2 , CH_4 , NH_3 and H_2S .

The biological activity at the surface of the lake and at the bottom of the lake may have a lot to do with what will be going on in the middle of the water column, especially near the interface of the aerobic and anaerobic zones. This area, called the **thermocline**, is biologically very active. Bacterial photosynthesis, which is anaerobic, occurs here, using longer wave lengths of light that will penetrate the water column and are not absorbed by all the plant chlorophyll above. The methanotrophs will stay just within the aerobic area taking up the CH_4 from the sediments as a carbon source, and returning it as CO_2 . Lithotrophic nitrogen- and sulfur-utilizing bacteria do something

analogous: they are aerobes that use NH_3 and H_2S from the sediments, returning them to NO_3^- and SO_4^{2-} .

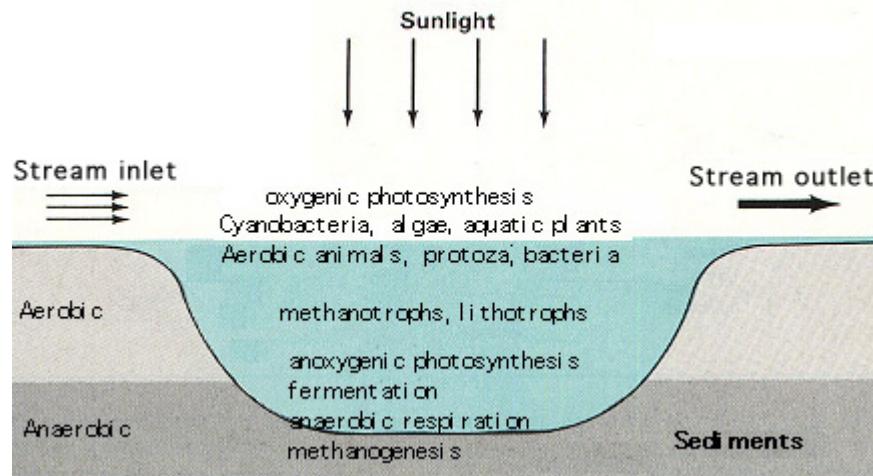
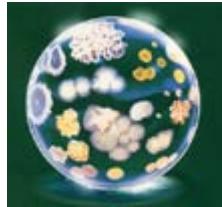


Figure 5. Ecology of a Stratified lake

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IMPORTANT GROUPS OF PROKARYOTES

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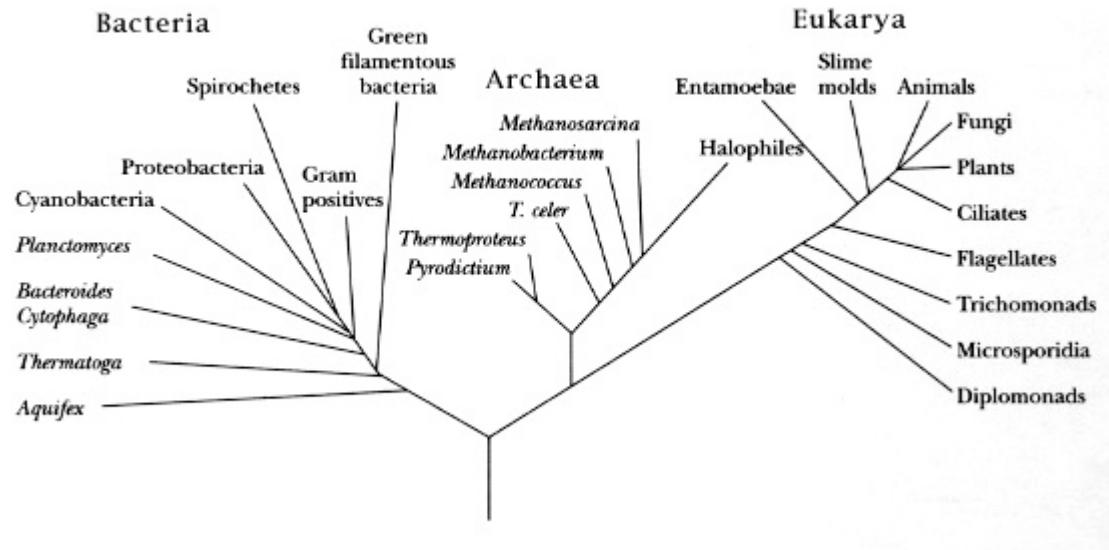


Figure 0. The Phylogenetic Tree of Life based on Comparative ssrRNA Sequencing. The Tree shows the prokaryotes in two Domains, Archaea and Bacteria. At a taxonomic level, most organisms at the tips of the Archaeal branches represent a unique Order; most organisms at the tips of the bacterial branches are classified into a unique Phylum. On the Archaeal limb, the three physiological groups are evident in the names: "thermo" and "pyro" for the extreme thermophiles; "methano" for the methanogens; and "halophiles" for the extreme halophiles. The most important, best known, and diverse groups (phyla) branching off of the Bacterial limb are the Cyanobacteria, Proteobacteria and Gram positives.

The **prokaryotes** (or prokaryotes) consist of millions of genetically-distinct unicellular organisms. What they lack in structural diversity, so well-known among eucaryotes (including the protista), they make up for in their physiological diversity. It is often a particular physiological trait that unifies and distinguishes a particular group of prokaryotes to microbiologists. In [Bergey's Manual of Determinative bacteriology \(1994\)](#), the identifiable groups of prokaryotes are assembled based on easily-observed phenotypic characteristics such as Gram stain, morphology (rods, cocci, etc), motility, structural features (e.g. spores, filaments, sheaths, appendages, etc.), and on distinguishing physiological features (e.g. anoxygenic photosynthesis, anaerobiosis, methanogenesis, lithotrophy, etc.).

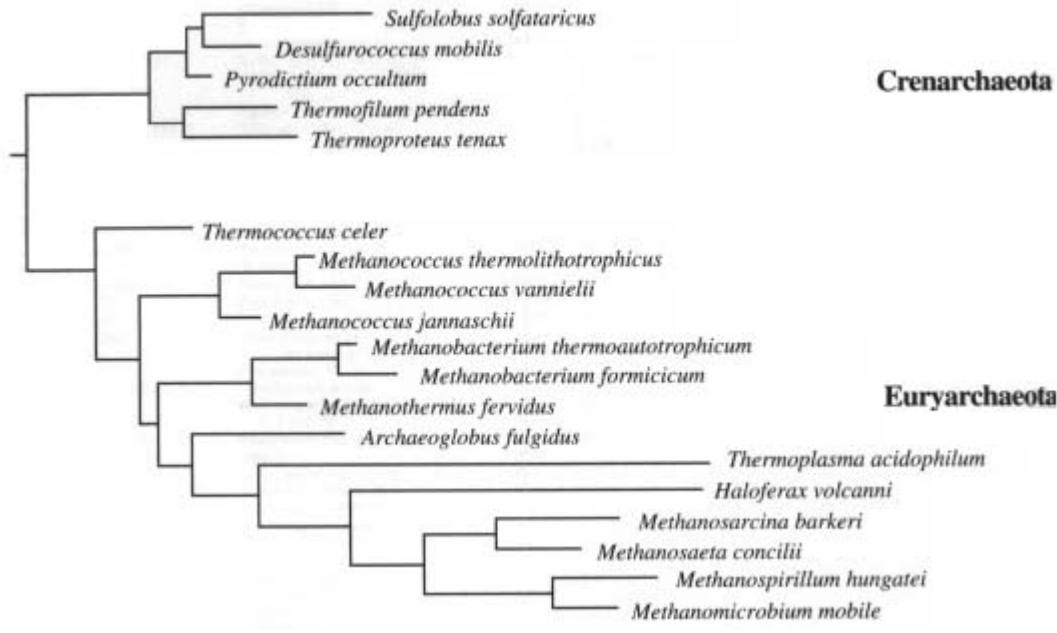
Nowadays, this type of artificial classification scheme has been abandoned in favor of hierachal taxonomic schemes based on comparative genetic analysis of the nucleotide sequences of the small subunit ribosomal RNA that is contained in all cellular organisms. In the Second edition of [Bergey's Manual of Systematic Bacteriology \(2001\)](#) as well as the current edition of [The Prokaryotes](#), phylogeny dominates the classification schemes. Such an approach generates the Phylogenetic Tree of Life (above) that lands the prokaryotes in two Domains, **Archaea** and **Bacteria**. At a taxonomic level, organisms at the tips of the archaeal branches represent Orders; The tips of the bacterial branches are Phyla. More information on the taxonomy, phylogeny and classification of prokaryotes is given in the references at the bottom of this page. Also, an excellent article online that integrates phylogeny with classification of prokaryotes is [Classification and Phylogeny by Gary Olsen](#).

In the ensuing description of prokaryotes, groups of organisms are placed under **artificial headings** based on common **structural, biochemical or ecological** properties. This does not imply close genetic relatedness among different genera in a group. Sometimes all of the members of a group do share a close genetic relatedness; in other cases, members of a group are genetically-unrelated, even to an extent that is greater than exists among all members of the Eucaryotic domain. Also herein, some prokaryotes are placed in more than one group, and some groups consist of both Archaea and Bacteria.

ARCHAEA

On the basis of ssrRNA analysis, the Archaea consist of three phylogenetically-distinct groups: **Crenarchaeota**, **Euryarchaeota** and **Korarchaeota**. However, for the Korarchaeota, only their nucleic acids have been detected, and no organisms have been isolated or cultured. Based on their physiology, the Archaea can be organized into three types: **methanogens** (prokaryotes that produce methane); **extreme halophiles** (prokaryotes that live at very high concentrations of salt (NaCl); and **extreme (hyper) thermophiles** (prokaryotes that live at very high temperatures). In addition to the unifying archaeal features that distinguish them from Bacteria (i.e., no murein in cell wall, ether-linked membrane lipids, etc.), the Archaea exhibit other unique structural or biochemical attributes which adapt them to their particular habitats. The **Crenarchaeota** consists mainly of hyperthermophilic sulfur-dependent prokaryotes and the **Euryarchaeota** contains the methanogens and extreme halophiles. ssrRNAs of the **Korarchaeota** have been obtained from hyperthermophilic environments similar to those inhabited by Crenarchaeota. None of the Korarchaeota have been cultured in the laboratory, although information about them can be inferred from their genome structure.

Figure 1. Phylogenetic tree of Archaea



Methanogens are obligate anaerobes that will not tolerate even brief exposure to air (O_2). Anaerobic environments are plentiful, however, and include marine and fresh-water sediments, bogs and deep soils, intestinal tracts of animals, and sewage treatment facilities. Methanogens have an incredible type of metabolism that can use H_2 as an energy source and CO_2 as a carbon source for growth. In the process of making cell material from H_2 and CO_2 , the methanogens produce methane (CH_4) in a unique energy-generating process. The end product (methane gas) accumulates in their environment. Methanogen metabolism created most the natural gas (fossil fuel) reserves that are tapped as energy sources for domestic or industrial use. Methanogens are normal inhabitants of the rumen (fore-stomach) of cows and other ruminant animals. A cow belches about 50 liters of methane a day during the process of eructation (chewing the cud). Methane is a significant greenhouse gas and is accumulating in the atmosphere at an alarming rate. When rain forests are destroyed and replaced by cows, it is "double-hit" on the greenhouse: (1) less CO_2 is taken up due to removal of the the autotrophic green plants; (2) additional CO_2 and CH_4 are produced as gases by the combined metabolism of the animal and symbiotic methanogens. Methanogens represent a microbial system that can be exploited to produce energy from waste materials. Large amounts of methane are produced during industrial sewage treatment processes, but the gas is usually wasted rather than

trapped for recycling.

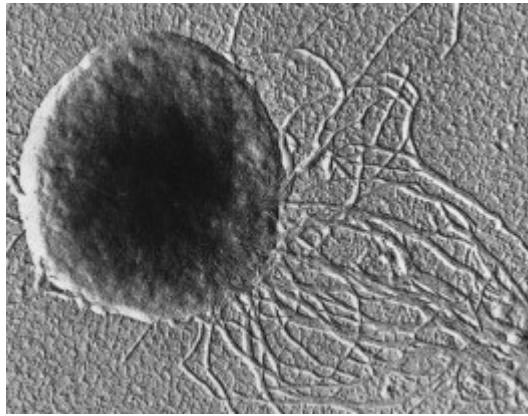


Figure 2. *Methanococcus jannischii* (Holger Jannisch). The archaean was originally isolated from a "white smoker" chimney at an oceanic depth of 2,600 meters on the East Pacific Rise. It can be grown in a mineral medium containing only H₂ and CO₂ as sources of energy and carbon for growth within a temperature range of 50 to 86 degrees. Cells are irregular cocci that are motile due to two bundles of polar flagella inserted near the same cellular pole, making it a rare example of a motile coccus.

Extreme halophiles live in natural environments such as the Dead Sea, the Great Salt Lake, or evaporating ponds of seawater where the salt concentration is very high (as high as 5 molar or 25 percent NaCl). The organisms require salt for growth and will not grow at low salt concentrations (Actually, the cells lyse at low NaCl concentrations). Their cell walls, ribosomes, and enzymes are stabilized by Na⁺. *Halobacterium halobium*, the prevalent species in the Great Salt Lake, adapts to the high-salt environment by the development of "purple membrane", formed by patches of light-harvesting pigment in the plasma membrane. The pigment is a type of rhodopsin called **bacteriorhodopsin** which reacts with light in a way that forms a proton gradient on the membrane allowing the synthesis of ATP. This is the only example in nature of non photosynthetic photophosphorylation. The organisms are heterotrophs that normally respire by aerobic means. The high concentration of NaCl in their environment limits the availability of O₂ for respiration so they are able to supplement their ATP-producing capacity by converting light energy into ATP using bacteriorhodopsin.



Figure 3. *Halobacterium salinarum* is an extreme halophile that grows at 4 to 5 M NaCl and does not grow below 3 M NaCl. This freeze etched preparation shows the surface structure of the cell membrane and reveals smooth patches of "purple membrane" (bacteriorhodopsin) imbedded in the plasma membrane.

Thermophiles and **extreme thermophiles** or "hyperthermophiles" come from several distinct phylogenetic lines of Archaea. These organisms require a very high temperature (80 degrees to 105 degrees) for growth. Their membranes and enzymes are unusually stable at high temperatures. Most of these Archaea require elemental sulfur for growth. Some are anaerobes that use sulfur as an electron acceptor for respiration in place of oxygen. Some are lithotrophs that oxidize sulfur as an energy source. Sulfur-oxidizers grow at low pH (less than pH 2), partly because they acidify their own environment by oxidizing S⁰ (sulfur) to SO₄ (sulfuric acid). Hyperthermophiles are inhabitants of hot, sulfur-rich environments usually associated with volcanism, such as hot springs, geysers and fumaroles in Yellowstone National Park and elsewhere, and thermal vents ("smokers") and cracks in the ocean floor. *Sulfolobus* was the first hyperthermophilic Archaeon discovered by Thomas D. Brock of the University of Wisconsin in 1970. His discovery, along with that of *Thermus aquaticus* (a thermophilic bacterium) in Yellowstone National Park, launched the field of hyperthermophile biology. (*Thermus aquaticus* is the source of the enzyme **taq polymerase** used in the polymerase chain reaction, PCR.) The bacterium has an optimum temperature for growth of 70 degrees. *Sulfolobus* grows in sulfur-rich, hot acid springs at temperatures as high as 90 degrees and pH

values as low as 1. *Thermoplasma*, also discovered by Brock, is a unique thermophile that is the sole representative of a distinct phylogenetic line of Archaea. *Thermoplasma* resembles the bacterial mycoplasmas in that it lacks a cell wall. *Thermoplasma* grows optimally at 55 degrees and pH 2. Interestingly, it has only been found in self-heating coal refuse piles, which are a man-made waste.

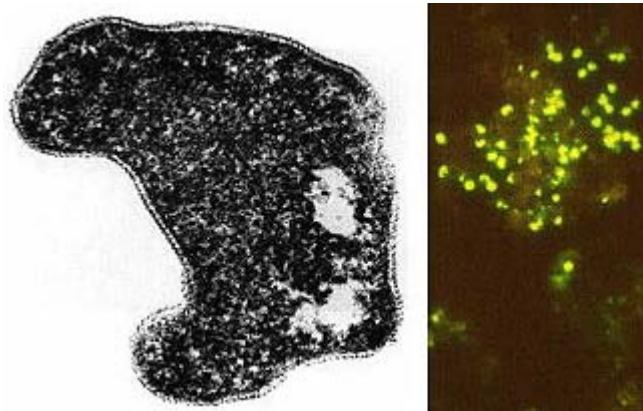


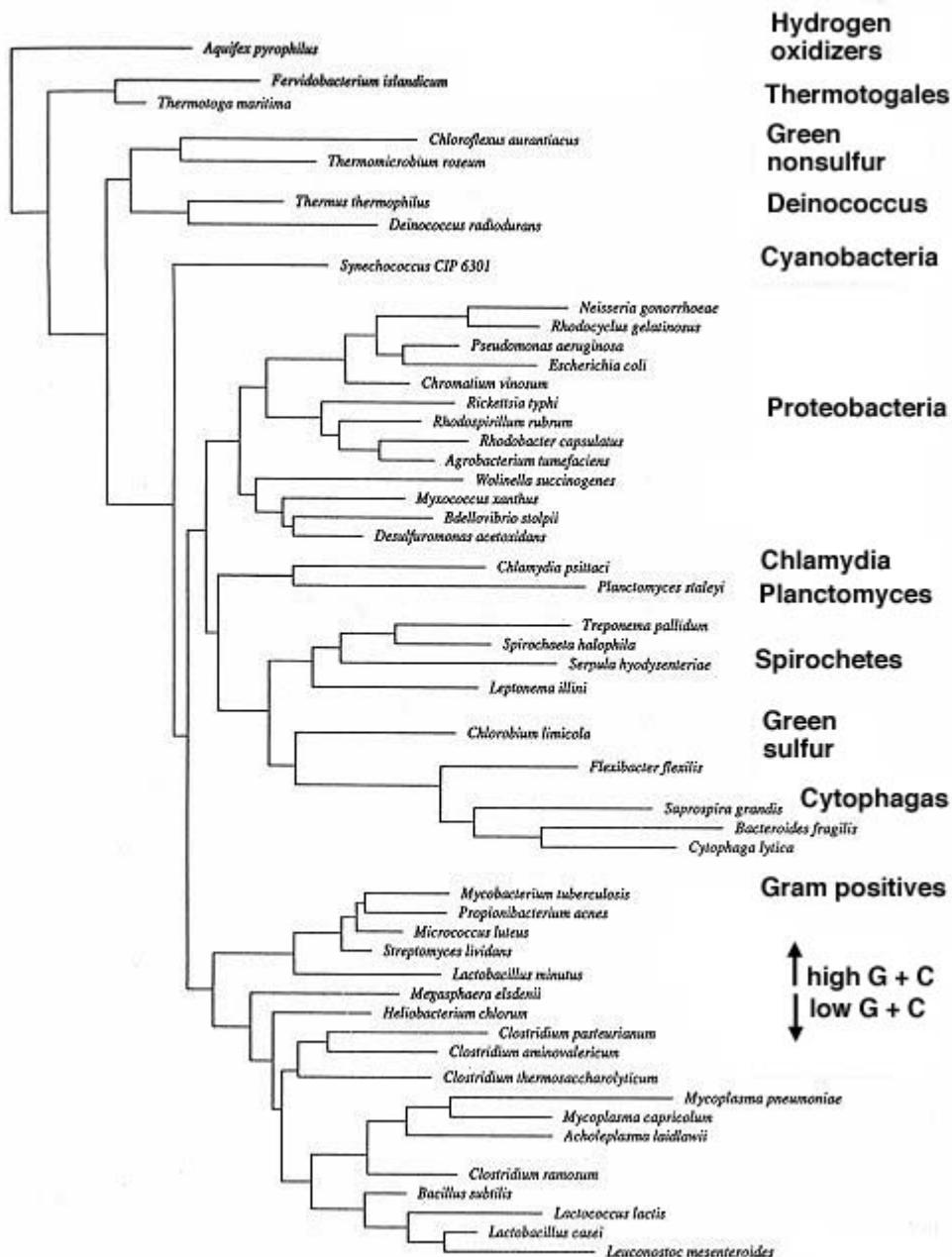
Figure 4. *Sulfolobus acidocaldarius* (T.D. Brock). *Sulfolobus* is an extreme thermophile that has been found in geothermally-heated acid springs, mud pots and surface soils with temperatures from 60 to 95 degrees C, and a pH of 1 to 5. Left: Electron micrograph of a thin section (85,000X). Under the electron microscope the organism appears as irregular spheres which are often lobed. Right: Fluorescent photomicrograph of cells attached to a sulfur crystal. Fimbrial-like appendages have been observed on the cells attached to solid surfaces such as sulfur crystals.

Although the Archaea are often inhabitants of unusual or extreme environments, there may be corresponding species of Bacteria, and even eucaryotes, in these habitats as well. No bacterium can produce methane, but in many anaerobic environments Bacteria are found in association with methanogens. With regard to acid tolerance, a bacterium, *Thiobacillus*, has been observed growing at pH near 0. A eucaryotic alga, *Cyanidium*, has also been found growing near pH 0. In superheated environments (greater than 100 degrees), Archaea may have an exclusive hold, but Bacteria have been isolated from boiling hot springs in Yellowstone National Park and other parts of the world. No bacterium grows at the highest salt concentration which supports the growth of the halobacteria, but osmophilic yeasts and fungi can grow at correspondingly low water activities where sugar is the solute in high concentration.

BACTERIA

Phylogenetic analysis of the **Bacteria** has demonstrated the existence of at least 13 distinct groups (Figure 5), 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.

Figure 5. Phylogenetic tree of Bacteria



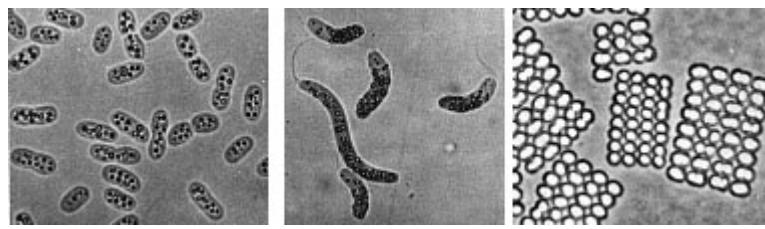
Photosynthetic purple and green bacteria. These bacteria conduct **anoxygenic photosynthesis**, also called **bacterial photosynthesis**. Bacterial photosynthesis differs from plant-type (oxygenic) photosynthesis in several ways. Bacterial photosynthesis does not produce O₂; in fact, it only occurs under 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 H₂O but may be H₂, H₂S or S⁰, 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 SO₄ as a photosynthetic electron donor.

The **purple and green bacteria** may use H₂S during photosynthesis in the same manner that cyanobacteria or algae or plants use H₂O as an electron donor for autotrophic CO₂ 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 CO₂ fixation.

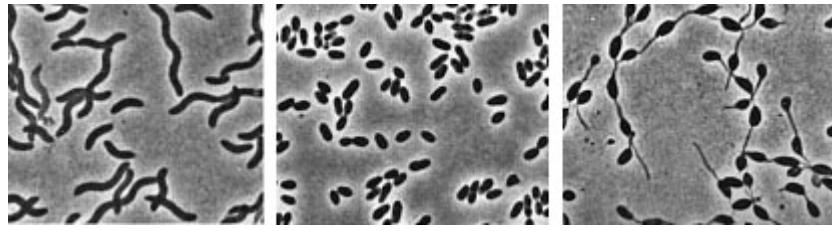
The bacterium that became an endosymbiont of eukaryotes and evolved into mitochondria is thought to be a relative of the purple nonsulfur bacteria. This conclusion is based on similar metabolic features of mitochondria and purple nonsulfur bacteria and on comparisons of the base sequences in their 16S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

Figure 6. Photomicrographs (phase contrast and ordinary illumination) of various photosynthetic bacteria (Norbert Pfennig). Magnifications are about 1400X. The purple and green bacteria exhibit a full range of prokaryotic morphologies, as these

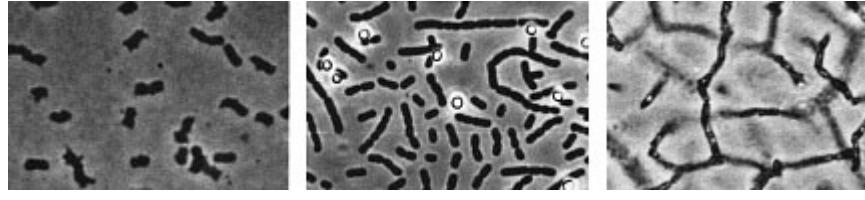
photomicrographs illustrate. Diversity among their phylogenetic relationships is also noted.



A. Purple sulfur bacteria (L to R): *Chromatium vinosum*, *Thiospirillum jenense*, *Thiopedia rosea*. The purple sulfur bacteria are classified among the Gammaproteobacteria, a class that also includes *Pseudomonas* and *E. coli*.



B. Purple nonsulfur bacteria (L to R): *Rhodospirillum rubrum*, *Rhodobacter sphaeroides*, *Rhodomicrobiun 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* in Figure 5.

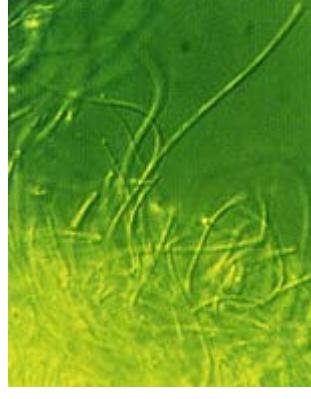


Figure 7. Green nonsulfur bacterium, *Chloroflexus* (T.D. Brock). *Chloroflexus* also represents a phylogenetically distinct group of green bacteria. *Chloroflexus* is a thermophilic, filamentous gliding bacterium.



Figure 8. Photosynthetic prokaryotes 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 9. 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 unicellular 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 prokaryotes. 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 eukaryotic 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 mollusks. 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 O₂ into the cell. Any O₂ that diffuses into the heterocyst is rapidly reduced by hydrogen, a byproduct of N₂ 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 fixation 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 eukaryotic 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 CO₂ 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 N₂ under certain conditions, they are one of the most significant free-living nitrogen-fixing prokaryotes. 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 prokaryotes, called **prochlorophytes** contain chlorophyll *a* and *b* but do **not** contain phycobilins. Prochlorophytes, therefore, resemble both cyanobacteria (because they are prokaryotic 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 prokaryotes 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 10. 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 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. Nonetheless, 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 **boluminescence**, 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 prokaryotes, 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 prokaryotes. 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 11). 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.

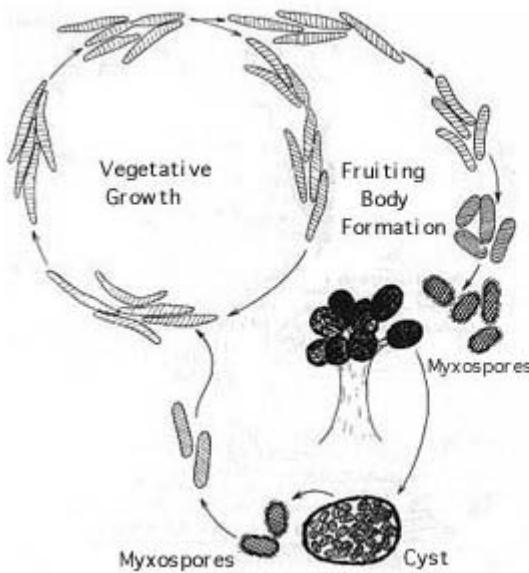


Figure 11. *Stigmatella aurantiaca*, a fruiting myxobacterium: L. Life Cycle R. Fruiting Body.

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 H₂ as an energy source, and many extreme thermophiles use H₂S or elemental sulfur as a source of energy for growth. Lithotrophic Bacteria are typically Gram-negative species that utilize inorganic substrates including H₂, NH₃, NO₂, H₂S, S, Fe⁺⁺, and CO. Ecologically, the most important lithotrophic Bacteria are the **nitrifying bacteria**, *Nitrosomonas* and *Nitrobacter* that together convert NH₃ to NO₂, and NO₂ to NO₃, and the **colorless sulfur bacteria**, such as *Thiobacillus*, that oxidize H₂S to S and S to SO₄. 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 12. 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 characteristics, 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.

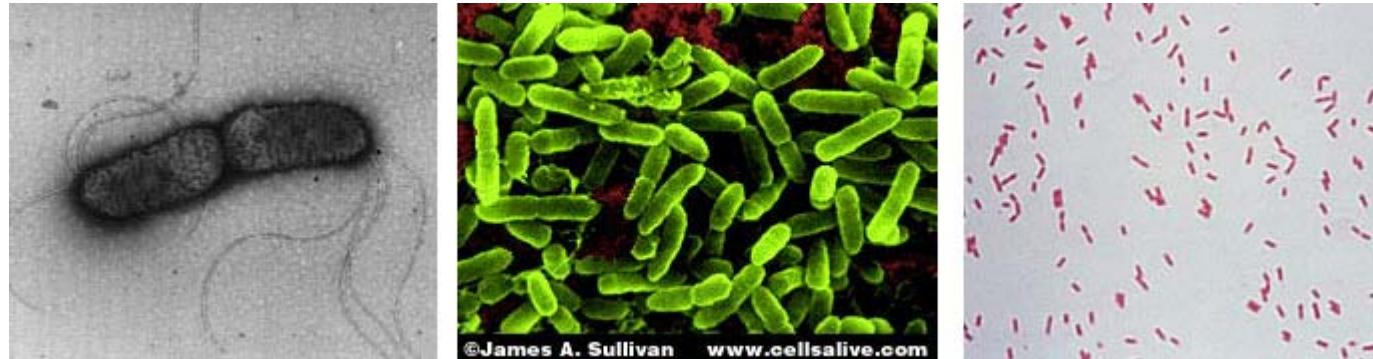


Figure 13. Profile of a pseudomonad: Gram-negative rods motile by polar flagella. A. Electron micrograph, negative stain. B. Scanning electron micrograph. C. Gram stain.

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. *Pseudomonas* species are discussed elsewhere in the text at [Opportunistic Infections caused by *Pseudomonas aeruginosa*](#) and [The Genus *Pseudomonas*](#).

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**. See [E. coli and Gastroenteritis, Urinary tract Infections and Neonatal Meningitis](#).

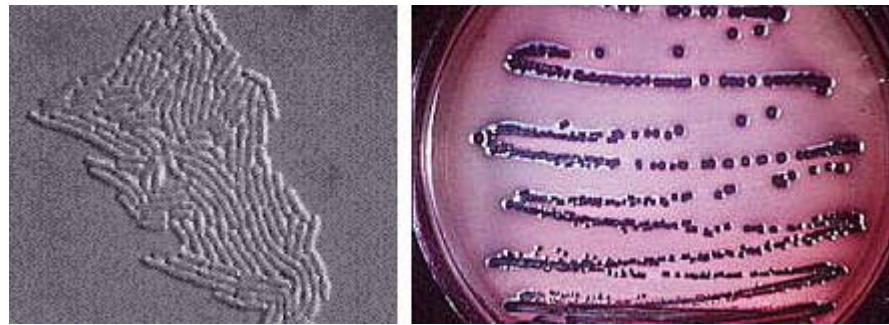


Figure 14. Left: *Escherichia coli* cells. **Right:** *E. coli* colonies on EMB Agar.

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 fever**. 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 scattered 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. See [**Gonorrhea and Meningitis**](#). Among the Gamma group, *Pseudomonas aeruginosa*, the enterics, and *Vibrio cholerae* have already been mentioned. Likewise, the agents of Legionaires' pneumonia (*Legionella pneumophila*), 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 prokaryotes, 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 N₂ (atmospheric nitrogen) to NH₃ (ammonia). It is a complicated enzymatic process mediated by the enzyme **nitrogenase**. Nitrogenase is found only in prokaryotes and is second only to RUBP carboxylase (the enzyme responsible for CO₂ 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 (NH₃) or nitrates (NO₃), and never from N₂. Nitrogenase extracts N₂ from the atmosphere and reduces it to NH₃ in a reaction that requires substantial reducing power (electrons) and energy (ATP). The NH₃ 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 prokaryotes, 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 prokaryotes 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 prokaryote 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 NH₃ in the process of **ammonification**. NH₃ is oxidized by lithotrophic *Nitrosomonas* species to NO₂ which is subsequently oxidized by *Nitrobacter* to NO₃. The overall conversion of NH₃ to NO₃ is called **nitrification**. NO₃ can be assimilated by cells as a source of nitrogen (**assimilatory nitrate reduction**), or certain bacteria can reduce NO₃ 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, NO₃ is first reduced to NO₂, which is subsequently reduced to N₂O or N₂ or NH₃ (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.

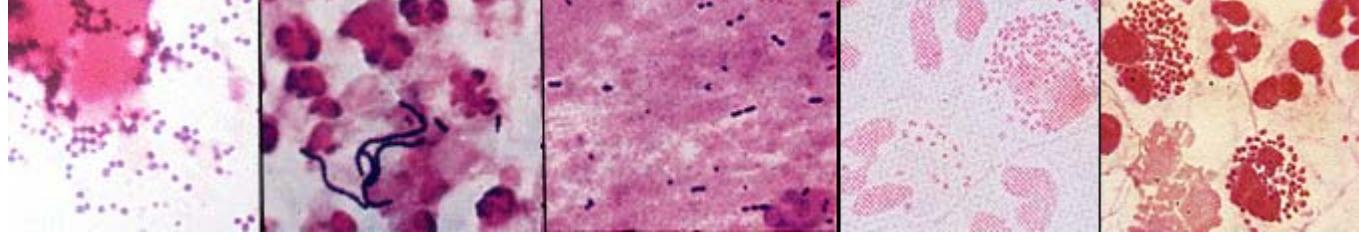


Figure 15. Gallery of pyogenic cocci, Gram stains of clinical specimens (pus), L to R: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*. The large cells with lobed nuclei are neutrophils. Pus is the outcome of the battle 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 pyogenes, 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 meningococcal 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.

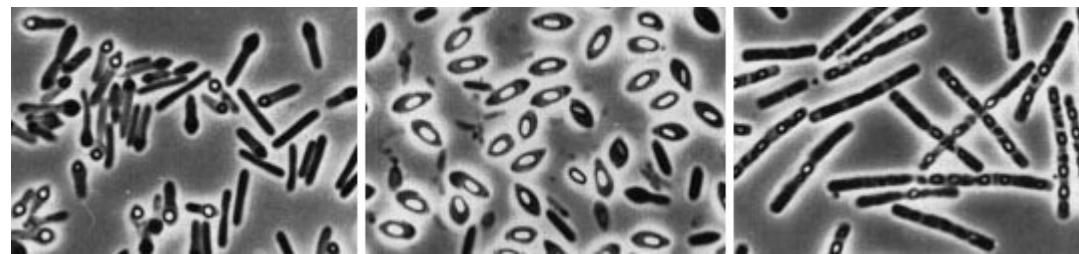


Figure 16. Endospore-forming bacilli (phase contrast illumination). Endospores are dehydrated, refractile cells appearing as points of bright light under phase microscopy. Endospore-forming bacteria are characterized by the location (position) of the endospore in the mother cell (sporangium) before its release. The spore may be central, terminal or subterminal, and the sporangium may or may not be swollen to accommodate the spore.

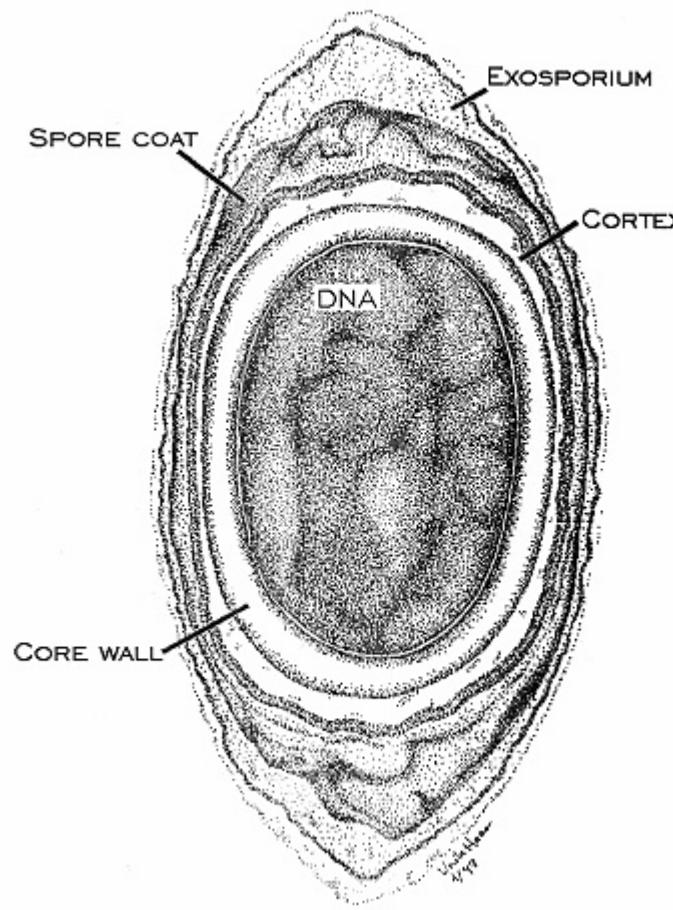


Figure 17. Anatomy of an endospore, cross section drawing by Viake Haas. Endospores differ from the vegetative cells that form them in a variety of ways. Several new surface layers develop outside the core (cell wall), including the cortex and spore coat. The cytoplasm is dehydrated and contains only 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.

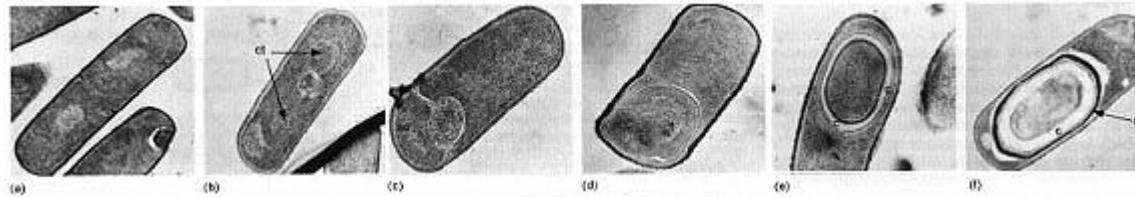


Figure 18. The sequential steps in the process of endospore formation in *Bacillus subtilis*.

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 increasingly recognized as an agent of food poisoning. *Clostridium botulinum* causes [botulism](#) a form of food-poisoning, and *Clostridium tetani* causes [tetanus](#).

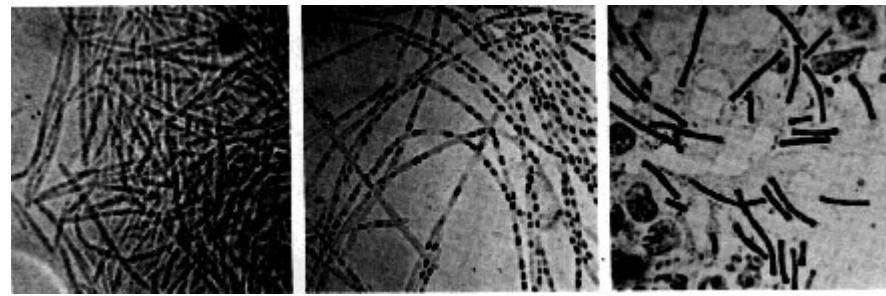


Figure 19. Robert Koch's original photomicrographs of *Bacillus anthracis*. In 1876, Koch established by careful microscopy that the bacterium was always present in the blood of animals that died of anthrax. He took a small amount of blood from such an animal and injected it into a healthy mouse, which 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 *Phytophthora*, 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.

Actinomycetes and related bacteria are a large group of Gram-positive bacteria that usually grow by filament formation, or at least show a tendency towards branching and filament formation. Many of the organisms can form resting structures called spores, but they are not the same as endospores. Branched forms superficially resemble molds and are a striking example of convergent evolution of a prokaryote and a eucaryote together in the soil habitat. Actinomycetes such as *Streptomyces* have a world-wide distribution in soils. They are important in aerobic decomposition of organic compounds and have an important role in biodegradation and the carbon cycle. Products of their metabolism, called **geosmins**, impart a characteristic earthy odor to soils. Actinomycetes are the main producers of antibiotics in industrial settings, being the source of most tetracyclines, macrolides (e.g. erythromycin), and aminoglycosides (e.g. streptomycin, gentamicin, etc.). Two bacteria in this diverse group are important pathogens of humans: *Mycobacterium tuberculosis* is the cause of [tuberculosis](#); *Corynebacterium diphtheriae* is the cause of [diphtheria](#). Also, many nonpathogenic mycobacteria and corynebacteria live in associations with animals.

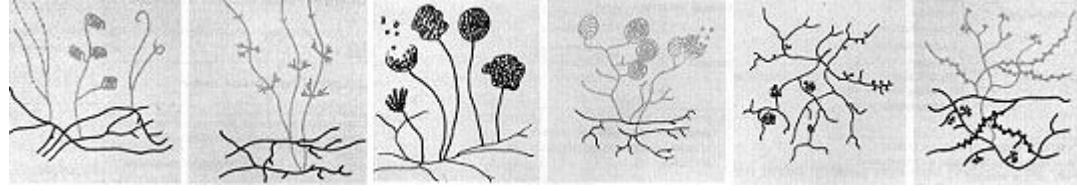


Figure 20. Schematic diagrams illustrating mycelial growth and spore formation in several genera of actinomycetes.

Rickettsias and chlamydiae are two unrelated groups of Bacteria that are **obligate intracellular parasites** of eucaryotic cells. Rickettsias cannot grow outside of a host cell because they have leaky membranes and are unable to obtain nutrients in an extracellular habitat. Chlamydiae are unable to produce ATP in amounts required to sustain metabolism outside of a host cell and are, in a sense, energy-parasites.

Rickettsias occur in nature in the gut lining of arthropods (ticks, fleas, lice, etc.). They are transmitted to vertebrates by an arthropod bite and produce such diseases as [typhus fever](#), [Rocky Mountain Spotted Fever](#), Q

fever and canine ehrlichiosis. Chlamydiae are tiny bacteria that infect birds and mammals. They may colonize and infect tissues of the eye and urogenital tract in humans. *Chlamydia trachomatis* causes several important diseases in humans: **chlamydia**, the most prevalent sexually transmitted disease in the U.S., **trachoma**, a leading cause of blindness worldwide, and **lymphogranuloma venereum**.

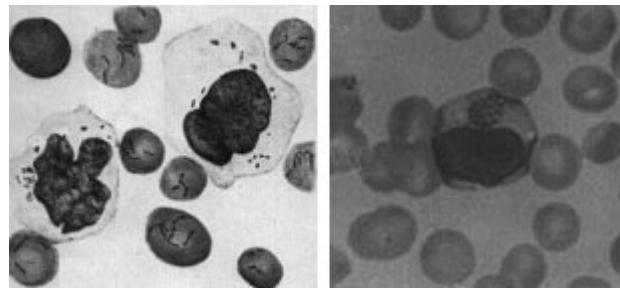


Figure 21. Mammalian cells infected with rickettsial organisms. L. *Bartonella bacilliformis* infection of human erythrocytes and blood monocytes. R. *Ehrlichia canis* infection of canine erythrocytes and blood monocytes. The distinct stained intracytoplasmic inclusion body in the monocyte is characteristic of the infection.

Mycoplasmas are a group of bacteria that lack a cell wall. The cells are bounded by a single triple-layered membrane. They may be free-living in soil and sewage, parasitic inhabitants of the mouth and urinary tract of humans, or pathogens in animals and plants. In humans, *Mycoplasma pneumoniae* causes **primary atypical pneumonia** ("walking pneumonia").

Mycoplasmas include the smallest known cells, usually about 0.2 - 0.3 micrometers in diameter. Mycoplasmas correspondingly have the smallest known genome of any cell. Their DNA is thought to contain about 650 genes, which is about one-fifth the number found in *E. coli* and other common bacteria. Mycoplasmas can survive without a cell wall because their cytoplasmic membrane is more stable than that of other prokaryotes. In one group of mycoplasmas, the membrane contains sterols which seem to be responsible for the stability. Also, mycoplasmas tend to inhabit environments of high osmolarity wherein the risk of osmotic shock and lysis of the cells is minimized.

Plant-pathogenic bacteria. Many economically-important diseases of plants are caused by members of the Bacteria. It is estimated that one-eighth of the crops worldwide are lost to diseases caused by bacteria, fungi or insects. Almost all kinds of plants can be affected by bacterial diseases, and many of these diseases can be extremely destructive.

Almost all plant-pathogenic bacteria are Gram-negative bacilli, usually affiliated with the pseudomonads or enterics (above). The symptoms of bacterial disease in plants are described by a number of terms such as spots, blights, soft rots, wilts, and galls. Bacterial spots of various sizes on stems, leaves, flowers and fruits are usually caused by *Pseudomonas* or *Xanthomonas* species. Bacteria may cause **spots** by producing toxins that kill cells at the site of infection. **Brights** are caused by rapidly developing necrosis (dead, discolored areas) on stems, leaves and flowers. Fire blight in apples and pears, caused by *Erwinia amylovora*, can kill young trees within a single season. Bacterial **soft rots** occur most commonly in fleshy vegetables such as potatoes or onions or fleshy fruits such as tomatoes and eggplants. The most destructive soft rots are caused by *Erwinia* species that attack fruits and vegetables at the post-harvest stage.

Bacterial vascular **wilts** mainly affect herbaceous plants. The bacteria invade the vessels of the xylem, where they multiply, interfering with the movement of water and inorganic nutrients and resulting in the wilting and the death of the plants. The bacteria commonly degrade portions of the vessel walls and can even cause the vessels to rupture. Once the walls have ruptured, the bacteria then spread to the adjacent parenchyma tissues, where they continue to multiply. In some bacterial wilts, the bacteria ooze to the surface of the stems or leaves through cracks formed over cavities filled with cellular debris, gums, and bacteria. More commonly, however, the bacteria do not reach the surface of the plant until the plant has been killed by the disease. Wilts of alfalfa and bean plants are caused by species of *Clavibacter*; bacterial wilt of cucurbits, such as squashes and watermelons, are caused by *Erwinia tracheiphila*; the black rot of crucifers such as cabbage is caused by *Xanthomonas campestris*. The most economically-important wilt of plants is caused by *Pseudomonas solanacearum* which affects 44 genera of plants, including such major crops as bananas, peanuts, tomatoes, potatoes, eggplants and tobacco. This disease occurs worldwide in tropical, subtropical, and warm temperate areas.

Mycoplasmas (discussed above) have been identified in more than 200 plant species and associated with more than 50 plant diseases, many with symptoms of yellowing. Among these plant-pathogens are the spiroplasmas (genus *Spiroplasma*), which are pleomorphic, ovoid or spiral-shaped cells which are motile by means of a rotary or screw-like motion. Intracellular fibrils are thought to be responsible for their movement. The organisms have been isolated from the fluids of vascular plants and from the gut of insects that feed on these fluids. Some have been cultured on artificial media, including *Spiroplasma citri*, which is isolated from the leaves of citrus plants, where it causes citrus stubborn disease, and from corn plants suffering from corn stunt disease. A number of other mycoplasma-like organisms (sometimes called **MLOs**) have been detected in diseased plants by electron microscopy, which has been taken as evidence that these organisms may be more involved in plant disease than previously realized.

The causative agent of a common plant disease, termed **crown gall**, is *Agrobacterium tumefaciens*. The disease is characterized by large **galls** or swellings that form on the plant at the site of infection, usually near the soil line. Crown gall is a problem in nurseries, affecting ornamental plants and fruit stock, and it may be a serious disease in grapes. Because of their role in the genetic engineering of plants, the molecular biology of these bacteria is intensively studied.

References:

1. Balows, A., H.G. Truper, M. Dworkin, W. Harder, and K.-H. Schleifer (eds.). *The Prokaryotes*, 2nd ed. Springer-Verlag, New York. 1992. **Published in four volumes. The most complete reference on the characteristics of prokaryotes. Includes procedures for the selective isolation and identification of virtually all known prokaryotes. The online edition at [The Prokaryotes](#) provides access to the full text which is a work in continuous progress.**

2. Holt, J.G. (editor-in-chief). *Bergey's Manual of Systematic Bacteriology*. 1st edition. *published in four volumes:*
 Volume 1 (1984)
 Gram-negative Bacteria of general, medical, or industrial importance
 Volume 2 (1986)
 Gram-positive Bacteria other than Actinomycetes
 Volume 3 (1989)
 Archaeobacteria, Cyanobacteria, and remaining Gram-negative Bacteria
 Volume 4 (1989)
 Actinomycetes
This has been the standard authoritative guide to bacterial taxonomy and identification throughout the nineties and continuing into the new millennium. It was the usual place to begin a literature survey or an identification process of a specific bacterial group. The newer (second) edition, 2001 (below), takes a hierarchical approach to classification based primarily on genetic similarities as reflected in 16S and 23S ribosomal RNA sequences. As the second edition becomes progressively more available, it is sure to replace this classic, although it will remain useful to aid in the identification of bacteria.

3. Holt, J.G. (ed). *Bergey's Manual of Determinative Bacteriology*. 9th Edition. 1994.
The book was compiled by abstracting the phenotypic information contained in the four volumes of Bergey's Manual of Systematic Bacteriology. The arrangement of the book is strictly phenotypic, with no attempt to offer a natural higher classification. The arrangement chosen is utilitarian and is intended to aid in the identification of bacteria. The bacteria are divided into 35 groups, which are comparable to the "Parts" in the eighth edition and the "Sections" in the Systematic volumes. These groups are not meant to be formal taxonomic ranks, but are a continuation of the tradition of dividing the bacteria into easily recognized phenotypic groups. This arrangement is most useful for diagnostic purposes.

4. Garrity G.M. (ed). *Bergey's Manual of Systematic Bacteriology*. 2nd Edition. 2001
 Volume 1 (2001)
 The Archaea and the deeply branching and phototrophic Bacteria
 Volume 2 (2001)
 The Proteobacteria
 Volume 3 (2002)
 The low G + C Gram-positive Bacteria
 Volume 4 (2002)
 The high G + C Gram-positive Bacteria

Volume 5 (2003)

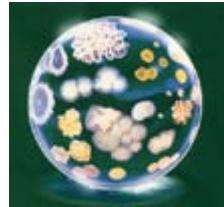
The Planctomycetes, Spriochaetes, Fibrobacteres, Bacteriodetes and Fusobacteria

The current "Bergey's Manual" in 5 volumes. The classification and taxonomy results from comparative sequencing of ribosomal RNA. Two Domains of Prokaryotes are identified, Archaea and Bacteria. The Domains are subdivided into Phylum, Class, Order, Family, Genus and Species. The Bacterial Domain contains 23 Phyla. A genus outline that includes an index of organisms is available online at [Bergey's Manual Genus Outline 2001](#).

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THE NATURE OF HOST-PARASITE INTERACTIONS

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The Nature of Host-parasite Interactions in Humans

Bacteria are consistently associated with the body surfaces of animals. There are many more bacterial cells on the surface of a human (including the gastrointestinal tract) than there are human cells that make up the animal. The bacteria that are consistently associated with an animal are called the **normal flora**. These bacteria have a full range of **symbiotic interactions** with their animal hosts.

In biology, symbiosis is defined as "life together", i.e. that two organisms live in an association with one -another. Thus, there are at least three types of relationships based on the quality of the association for the members of the symbiotic association.

Types of Symbiotic Associations

1. **Mutualism.** Both members of the association benefit.
2. **Commensalism.** There is no apparent benefit or harm to either member of the association.
3. **Parasitism.** One member of the association lives at the expense of the other member.

It is this type of a symbiotic association that draws our attention in this course. For many parasites are or can become pathogens, microorganisms the cause disease.

Bacterial Pathogenesis

A **pathogen** is a microorganism (or virus) that is able to produce disease. **Pathogenicity** is the ability of a microorganism to cause disease in another organism, namely the **host** for the pathogen. As implied above, pathogenicity is a manifestation of a host- parasite interaction.

In humans, some of the normal bacterial flora (e.g. *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus influenzae*) are **potential pathogens** that live in a commensal or parasitic relationship without producing disease. They do not cause disease in their host unless they have an opportunity brought on by some compromise or weakness in the host's anatomical barriers, tissue resistance or immunity. Furthermore, the bacteria are in a position to be transmitted from one host to another, giving them additional opportunities to colonize or infect.

There are some pathogens that do not associate with their host **EXCEPT** in the case of disease. These bacteria are **obligate pathogens**, even though some may rarely occur as normal flora, in asymptomatic or recovered carriers, or in some form where they cannot be eliminated by the host.

Opportunistic Pathogens

Bacteria which cause a disease in a compromised host which typically would not occur in a healthy (noncompromised) host are acting as **opportunistic pathogens**. A member of the normal flora can such as *Staphylococcus aureus* or *E. coli* can cause an **opportunistic infection**, but so can an environmental organism such as *Pseudomonas aeruginosa*. When a member of the normal flora causes an infectious disease, it might be referred

to as an **endogenous bacterial disease**, referring to a disease brought on by bacteria 'from within'.

Infection

The normal flora, as well as any "contaminating" bacteria from the environment, are all found on the body surfaces of the animal; the blood and internal tissues are sterile. If a bacterium, whether or not a component of the normal flora, breaches one of these surfaces, an **infection** is said to have occurred. Infection does not necessarily lead to infectious disease. In fact, infection probably rarely leads to infectious disease. Some bacteria rarely cause disease if they do infect; some bacteria will usually cause disease if they infect. But other factors, such as the route of entry, the number of infectious bacteria, and status of the host defenses, play a role in determining the outcome of infection.

Determinants of Virulence

Pathogenic bacteria are able to produce disease because they possess certain **structural** or **biochemical** or **genetic** traits that render them pathogenic or **virulent**. (The term **virulence** is best interpreted as referring to the **degree of pathogenicity**.) The sum of the characteristics that allow a bacterium to produce disease are the pathogen's **determinants of virulence**.

Some pathogens may rely on a single determinant of virulence, such as toxin production, to cause damage to their host. Thus, bacteria such as *Clostridium tetani* and *Corynebacterium diphtheriae*, which have hardly any invasive characteristics, are able to produce disease, the symptoms of which depend on a single genetic trait in the bacteria: the ability to produce a toxin. Other pathogens, such as *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa*, maintain a large repertoire of virulence determinants and consequently are able to produce a more complete range of diseases that affect different tissues in their host.

Properties of the Host

The **host** in a **host-parasite interaction** is the animal that maintains the parasite. The host and parasite are in a dynamic interaction, the outcome of which depends upon the properties of the parasite and of the host. The **bacterial parasite has its determinants of virulence** that allow it to invade and damage the host and to resist the defenses of the host. The **host has various degrees of resistance** to the parasite in the form of the **host defenses**.

Host Defenses

A healthy animal can defend itself against pathogens at different stages in the infectious disease process. The host defenses may be of such a degree that infection can be prevented entirely. Or, if infection does occur, the defenses may stop the process before disease is apparent. At other times, the defenses that are necessary to defeat a pathogen may not be effective until infectious disease is well into progress.

Typically the **host defense mechanisms are divided into two groups**:

1. Constitutive Defenses. Defenses common to all healthy animals. These defenses provide general protection against invasion by normal flora, or colonization, infection, and infectious disease caused by pathogens. The constitutive defenses have also been referred to as "natural" or "innate" resistance, since **they are inherent to the host**.

2. Inducible Defenses. Defense mechanisms that **must be induced or turned on by host exposure to a pathogen** (as during an infection). Unlike the constitutive defenses, they are not immediately ready to come into play until after the host is appropriately exposed to the parasite. The inducible defenses involve the immune responses to a pathogen causing an infection.

The inducible defenses are generally quite specifically directed against an invading pathogen. The constitutive defenses are not so specific, and are directed toward general strategic defense. The constitutive defenses, by themselves, may not be sufficient to protect the host against pathogens. Such pathogens that evade or overcome the relatively **nonspecific constitutive defenses** are usually susceptible to the more **specific inducible defenses**, once they have developed.

The Immune System

The inducible defenses are so-called because they are induced upon primary exposure to pathogen or one of its products. The **inducible defenses are a function of the immunological system** and the **immune responses**. The constitutive defenses are innate and immediately available for host defense. The inducible defenses must be triggered in a host and initially take time to develop. The type of resistance thus developed in the host is called **acquired immunity**. The term **immune** usually means the ability to resist infectious disease. **Immunity** refers to the relative state of resistance of the host to a specific pathogen.

Acquired immunity, itself, is sometimes divided into two types based on how it is acquired by the host.

In **active immunity**, the host undergoes an immunological response and produces the cells and factors responsible for the immunity, i.e., the host produces its own antibodies and/or immuno-reactive lymphocytes. Active immunity can persist a long time in the host, up to many years in humans.

In **passive immunity** there is acquisition by a host of immune factors which were produced in another animal, i.e., the host receives antibodies and/or immuno-reactive lymphocytes originally produced in another animal. Passive immunity is typically short-lived and usually persists only a few weeks or months.

Antigens

Antigens are chemical substances of relatively high molecular weight, that stimulate the immune response in animals. Bacteria are composed of various macromolecular components that are antigens or " **antigenic**" in their host and bacterial antigens interact with the host immunological system in a variety of ways.

Natural Antibodies

Studies on germ-free animals have confirmed that a normal bacterial flora in the gastrointestinal tract are necessary for full development of immunological (lymphatic) tissues in the intestine. Furthermore, the interaction between these immune tissues and intestinal bacteria results in the production of **serum and secretory antibodies** that are directed against bacterial antigens. These antibodies probably help protect the host from invasion by its own normal flora, and they can cross react with antigenically-related pathogens. For example, antibodies against normal *E. coli* could react with closely-related pathogenic *Shigella dysenteriae*. These type of antibodies are sometimes called **natural or cross-reactive antibodies**.

Bacterial Antigens made into Vaccines

In another way, **bacterial antigens** that are the components or products of pathogens are the **substances that induce the immune defenses** of the host to defend against, and to eliminate, the pathogen or disease. In the laboratory, these bacterial antigens can be manipulated or changed so that they will stimulate the immune response in the absence of infection or pathology. These isolated or modified antigens are the basis for **active immunization (vaccination)** against bacterial disease. Thus, a modified form of the tetanus toxin (tetanus toxoid), which has lost its toxicity but retains its antigenicity, is used to immunize against tetanus. Or, antigenic parts of the whooping cough bacterium, *Bordetella pertussis* can be used to induce active formation of antibodies that will react with the living organism and thereby prevent infection.

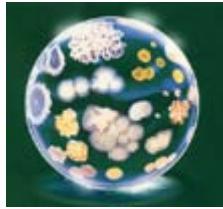
Antimicrobial Agents

One line of defense against bacterial infection is chemotherapy with antimicrobial agents such as **antibiotics**. The ecological relationships between animals and bacteria in the modern world are mediated by the omnipresence of antibiotics. Antibiotics are defined as substances produced by a microorganism that kill or inhibit other microorganisms. Originally, a group of soil bacteria, the *Streptomyces*, were the most innovative producers of antibiotics for clinical usage. They were the source of streptomycin, tetracycline, erythromycin and chloramphenicol, to name just a few antibiotics. Because **bacteria evolve rapidly toward resistance**, because **bacteria can exchange genes for antibiotic resistance**, perhaps because we have **overused and misused antibiotics**, many pathogens are emerging as resistant to antibiotics. There have already been reported infections by *Enterococcus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* that are refractory to all known antibiotics. Bacterial resistance to antimicrobial agents has become part of a pathogen's determinants of virulence. These are examples of genetic means by which bacteria exert their virulence.

The usage of antibiotics to control the growth of parasites is an artificial way to intervene in the natural process of the host-parasite interaction. But, of course, it is done for the obvious purpose of curing the disease. The body heals itself: most antibiotics just stop bacterial growth, and the host must rely entirely on its native defenses to accomplish the neutralization of bacterial toxins or the elimination of bacterial cells. The judicious use of antibiotics in the past five decades has saved millions of lives from infections caused by bacteria.

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The Bacterial Flora of Humans

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The Normal Flora

In a healthy animal, the internal tissues, e.g. blood, brain, muscle, etc., are normally free of microorganisms. On the other hand, the surface tissues, e.g. skin and mucous membranes, are constantly in contact with environmental organisms and become readily colonized by certain microbial species. The mixture of organisms regularly found at any anatomical site is referred to as the **normal flora**.

The normal flora of humans is exceedingly complex and consists of more than 200 species of bacteria. The makeup of the normal flora depends upon various factors, including genetics, age, sex, stress, nutrition and diet of the individual. The normal flora of humans consists of a few eukaryotic fungi and protists, and some methanogenic **Archaea** that colonize the lower intestinal tract, but the **Bacteria** are the most numerous and obvious microbial components of the normal flora. The distribution of the bacterial flora of humans is shown in Table 1. This table lists only a fraction of the total bacterial species that occur as normal flora of humans, and it does not express the total number or concentration of bacteria at any site.

TABLE 1. BACTERIA COMMONLY FOUND ON THE SURFACES OF THE HUMAN BODY

BACTERIUM	Skin	Conjunctiva	Nose	Pharynx	Mouth	Lower Intestine	Anterior urethra	Vagina
<i>Staphylococcus epidermidis</i> (1)	++	+		++	++	+	++	++
<i>Staphylococcus aureus</i> * (2)	+	+/-		+	+	++	+/-	+
<i>Streptococcus mitis</i>				+	++	+/-	+	+
<i>Streptococcus salivarius</i>				++	++			
<i>Streptococcus mutans</i> * (3)				+	++			
<i>Enterococcus faecalis</i> * (4)				+/-	+	++	+	+
<i>Streptococcus pneumoniae</i> * (5)	+/-		+/-	+	+			+/-
<i>Streptococcus pyogenes</i> * (6)	+/-	+/-		+	+	+/-		+/-
<i>Neisseria</i> sp. (7)	+		+	++	+		+	+
<i>Neisseria meningitidis</i> * (8)			+	++	+			+
<i>Veillonella</i> sp.					+	+/-		
<i>Enterobacteriaceae</i> * (<i>Escherichia coli</i>) (9)	+/-		+/-	+/-	+	++	+	+
<i>Proteus</i> sp.	+/-		+	+	+	+	+	+
<i>Pseudomonas aeruginosa</i> * (10)				+/-	+/-	+	+/-	
<i>Haemophilus influenzae</i> * (11)	+/-		+	+	+			
<i>Bacteroides</i> sp.*						++	+	+/-
<i>Bifidobacterium bifidum</i> (12)						++		
<i>Lactobacillus</i> sp. (13)				+	++	++		++
<i>Clostridium</i> sp.* (14)					+/-	++		

Clostridium tetani (15)							+/-	
Corynebacteria (16)	++	+	++	+	+	+	+	+
Mycobacteria	+		+/-	+/-		+	+	
Actinomycetes				+	+			
Spirochetes				+	++	++		
Mycoplasmas				+	+	+	+/-	+

++ = nearly 100 percent

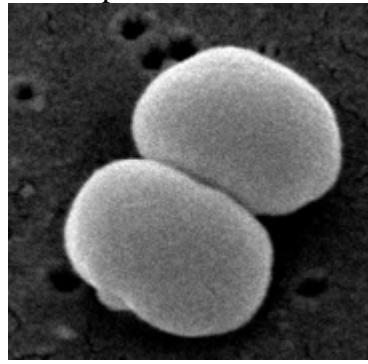
+ = common

+/- = rare

* = potential pathogen

Table 1 Notes

- (1) The staphylococci and corynebacteria occur at every site listed. *Staphylococcus epidermidis* is highly adapted to the diverse environments of its human host. *S. aureus* is a potential pathogen. It is a leading cause of bacterial disease in humans. It can be transmitted from the nasal membranes of an asymptomatic carrier to a susceptible host.

*S. epidermidis*. Scanning EM. CDC.

- (2) Many of the normal flora are either pathogens or opportunistic pathogens. The asterisks indicate members of the normal flora that may be considered major pathogens of humans.

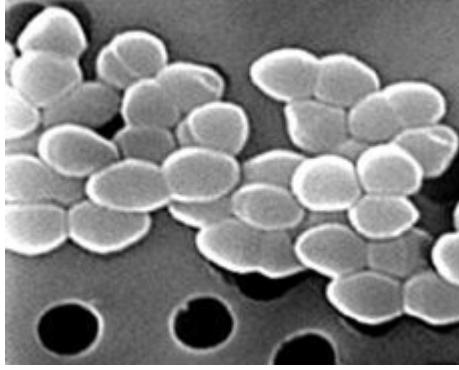
*S. aureus*. Gram stain.

- (3) *Streptococcus mutans* is the primary bacterium involved in plaque formation and initiation of dental caries. Viewed as an opportunistic infection, dental disease is one of the most prevalent and costly infectious diseases in the United States.



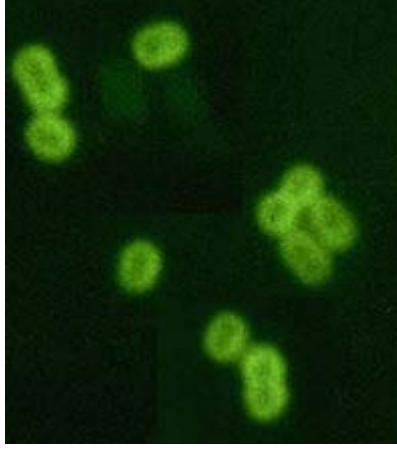
Streptococcus mutans. Gram stain. CDC

(4) *Enterococcus faecalis* was formerly classified as *Streptococcus faecalis*. The bacterium is such a regular a component of the intestinal flora, that many European countries use it as the standard indicator of fecal pollution, in the same way we use *E. coli* in the U.S. In recent years, *Enterococcus faecalis* has emerged as a significant, antibiotic-resistant, nosocomial pathogen.



Vancomycin Resistant *Enterococcus faecalis*. Scanning E.M. CDC

(5) *Streptococcus pneumoniae* is present in the upper respiratory tract of about half the population. If it invades the lower respiratory tract it can cause pneumonia. *Streptococcus pneumoniae* causes 95 percent of all bacterial pneumonia.



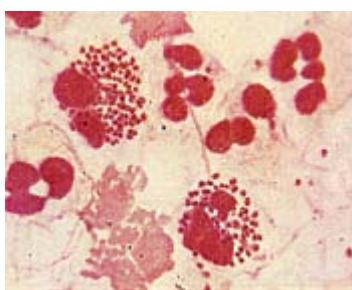
Streptococcus pneumoniae. Direct fluorescent antibody stain. CDC.

(6) *Streptococcus pyogenes* refers to the Group A, Beta-hemolytic streptococci.



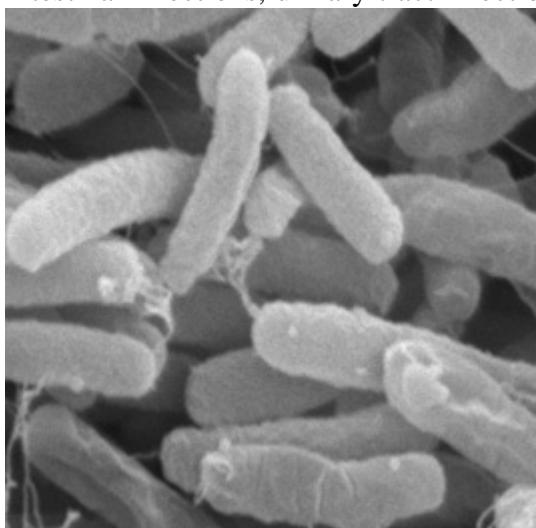
Streptococcus pyogenes. Gram stain.

(7) Gram-negative cocci, represented by various *Neisseria*, are frequent inhabitants of the upper respiratory tract, mainly the pharynx. *Neisseria meningitidis*, an important cause of bacterial meningitis, can colonize as well, until the host can develop active immunity against the pathogen.



Neisseria meningitidis. Gram stain.

(8) While *E. coli* is a consistent resident of the small intestine, many other enteric bacteria may reside here as well, including *Klebsiella*, *Enterobacter* and *Citrobacter*. Some strains of *E. coli* are pathogens that cause intestinal infections, urinary tract infections and neonatal meningitis.



E. coli. Scanning E.M. Shirley Owens. Center for Electron Optics. Michigan State University.

(9) *Pseudomonas aeruginosa* is the quintessential opportunistic pathogen of humans that can invade virtually any tissue. It is a leading cause of hospital-acquired (nosocomial) Gram-negative infections, but its source is often exogenous (from outside the host).



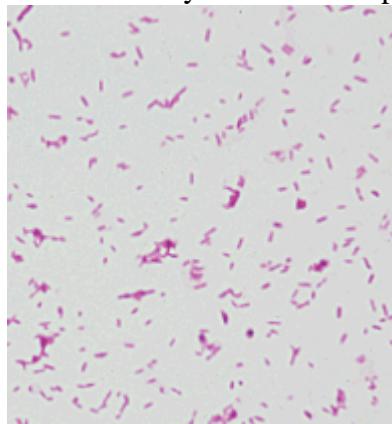
Colonies of *Pseudomonas aeruginosa* growing on an agar plate.

(10) *Haemophilus influenzae* is a frequent secondary invader to viral influenza, and was named accordingly. The bacterium was the leading cause of meningitis in infants and children until the recent development of the Hflu type B vaccine.



Haemophilus influenzae. Gram stain.

(11) The greatest number of bacteria are found in the lower intestinal tract, specifically the colon and the most prevalent bacteria are the *Bacteroides*, a group of Gram-negative, anaerobic, non-sporeforming bacteria. They have been implicated in the initiation colitis and colon cancer.



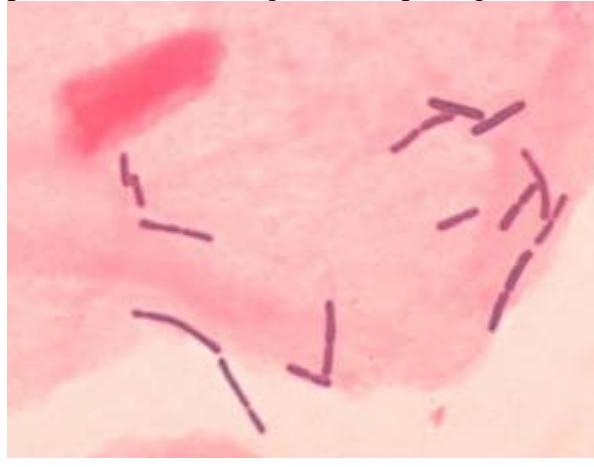
Bacteroides fragilis. Gram stain.

(12) Bifidobacteria are Gram-positive, non-sporeforming, lactic acid bacteria. They have been described as "friendly" bacteria in the intestine of humans. *Bifidobacterium bifidum* is the predominant bacterial species in the intestine of breast-fed infants, where it presumably prevents colonization by potential pathogens. These bacteria are sometimes used in the manufacture of yogurts and are frequently incorporated into probiotics.



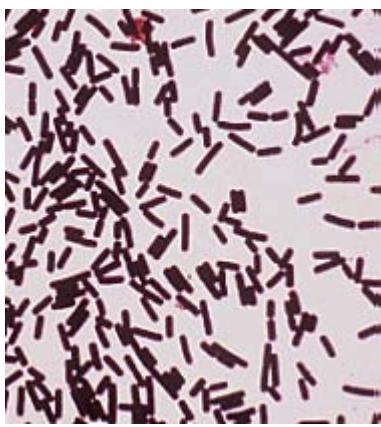
Bifidobacterium bifidum. Gram stain

(13) Lactobacilli in the oral cavity probably contribute to acid formation that leads to dental caries. *Lactobacillus acidophilus* colonizes the vaginal epithelium during child-bearing years and establishes the low pH that inhibits the growth of pathogens.



Lactobacillus species and a vaginal squamous epithelial cell. CDC

(14) There are numerous species of *Clostridium* that colonize the bowel. *Clostridium perfringens* is commonly isolated from feces. *Clostridium difficile* may colonize the bowel and cause "antibiotic-induced diarrhea" or pseudomembranous colitis.



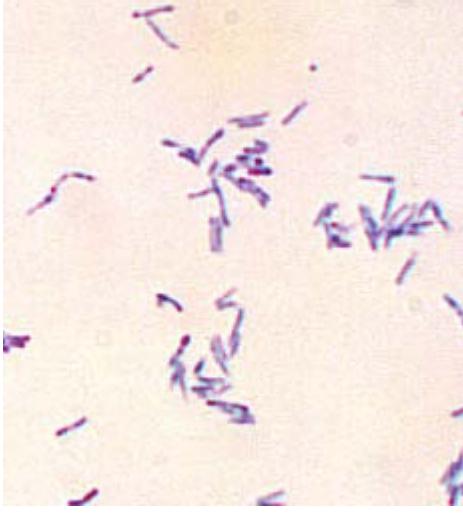
Clostridium perfringens. Gram stain.

(15) *Clostridium tetani* is included in the table as an example of a bacterium that is "transiently associated" with humans as a component of the normal flora. The bacterium can be isolated from feces of (up to) 25 percent of the population. The endospores are probably ingested with food and water, and the bacterium does not colonize the intestine.



Clostridium tetani. Gram stain.

(16) The corynebacteria, and certain related propionic acid bacteria, are consistent skin flora. Some have been implicated as a cause of acne. *Corynebacterium diphtheriae*, the agent of diphtheria, was considered a member of the normal flora before the widespread use of the diphtheria toxoid, which is used to immunize against the disease.



Corynebacterium diphtheriae. Methylene blue stain.

Very little is known about the nature of the associations between humans and their normal flora, but they are

thought to be dynamic interactions rather than associations of mutual indifference. Both host and bacteria are thought to derive benefit from each other, and the associations are, for the most part, **mutualistic**. The normal flora derives from the host a supply of nutrients, a stable environment and constant temperature, protection, and transport. The host obtains from the normal flora certain nutritional benefits, stimulation of the immune system, and colonization strategies that exclude potential pathogens at the site.

The normal flora are obviously adapted to their host (tissues), most probably by biochemical interactions between bacterial surface components (**ligands** or **adhesins**) and host cell molecular **receptors**. A great deal of information is available on the nature of adhesion of bacterial pathogens to animal cells and tissues, and reasonably similar mechanisms should apply to the normal flora.

In general, there are three explanations for why the normal bacterial flora are located at particular anatomical sites.

1. The normal flora exhibit a tissue preference or predilection for colonization. Certain species of bacteria are invariably in one locale and never in another (See Table 1 above). This is sometimes referred to as **tissue tropism** (See Table 2 below). One explanation for tissue tropism is that the host provides an essential growth factor needed by the bacterium. Of course, to explain why bacteria are not at an alternative site, the host inherently provides an inhospitable environment for the bacterium by the production of such substances as stomach acids, bile salts and lysozyme.
2. Many, perhaps most, of the normal flora are able to **specifically colonize a particular tissue** or surface using their own surface components (e.g. capsules, fimbriae, cell wall components, etc.) as specific ligands for attachment to specific receptors located at the colonization site (See Table 3)
3. Some of the indigenous bacteria are able to **construct bacterial biofilms** on a tissue surface, or they are able to colonize a biofilm built by another bacterial species. Many biofilms are a mixture of microbes, although one member is responsible for maintaining the biofilm and may predominate.

TABLE 2. EXAMPLES OF TISSUE TROPISM OF SOME BACTERIA ASSOCIATED WITH HUMANS

BACTERIUM	TISSUE
<i>Corynebacterium diphtheriae</i>	Throat
<i>Neisseria gonorrhoeae</i>	Urogenital epithelium
<i>Streptococcus mutans</i>	Tooth surfaces
<i>Streptococcus salivarius</i>	Tongue surfaces
<i>Vibrio cholerae</i>	Small intestine epithelium
<i>Escherichia coli</i>	Small intestine epithelium
<i>Staphylococcus aureus</i>	Nasal membranes
<i>Staphylococcus epidermidis</i>	Skin

TABLE 3. EXAMPLES OF SPECIFIC ATTACHMENTS OF BACTERIA TO HOST CELL OR TISSUE SURFACES

Bacterium	Bacterial ligand for attachment	Host cell or tissue receptor	Attachment site
<i>Streptococcus pyogenes</i>	Protein F	Amino terminus of fibronectin	Pharyngeal epithelium
<i>Streptococcus mutans</i>	Glycosyl transferase	Salivary glycoprotein	Pellicle of tooth
<i>Streptococcus salivarius</i>	Lipoteichoic acid	Unknown	Buccal epithelium of tongue

<i>Streptococcus pneumoniae</i>	Cell-bound protein	N-acetylhexosamine-galactose disaccharide	Mucosal epithelium
<i>Staphylococcus aureus</i>	Cell-bound protein	Amino terminus of fibronectin	Mucosal epithelium
<i>Neisseria gonorrhoeae</i>	N-methylphenyl-alanine pili	Glucosamine-galactose carbohydrate	Urethral/cervical epithelium
Enterotoxigenic <i>E. coli</i>	Type-1 fimbriae	Species-specific carbohydrate(s) (e.g. mannose)	Intestinal epithelium
Uropathogenic <i>E. coli</i>	Type 1 fimbriae	Complex carbohydrate	Urethral epithelium
Uropathogenic <i>E. coli</i>	P-pili (pap)	Globobiose linked to ceramide lipid	Upper urinary tract
<i>Bordetella pertussis</i>	Fimbriae ("filamentous hemagglutinin")	Galactose on sulfated glycolipids	Respiratory epithelium
<i>Vibrio cholerae</i>	N-methylphenylalanine pili	Fucose and mannose carbohydrate	Intestinal epithelium
<i>Treponema pallidum</i>	Peptide in outer membrane	Surface protein (fibronectin)	Mucosal epithelium
<i>Mycoplasma</i>	Membrane protein	Sialic acid	Respiratory epithelium
<i>Chlamydia</i>	Unknown	Sialic acid	Conjunctival or urethral epithelium

THE COMPOSITION OF THE NORMAL FLORA

The normal flora of corresponding anatomical sites in different animal species varies widely. Within a single species (e.g. humans) there is additional variation in the normal flora that is related to factors such as age, sex, diet and nutrition. Some bacteria are found regularly at particular anatomical locales; others are present only occasionally, or at certain times during life. Developmental changes in humans such as weaning, the eruption of the teeth, and the onset and cessation of ovarian functions, invariably affect the composition of the normal flora in the intestinal tract, the oral cavity, and the vagina, respectively. However, within the limits of these fluctuations, the bacterial flora of humans is sufficiently constant to give general description of the situation.

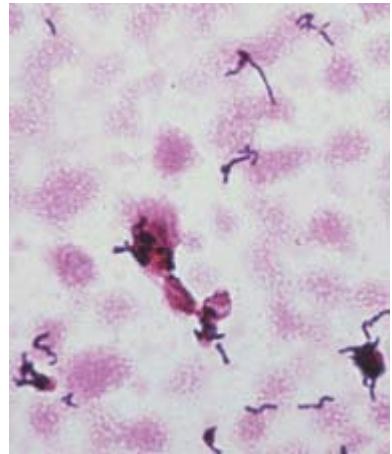
It has been calculated that the normal human houses about 10^{12} bacteria on the skin, 10^{10} in the mouth, and 10^{14} in the gastrointestinal tract. The latter number is far in excess of the number of eukaryotic cells in all organs which comprise the human host.

Normal Flora of the Skin. The adult human is covered with approximately 2 square meters of skin. The density and composition of the normal flora of the skin vary with anatomical locale. The high moisture content of the axilla, groin, and areas between the toes supports the activity and growth of relatively high densities of bacterial cells, but the density of bacterial populations at most other sites is fairly low, generally in 100s or 1000s per square cm. Qualitatively, the bacteria on the skin near any body orifice may be similar to those in the orifice.

The majority of skin microorganisms are found in the most superficial layers of the epidermis and the upper parts of the hair follicles. They consist largely of micrococci (*Staphylococcus epidermidis* and *Micrococcus* sp.) and corynebacteria. These are generally nonpathogenic and considered to be commensal, although mutualistic and parasitic roles have been assigned to them. Sometimes potentially pathogenic *Staphylococcus aureus* is found on the face and hands, particularly in individuals who are nasal carriers.

Normal Flora of the Conjunctiva. A variety of bacteria may be cultivated from the normal conjunctiva but the number of organisms is usually small. *Staphylococcus epidermidis* and certain coryneforms (*Propionibacterium acnes*) are dominant. *Staphylococcus aureus*, some streptococci, *Haemophilus* sp. and *Neisseria* sp. are occasionally found. The conjunctiva is kept moist and healthy by the continuous secretions from the lachrymal glands. Blinking wipes the conjunctiva every few seconds mechanically washing away foreign objects including bacteria. Lachrymal secretions (tears) also contain bactericidal substances including lysozyme. There is little or no opportunity for

microorganisms to colonize the conjunctiva without special mechanisms to attach to the epithelial surfaces and some ability to withstand attack by lysozyme. Pathogens which do infect the conjunctiva (e.g. *Neisseria gonorrhoeae* and *Chlamydia trachomatis*) are thought to be able to specifically attach to the conjunctival epithelium by means of sialic acid receptors on epithelial cells, but this is not certain.

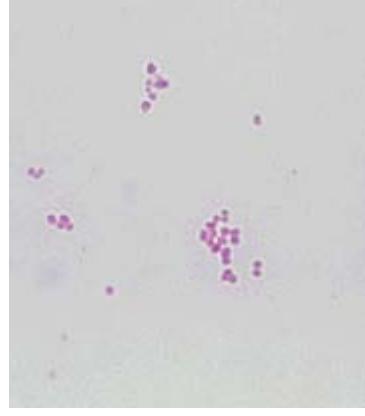


Propionibacterium acnes

Normal Flora of the Respiratory Tract. The nares (nostrils) are always heavily colonized, predominantly with *Staphylococcus epidermidis* and corynebacteria, and often (about 20% of the general population) with *Staphylococcus aureus*, this being the main carrier site of this important pathogen. The healthy sinuses, in contrast are sterile. A large number of bacterial species colonize the upper respiratory tract (nasopharynx). The predominant species are non-hemolytic and alpha-hemolytic streptococci and *Neisseria*, but sometimes pathogens such as *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Haemophilus influenzae* and *Neisseria meningitidis* colonize the pharynx.



Moraxella catarrhalis. Gram stain.

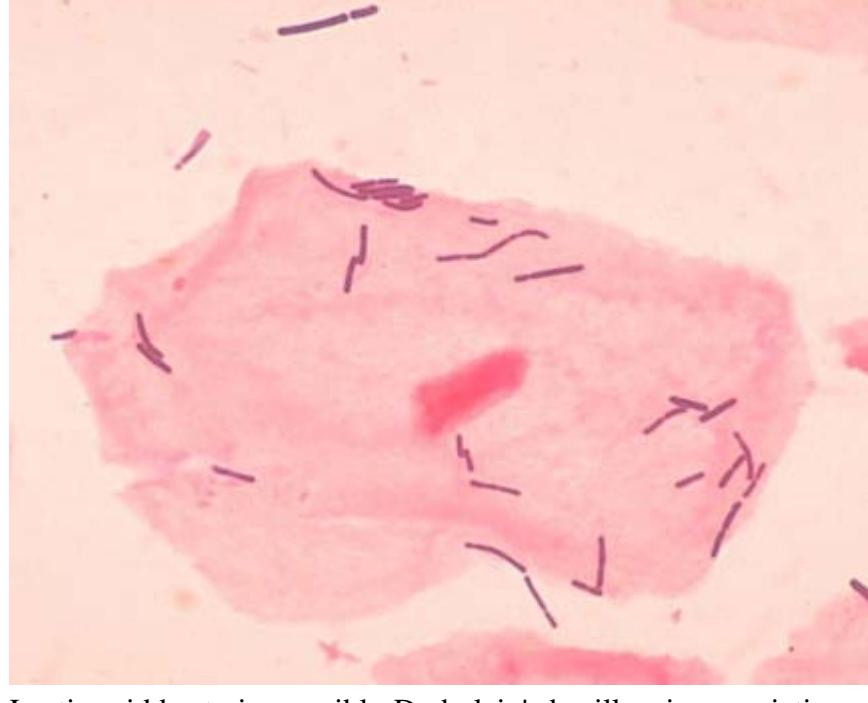


Veilonella. Gram stain.

The lower respiratory tract (trachea, bronchi, and pulmonary tissues) are virtually free of microorganisms, mainly because of the efficient cleansing action of the ciliated epithelium which lines the tract. Any bacteria reaching the lower respiratory tract are swept upward by the action of the mucociliary blanket that lines the bronchi, to be removed subsequently by coughing, sneezing, swallowing, etc. If the respiratory tract epithelium becomes damaged, as in bronchitis or viral pneumonia, the individual may become susceptible to infection by pathogens descending from the nasopharynx (e.g. *H. influenzae* or *S. pneumoniae*). The pathogen *Bordetella pertussis* is specifically able to colonize the tracheal epithelium of humans, allowing it to produce the disease, pertussis (whooping cough).

Normal flora of the Urogenital Tract. Urine is normally sterile, and since the urinary tract is flushed with urine every few hours, microorganisms have problems gaining access and becoming established. The flora of the anterior urethra, as indicated principally by urine cultures, suggests that the area may be inhabited by a relatively consistent normal flora consisting of *Staphylococcus epidermidis*, *Enterococcus faecalis* and some alpha-hemolytic streptococci. Their numbers are not plentiful, however. In addition, some enteric bacteria (e.g. *E. coli*, *Proteus*) and corynebacteria, which are probably contaminants from the skin, vulva or rectum, may occasionally be found at the anterior urethra.

The vagina becomes colonized soon after birth with corynebacteria, staphylococci, nonpyogenic streptococci, *E. coli*, and a lactic acid bacterium historically named "Doderlein's bacillus" (*Lactobacillus acidophilus*). During reproductive life, from puberty to menopause, the vaginal epithelium contains glycogen due to the actions of circulating estrogens. Doderlein's bacillus predominates, being able to metabolize the glycogen to lactic acid. The lactic acid and other products of metabolism inhibit colonization by all except Doderlein's bacillus and a select number of lactic acid bacteria. The resulting low pH of the vaginal epithelium prevents establishment of most bacteria as well as the potentially-pathogenic yeast, *Candida albicans*. This is a striking example of the protective effect of the normal bacterial flora for their human host.

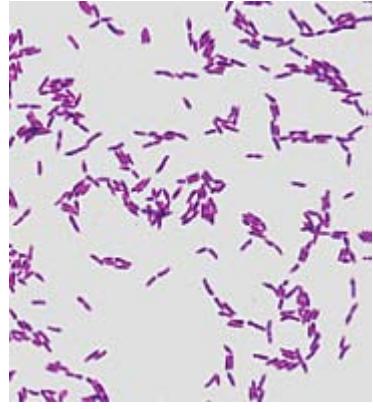


Lactic acid bacteria, possibly Doderlein's bacillus, in association with a vaginal epithelial cell.

Normal Flora of the Human Oral Cavity. The presence of nutrients, epithelial debris, and secretions makes the mouth a favorable habitat for a great variety of bacteria. Oral bacteria include streptococci, lactobacilli, staphylococci and corynebacteria, with a great number of anaerobes, especially bacteroides.

The mouth presents a succession of different ecological situations with age, and this corresponds with changes in the composition of the normal flora. At birth the oral cavity is composed solely of the soft tissues of the lips, cheeks, tongue and palate, which are kept moist by the secretions of the salivary glands. At birth the oral cavity is sterile but rapidly becomes colonized from the environment, particularly from the mother in the first feeding.

Streptococcus salivarius is dominant and may make up 98% of the total oral flora until the appearance of the teeth (6 - 9 months in humans). The eruption of the teeth during the first year leads to colonization by *S. mutans* and *S. sanguis*. These bacteria require a nondesquamating (nonepithelial) surface in order to colonize. They will persist as long as teeth remain. Other strains of streptococci adhere strongly to the gums and cheeks but not to the teeth. The creation of the gingival crevice area (supporting structures of the teeth) increases the habitat for the variety of anaerobic species found. The complexity of the oral flora continues to increase with time, and *Bacteroides* and spirochetes colonize around puberty.



Lactobacillus acidophilus. Gram Stain.

Clearly, the normal bacterial flora of the oral cavity benefit from their associations with their host. Are there benefits as well to the host? Perhaps. The normal flora occupy available colonization sites which makes it more difficult for other microorganisms (nonindigenous species) to become established. Also, the oral flora contribute to host nutrition through the synthesis of vitamins, and they contribute to immunity by inducing low levels of circulating and secretory antibodies that may cross react with pathogens. Finally, the oral bacteria exert microbial antagonism against nonindigenous species by production of inhibitory fatty acids, peroxides, bacteriocins, etc.

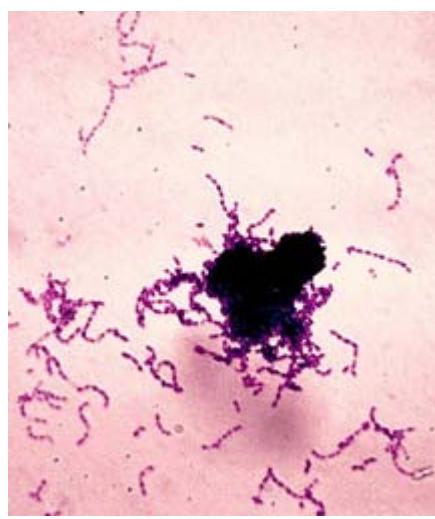
The oral flora of humans may harm their host since some of these bacteria are parasites or opportunistic pathogens. If certain oral bacteria are able to invade tissues not normally accessible to them, characteristic diseases result. For example, oral organisms gaining entrance into tissues (e.g. via surgical wounds) may cause abscesses of alveolar bone, lung, brain or the extremities. Such infections usually contain mixtures of bacteria with *Bacteroides melaninogenicus* often playing a dominant role. Also, oral streptococci may be introduced into wounds created by dental manipulation or treatment. If this occurs in an individual with damaged heart valves due to rheumatic fever (previously induced by streptococci), the oral streptococci may adhere to the damaged heart valves and initiate subacute bacterial endocarditis.

DENTAL PLAQUE, DENTAL CARIES AND PERIODONTAL DISEASE

Dental plaque, dental caries and periodontal disease in humans also result from actions initiated by the normal bacterial flora. This is arguably the most significant and costly negative effect resulting from human symbioses with bacteria.

Dental Plaque, which is material adhering to the teeth, consists of bacterial cells (60-70% the volume of the plaque), salivary polymers, and bacterial extracellular products. Plaque is a naturally-constructed biofilm, in which the consortia of bacteria may reach a thickness of 300-500 cells on the surfaces of the teeth. These accumulations subject the teeth and gingival tissues to high concentrations of bacterial metabolites, which result in dental disease.

By far the dominant bacterial species in dental plaque are *Streptococcus sanguis* and *Streptococcus mutans*, both of which are considered responsible for plaque.



Streptococcus mutans. Gram stain. CDC.

Plaque formation is initiated by a weak attachment of the streptococcal cells to salivary glycoproteins forming a pellicle on the surface of the teeth. This is followed by a stronger attachment by means of extracellular sticky polymers of glucose (glucans) which are synthesized by the bacteria from dietary sugars (principally sucrose). An enzyme on the cell surface of *Streptococcus mutans*, glycosyl transferase, is apparently involved in initial attachment of the bacterial cells to the tooth surface and in the conversion of sucrose to dextran and levan polymers (glucans) which form the extracellular matrix of plaque. Attachment of *S. mutans* and the formation of glucans is mediated by glycosyl transferase. The specificity of the adhesion has been proven by the fact that the attachment can be prevented by specific antibody to the enzyme.

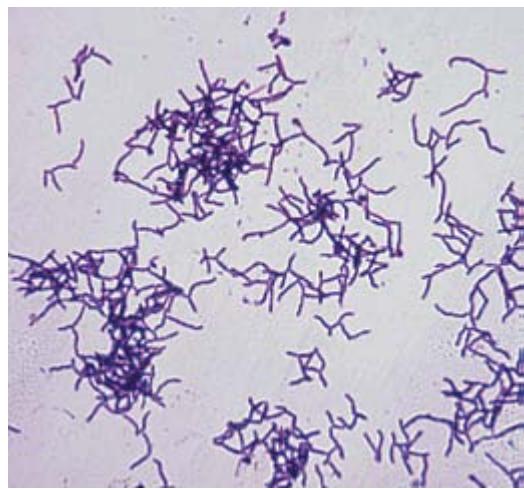
Dental Caries is the destruction of the enamel, dentin or cementum of teeth due to bacterial activities. Caries are initiated by direct demineralization of the enamel of teeth due to lactic acid and other organic acids which accumulate in dental plaque. Lactic acid bacteria in the plaque produce lactic acid from the fermentation of sugars and other carbohydrates in the diet of the host. *Streptococcus mutans* has most consistently been associated with the initiation of dental caries, but other lactic acid bacteria are probably involved as well. These organisms normally colonize the occlusal fissures and contact points between the teeth, and this correlates with the incidence of decay on these surfaces.

Streptococcus mutans has a number of physiological and biochemical properties which implicate it in the initiation of dental caries.

1. It is a regular component of the normal oral flora of humans which occurs in relatively large numbers. It readily colonizes tooth surfaces: salivary components (mucins, which are glycoproteins) form a thin film on the tooth called the enamel pellicle. The adsorbed mucins are thought to serve as molecular receptors for ligands on the bacterial cell surface.
2. It contains the enzyme glycosyl transferase that probably serves as the bacterial ligand for attachment, and that polymerizes glucose obtained from dietary sucrose to glucans which leads directly to the formation of plaque.
3. It produces lactic acid from the utilization of dietary carbohydrate which demineralizes tooth enamel. *S. mutans* produces more lactic acid and is more acid-tolerant than most other streptococci.
4. It stores polysaccharides made from dietary sugars which can be utilized as reserve carbon and energy sources for production of lactic acid. The extracellular glucans formed by *S. mutans* are, in fact, bacterial capsular polysaccharides that function as carbohydrate reserves. The organisms can also form intracellular polysaccharides from sugars which are stored in cells and then metabolized to lactic acid.

Streptococcus mutans appears to be important in the initiation of dental caries because its activities lead to colonization of the tooth surfaces, plaque formation, and localized demineralization of tooth enamel. It is not however, the only cause of dental decay. After initial weakening of the enamel, various oral bacteria gain access to interior regions of the tooth. *Lactobacilli*, *Actinomyces*, and various proteolytic bacteria are commonly found in human carious dentin and cementum, which suggests that they are secondary invaders that contribute to the

progression of the lesions.



Actinomyces israelii

Periodontal Diseases are bacterial infections that affect the supporting structures of the teeth (gingiva, cementum, periodontal membrane and alveolar bone). The most common form, **gingivitis**, is an inflammatory condition of the gums. It is associated with accumulations of bacterial plaque in the area. Increased populations of *Actinomyces* have been found, and they have been suggested as the cause.

Diseases that are confined to the gum usually do not lead to loss of teeth, but there are other more serious forms of periodontal disease that affect periodontal membrane and alveolar bone resulting in tooth loss. Bacteria in these lesions are very complex populations consisting of Gram-positive organisms (including *Actinomyces* and *streptococci*) and Gram-negative organisms (including spirochetes and *Bacteroides*). The mechanisms of tissue destruction in periodontal disease are not clearly defined but hydrolytic enzymes, endotoxins, and other toxic bacterial metabolites seem to be involved.

TABLE 4. FREQUENTLY ENCOUNTERED BACTERIA IN PLAQUE, DENTAL CARIES, GINGIVITIS AND PERIODONTITIS

BACTERIUM	Plaque	Dental caries	Gingivitis	Periodontitis
<i>Streptococcus sanguis</i>	++	++	++	+
<i>S. mutans</i>	++	++	0	0
<i>S. salivarius</i>	0	0	0	0
<i>Actinomyces viscosis</i>	+	+	++	+
<i>A. israelii</i>	+	+	++	++
<i>Lactobacillus</i> sp.	+	+	0	0
<i>Propionibacterium acnes</i>	0	+	+	++
<i>Bacteroides</i> sp.	0	0	+	++
<i>Selenomonas sputigena</i>	0	0	+	++
Large spirochetes	0	0	0	++

++ = Frequently encountered in high proportions; + = Frequently encountered in low to moderate proportions; 0 = Sometimes encountered in low proportions or not detectable.

Modified from Davis, et al.: Microbiology. 4th ed. J. B. Lippincott. Philadelphia, 1990.

Normal Flora of the Gastrointestinal Tract.



Colonies of *E. coli* growing on EMB agar.

The bacterial flora of the GI tract of animals has been studied more extensively than that of any other site. The composition differs between various animal species, and within an animal species. In humans, there are differences in the composition of the flora which are influenced by age, diet, cultural conditions, and the use of antibiotics. The latter greatly perturbs the composition of the intestinal flora. The following table shows the distribution of some common intestinal bacteria in various animal species including humans.

TABLE 5. NUMBERS OF VIABLE BACTERIA FOUND IN THE FECES OF ADULT ANIMALS (Log # viable cells per gram feces) *

Animal	<i>E. coli</i>	<i>C. perfringens</i>	Enterococci	<i>Bacteroides</i>	Lactobacilli
Cattle	4.3	2.3	5.3	0	2.4
Sheep	6.5	4.3	6.1	0	3.9
Horses	4.1	0	6.8	0	7.0
Pigs	6.5	3.6	6.4	5.7	8.4
Chickens	6.6	2.4	7.5	0	8.5
Rabbits	2.7	0	4.3	8.6	0
Dogs	7.5	8.4	7.6	8.7	4.6
Cats	7.6	7.4	8.3	8.9	8.8
Mice	6.8	0	7.9	8.9	9.1
Humans	6.7	3.2	5.2	9.7	8.8

* Median values from 10 animals

Modified from Rosebury, T. : Microorganisms Indigenous to Man. McGraw-Hill. New York. 1962.

In the upper GI tract of adult humans, the esophagus contains only the bacteria swallowed with saliva and food. Because of the high acidity of the gastric juice very few bacteria (mainly acid-tolerant lactobacilli) can be cultured from the normal stomach. However, at least half the population in the United States is colonized by a pathogenic bacterium, *Helicobacter pylori*. Since the 1980s, this bacterium has been known to be the cause of gastric ulcers, and it is probably a cause of gastric and duodenal cancer as well.



Helicobacter pylori. ASM

The proximal small intestine has a relatively sparse Gram-positive flora, consisting mainly of lactobacilli and

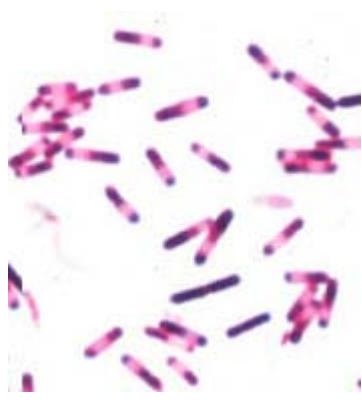
Enterococcus faecalis. This region has about 10^5 - 10^7 bacteria per ml of fluid. The distal part of the small intestine contains greater numbers of bacteria (10^8 /ml) and additional species including coliforms and *Bacteroides*, in addition to lactobacilli and enterococci. The flora of the large intestine (colon) is qualitatively similar to that found in feces. Populations of bacteria in the colon reach levels of 10^{11} /ml feces. Coliforms become more prominent, and enterococci, clostridia and lactobacilli can be regularly found, but the predominant species are anaerobic *Bacteroides* and anaerobic lactic acid bacteria in the genus *Bifidobacterium* (*Bifidobacterium bifidum*). These organisms may outnumber *E. coli* by 1,000:1 to 10,000:1. It is now known that significant numbers of anaerobic methanogenic bacteria (up to 10^{10} /gm) also reside in the colon of humans. The range of incidence of certain bacteria in the large intestine of humans is shown below.

TABLE 6. BACTERIA FOUND IN THE LARGE INTESTINE OF HUMANS

BACTERIUM	Range of Incidence
<i>Bacteroides fragilis</i>	100
<i>Bacteroides melaninogenicus</i>	100
<i>Bacteroides oralis</i>	100
<i>Lactobacillus</i>	20-60
<i>Clostridium perfringens</i>	25-35
<i>Clostridium septicum</i>	5-25
<i>Clostridium tetani</i>	1-35
<i>Bifidobacterium bifidum</i>	30-70
<i>Staphylococcus aureus</i>	30-50
<i>Enterococcus faecalis</i>	100
<i>Escherichia coli</i>	100
<i>Salmonella enteritidis</i>	3-7
<i>Salmonella typhi</i>	0.00001
<i>Klebsiella sp.</i>	40-80
<i>Enterobacter sp.</i>	40-80
<i>Proteus mirabilis</i>	5-55
<i>Pseudomonas aeruginosa</i>	3-11
<i>Peptostreptococcus sp.</i>	common
<i>Peptococcus sp.</i>	moderate
Methanogens (Archaea)	common

Modified from Youmans, et al.: The Biologic and Clinical Basis of Infectious Disease. W. B. Saunders Co. Philadelphia. 1985.

At birth the entire intestinal tract is sterile, but bacteria enter with the first feed. The initial colonizing bacteria vary with the food source of the infant. In breast-fed infants bifidobacteria account for more than 90% of the total intestinal bacteria. *Enterobacteriaceae* and enterococci are regularly present, but in low proportions, while *bacteroides*, *staphylococci*, *lactobacilli* and *clostridia* are practically absent. In bottle-fed infants, bifidobacteria are not predominant. When breast-fed infants are switched to a diet of cow's milk or solid food, bifidobacteria are progressively joined by enterics, *bacteroides*, enterococci *lactobacilli* and *clostridia*. Apparently, human milk contains a growth factor that enriches for growth of bifidobacteria, and these bacteria play an important role in preventing colonization of the infant intestinal tract by non indigenous or pathogenic species.



Clostridium difficile. Gram stain.

The composition of the flora of the gastrointestinal tract varies along the tract (at longitudinal levels) and across the tract (at horizontal levels) where certain bacteria attach to the gastrointestinal epithelium and others occur in the lumen. There is frequently a very close association between specific bacteria in the intestinal ecosystem and specific gut tissues or cells (evidence of tissue tropism). Many bacteria adhere specifically to the gastrointestinal epithelial surfaces, and this has been shown in many animal species including humans, cows, dogs, pigs, mice and chickens. Gram-positive bacteria, such as the streptococci and lactobacilli, are thought to adhere to the gastrointestinal epithelium using polysaccharide capsules or wall lipoteichoic acids to attach to specific receptors on the epithelial cells. Likewise, Gram-negative bacteria such as the enterics may attach by means of specific fimbriae on the bacterial cell which bind to glycoproteins on the epithelial cell surface.

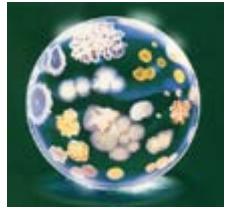
THE BENEFITS OF THE NORMAL FLORA

The indigenous bacteria of the gastrointestinal tract of an animal, perhaps mainly as a consequence of their great numbers, seem to have the greatest overall impact on their host. The nature of the interactions between an animal host and its normal flora has been inferred from the study of **germ-free animals** (animals which lack any bacterial flora) compared to **conventional animals** (animals which have a typical normal flora). Following are the primary beneficial effects of the normal flora that are derived from these studies.

- 1. The normal flora synthesize and excrete vitamins** in excess of their own needs, which can be absorbed as nutrients by the host. For example, enteric bacteria secrete Vitamin K and Vitamin B12, and lactic acid bacteria produce certain B-vitamins. Germ-free animals may be deficient in Vitamin K to the extent that it is necessary to supplement their diets.
- 2. The normal flora prevent colonization by pathogens** by competing for attachment sites or for essential nutrients. This is thought to be their most important beneficial effect, which has been demonstrated in the oral cavity, the intestine, the skin, and the vaginal epithelium. In some experiments, germ-free animals can be infected by 10 *Salmonella* bacteria, while the infectious dose for conventional animals is near 10^6 cells.
- 3. The normal flora may antagonize other bacteria** through the production of substances which inhibit or kill nonindigenous species. The intestinal bacteria produce a variety of substances ranging from relatively nonspecific fatty acids and peroxides to highly specific bacteriocins, which inhibit or kill other bacteria.
- 4. The normal flora stimulate the development of certain tissues**, i.e., the caecum and certain lymphatic tissues (Peyer's patches) in the GI tract. The caecum of germ-free animals is enlarged, thin-walled, and fluid-filled, compared to that organ in conventional animals. Also, based on the ability to undergo immunological stimulation, the intestinal lymphatic tissues of germ-free animals are poorly-developed compared to conventional animals.
- 5. The normal flora stimulate the production of cross-reactive antibodies.** Since the normal flora behave as antigens in an animal, they induce an immunological response, in particular, an antibody-mediated immune (AMI) response. Low levels of antibodies produced against components of the normal flora are known to cross react with certain related pathogens, and thereby prevent infection or invasion. Antibodies produced against antigenic components of the normal flora are sometimes referred to as "natural" antibodies, and such antibodies are lacking in germ-free animals.

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The Mechanisms of Bacterial Pathogenicity

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Introduction

A **pathogen** is a microorganism that is able to cause disease in a plant, animal or insect. **Pathogenicity** is the ability to produce disease in a host organism. Microbes express their pathogenicity by means of their **virulence**, a term which refers to the **degree of pathogenicity** of the microbe. Hence the **determinants of virulence** of a pathogen are any of its **genetic** or **biochemical** or **structural** features that enable it to produce disease. in a host.

The relationship between a host and a pathogen is dynamic, since each modifies the activities and functions of the other. The outcome of an infection depends on the **virulence of the pathogen** and the relative degree of resistance or susceptibility of the host, due mainly to the effectiveness of the **host defense mechanisms**.

The Underlying Mechanisms of Bacterial Pathogenicity

Two broad qualities of pathogenic bacteria underlie the means by which they cause disease:

1. The ability to invade tissues: Invasiveness, which encompasses mechanisms for **colonization** (adherence and initial multiplication), **ability to bypass or overcome host defense mechanisms**, and **the production of extracellular substances which facilitate invasion**.

2. The ability to produce toxins: Toxigenesis. Bacteria produce two types of toxins called **exotoxins** and **endotoxins**. Exotoxins are released from bacterial cells and may act at tissue sites removed from the site of bacterial growth. Endotoxins are cell-associated substances that are structural components of the cell walls of Gram-negative bacteria. However, endotoxins may be released from growing bacterial cells or from cells which are lysed as a result of effective host defense (e.g. lysozyme) or the activities of certain antibiotics (e.g. penicillins and cephalosporins). Hence, bacterial toxins, both soluble and cell-associated, may be transported by blood and lymph and cause cytotoxic effects at tissue sites remote from the original point of invasion or growth. Some bacterial toxins may also act at the site of colonization and play a role in invasion.

COLONIZATION

The first stage of microbial infection is **colonization**: the establishment of the pathogen at the appropriate portal of entry. Pathogens usually colonize host tissues that are in contact with the external environment. Sites of entry in human hosts include the urogenital tract, the digestive tract, the respiratory tract and the conjunctiva. Organisms that infect these regions have usually developed tissue adherence mechanisms and some ability to overcome or withstand the constant pressure of the host defenses on the surface.

Bacterial Adherence to Mucosal Surfaces. In its simplest form, bacterial adherence or attachment to a eukaryotic cell or tissue surface requires the participation of two factors: a **receptor** and an **adhesin**. The receptors so far defined are usually specific carbohydrate or peptide residues on the eukaryotic cell surface. The bacterial adhesin is typically a macromolecular component of the bacterial cell surface which interacts with the host cell receptor. Adhesins and receptors usually interact in a complementary and specific fashion. Table 1 is a list of terms that are used in medical microbiology to refer to microbial adherence to surfaces or tissues.

TABLE 1. TERMS USED TO DESCRIBE ADHERENCE FACTORS IN HOST-PARASITE

INTERACTIONS

ADHERENCE FACTOR	DESCRIPTION
Adhesin	A surface structure or macromolecule that binds a bacterium to a specific surface
Receptor	A complementary macromolecular binding site on a (eukaryotic) surface that binds specific adhesins or ligands
Lectin	Any protein that binds to a carbohydrate
Ligand	A surface molecule that exhibits specific binding to a receptor molecule on another surface
Mucous	The mucopolysaccharide layer of glucosaminoglycans covering animal cell mucosal surfaces
Fimbriae	Filamentous proteins on the surface of bacterial cells that may behave as adhesins for specific adherence
Common pili	Same as fimbriae
Sex pilus	A specialized pilus that binds mating prokaryotes together for the purpose of DNA transfer
Type 1 fimbriae	Fimbriae in <i>Enterobacteriaceae</i> which bind specifically to mannose terminated glycoproteins on eukaryotic cell surfaces
Glycocalyx	A layer of exopolysaccharide fibers on the surface of bacterial cells which may be involved in adherence to a surface
Capsule	A detectable layer of polysaccharide (rarely polypeptide) on the surface of a bacterial cell which may mediate specific or nonspecific attachment
Lipopolysaccharide (LPS)	A distinct cell wall component of the outer membrane of Gram-negative bacteria with the potential structural diversity to mediate specific adherence. Probably functions as an adhesin
Teichoic acids and lipoteichoic acids (LTA)	Cell wall components of Gram-positive bacteria that may be involved in nonspecific or specific adherence

Specific Adherence of Bacteria to Cell and Tissue Surfaces

Several types of observations provide indirect evidence for **specificity of adherence** of bacteria to host cells or tissues:

1. **Tissue tropism**: particular bacteria are known to have an apparent preference for certain tissues over others, e.g. *S. mutans* is abundant in dental plaque but does not occur on epithelial surfaces of the tongue; the reverse is true for *S. salivarius* which is attached in high numbers to epithelial cells of the tongue but is absent in dental plaque.

2. **Species specificity**: certain pathogenic bacteria infect only certain species of animals, e.g. *N. gonorrhoeae* infections are limited to humans; Enteropathogenic *E. coli* K-88 infections are limited to pigs; *E. coli* CFA I and CFA II infect humans; *E. coli* K-99 strain infects calves.; Group A streptococcal infections occur only in humans.

3. **Genetic specificity within a species**: certain strains or races within a species are genetically immune to a pathogen , e.g. Certain pigs are not susceptible to *E. coli* K-88 infections; Susceptibility to *Plasmodium vivax* infection (malaria) is dependent on the presence of the Duffy antigens on the host's redblood cells.

Although other explanations are possible, the above observations might be explained by the existence of specific interactions between microorganisms and eukaryotic tissue surfaces which allow microorganisms to become established on the surface.

Mechanisms of Adherence to Cell or Tissue Surfaces

The mechanisms for adherence may involve two steps:

1. **nonspecific adherence: reversible attachment** of the bacterium to the eukaryotic surface (sometimes called

"docking")

2. specific adherence: reversible permanent attachment of the microorganism to the surface (sometimes called "anchoring").

The usual situation is that reversible attachment precedes irreversible attachment but in some cases, the opposite situation occurs or specific adherence may never occur

Nonspecific adherence involves nonspecific attractive forces which allow approach of the bacterium to the eukaryotic cell surface. Possible interactions and forces involved are:

1. hydrophobic interactions
2. electrostatic attractions
3. atomic and molecular vibrations resulting from fluctuating dipoles of similar frequencies
4. Brownian movement
5. recruitment and trapping by biofilm polymers interacting with the bacterial glycocalyx (capsule)

Specific adherence involves permanent formation of many specific lock-and-key bonds between complementary molecules on each cell surface. Complementary receptor and adhesin molecules must be accessible and arranged in such a way that many bonds form over the area of contact between the two cells. Once the bonds are formed, attachment under physiological conditions becomes virtually irreversible.

Several types of experiments provide **direct evidence that receptor and/or adhesin molecules mediate specificity** of adherence of bacteria to host cells or tissues. These include:

1. The bacteria will bind isolated receptors or receptor analogs.
2. The isolated adhesins or adhesin analogs will bind to the eukaryotic cell surface.
3. Adhesion (of the bacterium to the eukaryotic cell surface) is inhibited by:
 - a. isolated adhesin or receptor molecules
 - b. adhesin or receptor analogs
 - c. enzymes and chemicals that specifically destroy adhesins or receptors
 - d. antibodies specific to surface components (i.e., adhesins or receptors)

Some Specific Bacterial Adhesins and their Receptors

The adhesins of *E. coli* are their common pili or fimbriae. A single strain of *E. coli* is known to be able to express several distinct types of fimbriae encoded by distinct regions of the chromosome or plasmids. This genetic diversity permits an organism to adapt to its changing environment and exploit new opportunities presented by different host surfaces. Many of the adhesive fimbriae of *E. coli* have probably evolved from fimbrial ancestors resembling Type-1 and type 4 fimbriae.

Type-1 fimbriae enable *E. coli* to bind to D-mannose residues on eukaryotic cell surfaces. Type-1 fimbriae are said to be "mannose-sensitive" since exogenous mannose blocks binding to receptors on red blood cells. Although the primary 17kDa fimbrial subunit is the major protein component of Type-1 fimbriae, the mannose-binding site is not located here, but resides in a minor protein (28-31kDa) located at the tips or inserted along the length of the fimbriae. By genetically varying the minor "tip protein" adhesin, the organisms can gain ability to adhere to different receptors. For example, tip proteins on pyelonephritis-associated (pap) pili recognize a galactose-galactose disaccharide, while tip proteins on S-fimbriae recognize sialic acid.

Pseudomonas, Vibrio and *Neisseria* possess a fimbrial protein subunit which contains methylated phenylalanine at

its amino terminus. These "N-methylphenylalanine pili" have been established as virulence determinants in pathogenesis of *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients. These type of fimbriae occur in *Neisseria gonorrhoeae* and their receptor is thought to be an oligosaccharide.

The adhesins of *Streptococcus pyogenes* are controversial. In 1972, Gibbons and his colleagues demonstrated that attachment of streptococci to the oral mucosa of mice is dependent on M protein. Olfek and Beachey argued that lipoteichoic acid (LTA), rather than M protein, was responsible for streptococcal adherence to buccal epithelial cells. In 1996, Hasty and Courtney proposed a two-step model of attachment that involved both M protein and teichoic acids. They suggested that LTA loosely tethers streptococci to epithelial cells, and then M protein secures a firmer, irreversible association. In 1992, protein F was discovered and found to be a fibronectin binding protein. More recently, in 1998, M proteins M1 and M3 were also found to bind to fibronectin. Apparently, *S. pyogenes* produces multiple adhesins with varied specificities.

Staphylococcus aureus also binds to the amino terminus of fibronectin by means of a fibronectin-binding protein which occurs on the bacterial surface. Apparently *S. aureus* and Group A streptococci use different mechanisms but adhere to the same receptor on epithelial surfaces.

Treponema pallidum has three related surface adhesins (P1, P2 and P3) which bind to a four-amino acid sequence (Arg-Gly-Asp-Ser) of the cell-binding domain of fibronectin. It is not clear if *T. pallidum* uses fibronectin to attach to host surfaces or coats itself with fibronectin to avoid host defenses (phagocytes and immune responses).

TABLE 2. EXAMPLES OF SPECIFIC ATTACHMENTS OF BACTERIA TO HOST CELL OR TISSUE SURFACES

Bacterium	Adhesin	Receptor	Attachment site	Disease
<i>Streptococcus pyogenes</i>	Protein F	Amino terminus of fibronectin	Pharyngeal epithelium	Sore throat
<i>Streptococcus mutans</i>	Glycosyl transferase	Salivary glycoprotein	Pellicle of tooth	Dental caries
<i>Streptococcus salivarius</i>	Lipoteichoic acid	Unknown	Buccal epithelium of tongue	None
<i>Streptococcus pneumoniae</i>	Cell-bound protein	N-acetylhexosamine-galactose disaccharide	Mucosal epithelium	pneumonia
<i>Staphylococcus aureus</i>	Cell-bound protein	Amino terminus of fibronectin	Mucosal epithelium	Various
<i>Neisseria gonorrhoeae</i>	N-methylphenylalanine pili	Glucosamine-galactose carbohydrate	Urethral/cervical epithelium	Gonorrhea
<i>Enterotoxigenic E. coli</i>	Type-1 fimbriae	Species-specific carbohydrate(s)	Intestinal epithelium	Diarrhea
<i>Uropathogenic E. coli</i>	Type 1 fimbriae	Complex carbohydrate	Urethral epithelium	Urethritis
<i>Uropathogenic E. coli</i>	P-pili (pap)	Globobiose linked to ceramide lipid	Upper urinary tract	Pyelonephritis
<i>Bordetella pertussis</i>	Fimbriae ("filamentous hemagglutinin")	Galactose on sulfated glycolipids	Respiratory epithelium	Whooping cough
<i>Vibrio cholerae</i>	N-methylphenylalanine pili	Fucose and mannose carbohydrate	Intestinal epithelium	Cholera
<i>Treponema</i>	Peptide in outer	Surface	Mucosal	Syphilis

<i>pallidum</i>	membrane	protein(fibronectin)	epithelium	
Mycoplasma	Membrane protein	Sialic acid	Respiratory epithelium	Pneumonia
Chlamydia	Unknown	Sialic acid	Conjunctival or urethral epithelium	Conjunctivitis or urethritis

INVASION

The invasion of a host by a pathogen may be aided by the production of bacterial extracellular substances which act against the host by breaking down primary or secondary defenses of the body. Medical microbiologists have long referred to these substances as **invasins**. Invasins are proteins (enzymes) that act locally to damage host cells and/or have the immediate effect of facilitating the growth and spread of the pathogen. The damage to the host as a result of this invasive activity may become part of the pathology of an infectious disease.

The extracellular proteins produced by bacteria which promote their invasion are not clearly distinguished from some extracellular protein toxins ("exotoxins") which also damage the host. Invasins usually act at a short range (in the immediate vicinity of bacterial growth) and may not actually kill cells in their range of activity; exotoxins are often cytotoxic and may act at remote sites (removed from the site of bacterial growth). Also, exotoxins typically are more specific and more potent in their activity than invasins. Even so, some classic exotoxins (e.g. diphtheria toxin, anthrax toxin) may play some role in invasion in the early stages of an infection, and some invasins (e.g. staphylococcal leukocidin) have a relatively specific cytopathic effect.

A Survey of Bacterial Invasins

Spreading Factors

"Spreading Factors" is a descriptive term for a family of bacterial enzymes that affect the physical properties of tissue matrices and intercellular spaces, thereby promoting the spread of the pathogen.

Hyaluronidase is the original spreading factor. It is produced by streptococci, staphylococci, and clostridia. The enzyme attacks the interstitial cement ("ground substance") of connective tissue by depolymerizing hyaluronic acid.

Collagenase is produced by *Clostridium histolyticum* and *Clostridium perfringens*. It breaks down collagen, the framework of muscles, which facilitates gas gangrene due to these organisms.

Neuraminidase is produced by intestinal pathogens such as *Vibrio cholerae* and *Shigella dysenteriae*. It degrades neuraminic acid (also called sialic acid), an intercellular cement of the epithelial cells of the intestinal mucosa.

Streptokinase and **Staphylokinase** are produced by streptococci and staphylococci, respectively. Kinase enzymes convert inactive plasminogen to plasmin which digests fibrin and prevents clotting of the blood. The relative absence of fibrin in spreading bacterial lesions allows more rapid diffusion of the infectious bacteria.

Enzymes that Cause Hemolysis and/or Leucolysis

These enzymes usually act on the animal cell membrane by insertion into the membrane (forming a pore that results in cell lysis), or by enzymatic attack on phospholipids, which destabilizes the membrane. They may be referred to as **lecithinases** or **phospholipases**, and if they lyse red blood cells they are sometimes called **hemolysins**.

Leukocidins, produced by staphylococci and **streptolysin** produced by **streptococci** specifically lyse phagocytes and their granules. These latter two enzymes are also considered to be bacterial exotoxins.

Phospholipases, produced by *Clostridium perfringens* (i.e., alpha toxin), hydrolyze phospholipids in cell membranes by removal of polar head groups.

Lecithinases, also produced by *Clostridium perfringens*, destroy lecithin (phosphatidylcholine) in cell membranes.

Hemolysins, notably produced by staphylococci (i.e., alpha toxin), streptococci (i.e., streptolysin) and various clostridia, may be channel-forming proteins or phospholipases or lecithinases that destroy red blood cells and other

cells (i.e., phagocytes) by lysis.

Staphylococcal coagulase

Coagulase, formed by *Staphylococcus aureus*, is a cell-associated and diffusible enzyme that converts fibrinogen to fibrin which causes clotting. Coagulase activity is almost always associated with pathogenic *S. aureus* and almost never associated with nonpathogenic *S. epidermidis*, which has led to much speculation as to its role as a determinant of virulence. Possibly, cell bound coagulase could provide an antigenic disguise if it clotted fibrin on the cell surface. Or a staphylococcal lesion encased in fibrin (e.g. a boil or pimple) could make the bacterial cells resistant to phagocytes or tissue bactericides or even drugs which might be unable to diffuse to their bacterial target.

Extracellular Digestive Enzymes

Heterotrophic bacteria, in general, produce a wide variety of extracellular enzymes including **proteases**, **lipases**, **glycohydrolases**, **nucleases**, etc., which are not clearly shown to have a direct role in invasion or pathogenesis. These enzymes presumably have other functions related to bacterial nutrition or metabolism, but may aid in invasion either directly or indirectly.

Toxins With Short-Range Effects Related to Invasion

Bacterial protein toxins which have adenylate cyclase activity, are thought to have immediate effects on host cells that promote bacterial invasion. One component of the anthrax toxin (**EF** or **Edema Factor**) is an **adenylate cyclase** that acts on nearby cells to cause increased levels of cyclic AMP and disruption of cell permeability. One of the toxins of *Bordetella pertussis*, the agent of whooping cough, has a similar effect. These toxins may contribute to invasion through their effects on macrophages or lymphocytes in the vicinity which are playing an essential role to contain the infection.

The following table summarizes the activities of many bacterial proteins that are noted for their contribution to bacterial invasion of tissues.

TABLE 3. SOME EXTRACELLULAR BACTERIAL PROTEINS THAT ARE CONSIDERED INVASINS

Invasin	Bacteria Involved	Activity
Hyaluronidase	Streptococci, staphylococci and clostridia	Degrades hyaluronic acid of connective tissue
Collagenase	<i>Clostridium</i> species	Dissolves collagen framework of muscles
Neuraminidase	<i>Vibrio cholerae</i> and <i>Shigella dysenteriae</i>	Degrades neuraminic acid of intestinal mucosa
Coagulase	<i>Staphylococcus aureus</i>	Converts fibrinogen to fibrin which causes clotting
Kinases	Staphylococci and streptococci	Converts plasminogen to plasmin which digests fibrin
Leukocidin	<i>Staphylococcus aureus</i>	Disrupts neutrophil membranes and causes discharge of lysosomal granules
Streptolysin	<i>Streptococcus pyogenes</i>	Repels phagocytes and disrupts phagocyte membrane and causes discharge of lysosomal granules
Hemolysins	Streptococci, staphylococci and clostridia	Phospholipases or lecithinases that destroy red blood cells (and other cells) by lysis
Lecithinases	<i>Clostridium perfringens</i>	Destroy lecithin in cell membranes
Phospholipases	<i>Clostridium perfringens</i>	Destroy phospholipids in cell membrane
Anthrax EF	<i>Bacillus anthracis</i>	One component (EF) is an adenylate cyclase which causes increased levels of intracellular cyclic AMP

Pertussis AC

Bordetella pertussis

One toxin component is an adenylate cyclase that acts locally producing an increase in intracellular cyclic AMP

EVASION OF HOST DEFENSES

Some pathogenic bacteria are inherently able to resist the bactericidal components of host tissues. For example, the poly-D-glutamate capsule of *Bacillus anthracis* protects the organisms against cell lysis by cationic proteins in sera or in phagocytes. The outer membrane of Gram-negative bacteria is a formidable permeability barrier that is not easily penetrated by hydrophobic compounds such as bile salts which are harmful to the bacteria. Pathogenic mycobacteria have a waxy cell wall that resists attack or digestion by most tissue bactericides. And intact lipopolysaccharides (LPS) of Gram-negative pathogens may protect the cells from complement-mediated lysis or the action of lysozyme.

Most successful pathogens, however, possess additional structural or biochemical features which allow them to resist the main lines of host internal defense against them, i.e., the phagocytic and immune responses of the host.

Overcoming Host Phagocytic Defenses

Microorganisms invading tissues are first and foremost exposed to phagocytes. Bacteria that readily attract phagocytes, and that are easily ingested and killed, are generally unsuccessful as parasites. In contrast, most bacteria that are successful as parasites interfere to some extent with the activities of phagocytes or in some way avoid their attention.

Microbial strategies to avoid phagocytic killing are numerous and diverse, but are usually aimed at blocking one or more steps in the phagocytic process. Recall the steps in phagocytosis:

1. Contact between phagocyte and microbial cell
2. Engulfment
3. Phagosome formation
4. Phagosome-lysosome fusion
5. Killing and digestion

Avoiding Contact with Phagocytes

Bacteria can avoid the attention of phagocytes in a number of ways.

1. Invade or remain confined in regions inaccessible to phagocytes. Certain internal tissues (e.g. the lumen of glands) and surface tissues (e.g. the skin) are not patrolled by phagocytes.
2. Avoid provoking an overwhelming inflammatory response. Some pathogens induce minimal or no inflammation required to focus the phagocytic defenses.
3. Inhibit phagocyte chemotaxis. e.g. Streptococcal streptolysin (which also kills phagocytes) suppresses neutrophil chemotaxis, even in very low concentrations. Fractions of *Mycobacterium tuberculosis* are known to inhibit leukocyte migration. *Clostridium* ϕ toxin inhibits neutrophil chemotaxis.
4. Hide the antigenic surface of the bacterial cell. Some pathogens can cover the surface of the bacterial cell with a component which is seen as "self" by the host phagocytes and immune system. Phagocytes cannot recognize bacteria upon contact and the possibility of opsonization by antibodies to enhance phagocytosis is minimized. For example, pathogenic *Staphylococcus aureus* produces cell-bound coagulase which clots fibrin on the bacterial surface. *Treponema pallidum* binds fibronectin to its surface. Group A streptococci are able to synthesize a capsule composed of hyaluronic acid.

Inhibition of Phagocytic Engulfment

Some bacteria employ strategies to **avoid engulfment (ingestion)** if phagocytes do make contact with them. Many important pathogenic bacteria bear on their surfaces substances that inhibit phagocytic adsorption or engulfment. Clearly it is the bacterial surface that matters. Resistance to phagocytic ingestion is usually due to a component of the bacterial cell wall, or fimbriae, or a capsule enclosing the bacterial wall. Classical examples of antiphagocytic substances on the bacterial surface include:

Polysaccharide capsules of *S. pneumoniae*, *Haemophilus influenzae*, *Treponema pallidum* and *Klebsiella pneumoniae*

M protein and fimbriae of Group A streptococci

Surface slime (polysaccharide) produced by *Pseudomonas aeruginosa*

O antigen associated with LPS of *E. coli*

K antigen of *E. coli* or the analogous Vi antigen of *Salmonella typhi*

Cell-bound or soluble Protein A produced by *Staphylococcus aureus*

Survival Inside of Phagocytes

Some bacteria survive inside of phagocytic cells, in either neutrophils or macrophages. Bacteria that can resist killing and survive or multiply inside of phagocytes are considered intracellular parasites. The environment of the phagocyte may be a protective one, protecting the bacteria during the early stages of infection or until they develop a full complement of virulence factors. The intracellular environment guards the bacteria against the activities of extracellular bactericides, antibodies, drugs, etc.

Most intracellular parasites have special (genetically-encoded) mechanisms to get themselves into their host cell as well as special mechanisms to survive once they are inside. Intracellular parasites usually survive by virtue of mechanisms which interfere with the bactericidal activities of the host cell. Some of these bacterial mechanisms include:

1. Inhibition of phagosome-lysosome fusion. The bacteria survive inside of phagosomes because they prevent the discharge of lysosomal contents into the phagosome environment. Specifically phagolysosome formation is inhibited in the phagocyte. This is the strategy employed by *Salmonella*, *M. tuberculosis*, *Legionella* and the *Chlamydiae*.

2. Survival inside the phagolysosome. With some intracellular parasites, phagosome-lysosome fusion occurs but the bacteria are resistant to inhibition and killing by the lysosomal constituents. Also, some extracellular pathogens can resist killing in phagocytes utilizing similar resistance mechanisms. Little is known of how bacteria can resist phagocytic killing within the phagocytic vacuole, but it may be due to the surface components of the bacteria or due to extracellular substances that they produce which interfere with the mechanisms of phagocytic killing. *Bacillus anthracis*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* all possess mechanisms to survive intracellular killing in macrophages.

3. Escape from the phagosome. Early escape from the phagosome vacuole is essential for growth and virulence of some intracellular pathogens. This is a very clever strategy employed by the Rickettsias which produce a phospholipase enzyme that lyses the phagosome membrane within thirty seconds of after ingestion.

Products of Bacteria that Kill or Damage Phagocytes

One obvious strategy in defense against phagocytosis is direct attack by the bacteria upon the professional phagocytes. Any of the substances that pathogens produce that cause damage to phagocytes have been referred to as "aggressins". Most of these are actually extracellular enzymes or toxins that kill phagocytes. Phagocytes may be killed by a pathogen before or after ingestion.

Killing phagocytes before ingestion. Many Gram-positive pathogens, particularly the pyogenic cocci, secrete extracellular enzymes which kill phagocytes. Many of these enzymes are called "hemolysins" because their activity in the presence of red blood cells results in the lysis of the rbc's.

Pathogenic streptococci produce streptolysin. Streptolysin O binds to cholesterol in membranes. The effect on neutrophils is to cause lysosomal granules to explode, releasing their contents into the cell cytoplasm.

Pathogenic staphylococci produce leukocidin, which also acts on the neutrophil membrane and causes discharge of lysosomal granules.

Other examples of bacterial extracellular proteins that inhibit phagocytosis include the Exotoxin A of *Pseudomonas aeruginosa* which kills macrophages, and the bacterial exotoxins that are adenylate cyclases (e.g. anthrax toxin EF and pertussis AC) which decrease phagocytic activity.

Killing phagocytes after ingestion. Some bacteria exert their toxic action on the phagocyte after ingestion has taken place. They may grow in the phagosome and release substances which can pass through the phagosome membrane and cause discharge of lysosomal granules, or they may grow in the phagolysosome and release toxic substances which pass through the phagolysosome membrane to other target sites in the cell. Many bacteria which are the intracellular parasites of macrophages (e.g Mycobacteria, *Brucella*, *Listeria*) usually destroy macrophages in the end, but the mechanisms are not understood.

Overcoming Host Phagocytic Defenses

On epithelial surfaces the main antibacterial immune defense of the host is the protection afforded by secretory antibody (IgA). Once the epithelial surfaces have been penetrated, however, the major host defenses of inflammation, complement, phagocytosis, Antibody-mediated Immunity (AMI), and Cell-mediated Immunity (CMI) are encountered. If there is a way for a pathogen to successfully bypass or overcome these host defenses, then some bacterial pathogen has probably discovered it. Bacteria evolve very rapidly in relation to their host, so that most of the feasible anti-host strategies are likely to have been tried out and exploited. Ability to defeat the immune defenses may play a major role in the virulence of a bacterium and in the pathology of disease. Several strategic bacterial defenses are described below.

Immunological Tolerance to a Bacterial Antigen

Tolerance is a property of the host in which there is an immunologically-specific reduction in the immune response to a given Ag. Tolerance to a bacterial Ag does not involve a general failure in the immune response but a particular deficiency in relation to the specific antigen(s) of a given bacterium. If there is a depressed immune response to relevant antigens of a parasite, the process of infection is facilitated. Tolerance can involve either AMI or CMI or both arms of the immunological response.

Tolerance to an Ag can arise in a number of ways, but three are possibly relevant to bacterial infections.

1. Fetal exposure to Ag

2. High persistent doses of circulating Ag

3. **Molecular mimicry.** If a bacterial Ag is very similar to normal host "antigens", the immune responses to this Ag may be weak giving a degree of tolerance. Resemblance between bacterial Ag and host Ag is referred to as molecular mimicry. In this case the antigenic determinants of the bacterium are so closely related chemically to host "self" components that the immunological cells cannot distinguish between the two and an immune response cannot be raised. Some bacterial capsules are composed of polysaccharides (hyaluronic acid, sialic acid) so similar to host tissue polysaccharides that they are not immunogenic.

Antigenic Disguise

Bacteria may be able to coat themselves with host proteins (fibrin, fibronectin, antibody molecules) or with host polysaccharides (sialic acid, hyaluronic acid) so that they are able to hide their own antigenic surface components from the immunological system.

Immunosuppression

Some pathogens (mainly viruses and protozoa, rarely bacteria) cause immunosuppression in the infected host. This means that the host shows depressed immune responses to antigens in general, including those of the infecting

pathogen. Suppressed immune responses are occasionally observed during chronic bacterial infections such as leprosy and tuberculosis.

Persistence of a Pathogen at Bodily Sites Inaccessible to the Immune Response

Some pathogens can avoid exposing themselves to immune forces.

Intracellular pathogens can evade host immune responses as long as they stay inside of infected cells and they do not allow microbial Ag to form on the cell surface. Macrophages support the growth of the bacteria and at the same time give them protection from immune responses.

Some pathogens persist on the luminal surfaces of the GI tract, oral cavity and the urinary tract, or the lumen of the salivary gland, mammary gland or the kidney tubule.

Induction of Ineffective Antibody

Many types of antibody are formed against a given Ag, and some bacterial components may display various antigenic determinants. Antibodies tend to range in their capacity to react with Ag (the ability of specific Ab to bind to an Ag is called **avidity**). If Abs formed against a bacterial Ag are of low avidity, or if they are directed against unimportant antigenic determinants, they may have only weak antibacterial action. Such "ineffective" (non-neutralizing) Abs might even aid a pathogen by combining with a surface Ag and blocking the attachment of any functional Abs that might be present.

Antibodies Absorbed by Soluble Bacterial Antigens

Some bacteria can liberate antigenic surface components in a soluble form into the tissue fluids. These soluble antigens are able to combine with and "neutralize" antibodies before they reach the bacterial cells. For example, small amounts of endotoxin (LPS) may be released into surrounding fluids by Gram-negative bacteria.

Antigenic Variation

One way bacteria can avoid forces of the immune response is by periodically changing antigens, i.e., undergoing antigenic variation. Some bacteria avoid the host antibody response by changing from one type of fimbriae to another, by switching fimbrial tips. This makes the original AMI response obsolete by using new fimbriae that do not bind the previous antibodies. Pathogenic bacteria can vary (change) other surface proteins that are the targets of antibodies. Antigenic variation is prevalent among pathogenic viruses as well.

Changing antigens during the course of an infection

Antigens may vary or change within the host during the course of an infection, or alternatively antigens may vary among multiple strains (antigenic types) of a parasite in the population. Antigenic variation is an important mechanism used by pathogenic microorganisms for escaping the neutralizing activities of antibodies. Antigenic variation usually results from site-specific inversions or gene conversions or gene rearrangements in the DNA of the microorganisms.

Changing antigens between infections

Many pathogenic bacteria exist in nature as multiple antigenic types or serotypes, meaning that they are variant strains of the same pathogenic species. For example, there are multiple serotypes of *Salmonella typhimurium* based on differences in cell wall (O) antigens or flagellar (H) antigens. There are 80 different antigenic types of *Streptococcus pyogenes* based on M-proteins on the cell surface. There are over one hundred strains of *Streptococcus pneumoniae* depending on their capsular polysaccharide antigens. Based on minor differences in surface structure chemistry there are multiple serotypes of *Vibrio cholerae*, *Staphylococcus aureus*, *Escherichia coli*, *Neisseria gonorrhoeae* and an assortment of other bacterial pathogens.

TOXIGENESIS

Two types of bacterial toxins

At a chemical level there are two types of bacterial toxins:

lipopolysaccharides, which are associated with the cell walls of Gram-negative bacteria.

proteins, which may be released into the extracellular environment of pathogenic bacteria.

The lipopolysaccharide (LPS) component of the Gram-negative bacterial outer membrane bears the name endotoxin because of its association with the cell wall of bacteria.

Most of the protein toxins are thought of as exotoxins, since they are "released" from the bacteria and act on host cells at a distance.

BACTERIAL PROTEIN TOXINS

The protein toxins are typically soluble proteins secreted by living bacteria during exponential growth. The production of protein toxins is generally specific to a particular bacterial species (e.g. only *Clostridium tetani* produces tetanus toxin; only *Corynebacterium diphtheriae* produces the diphtheria toxin). Usually, virulent strains of the bacterium produce the toxin (or range of toxins) while nonvirulent strains do not, such that the toxin is the major determinant of virulence. Both Gram-positive and Gram-negative bacteria produce soluble protein toxins. Bacterial protein toxins are the most potent poisons known and may show activity at very high dilutions.

The protein **toxins resemble enzymes** in a number of ways. Like enzymes, bacterial exotoxins:

are **proteins**

are **denatured by heat**, acid, proteolytic enzymes

have a **high biological activity** (most act catalytically)

exhibit **specificity** of action

As enzymes attack specific substrates, so bacterial protein toxins are **highly specific** in the substrate utilized and in their mode of action. The substrate (in the host) may be a component of tissue cells, organs, or body fluid. Usually the site of damage caused by the toxin indicates the location of the substrate for that toxin. Terms such as "enterotoxin", "neurotoxin", "leukocidin" or "hemolysin" are sometimes used to indicate the target site of some well-defined protein toxins.

Certain protein toxins have very specific **cytotoxic activity** (i.e., they attack specific cells, for example, tetanus or botulinum toxins), but some (as produced by staphylococci, streptococci, clostridia, etc.) have fairly broad cytotoxic activity and cause nonspecific death of tissues (necrosis). Toxins that are phospholipases may be relatively nonspecific in their cytotoxicity because they cleave phospholipids which are components of host cell membranes resulting in the death of the cell by leakage of cellular contents. This is also true of pore-forming "hemolysins" and "leukocidins".

A few protein toxins obviously bring about the death of the host and are known as "lethal toxins", and even though the tissues affected and the target sites may be known, the precise mechanism by which death occurs is not understood (e.g. anthrax toxin).

As "foreign" substances to the host, most of the protein toxins are **strongly antigenic**. In vivo, **specific antibody (antitoxin) neutralizes the toxicity** of these bacterial proteins. However, in vitro, specific antitoxin may not fully inhibit their enzymatic activity. This suggests that the antigenic determinant of the toxin is distinct from the active (enzymatic) portion of the protein molecule. The degree of neutralization of the enzymatic site may depend on the distance from the antigenic site on the molecule. However, since the toxin is fully neutralized in vivo, this suggests that other (host) factors must play a role.

Protein toxins are inherently unstable: in time they lose their toxic properties but retain their antigenic ones. This was first discovered by Ehrlich and he coined the term toxoid for this product. **Toxoids** are detoxified toxins which retain their antigenicity and their immunizing capacity. The formation of toxoids can be accelerated by treating toxins with a variety of reagents including formalin, iodine, pepsin, ascorbic acid, ketones, etc. The mixture is

maintained at 37° at pH range 6 to 9 for several weeks. The resulting toxoids can be used for artificial immunization against diseases caused by pathogens where the primary determinant of bacterial virulence is toxin production. Toxoids are the immunizing agents against diphtheria and tetanus that are part of the DPT vaccine.

A + B Subunit Arrangement of Protein Toxins

Many protein toxins, notably those that act intracellularly (with regard to host cells), consist of two components: one component (subunit A) is responsible for the enzymatic activity of the toxin; the other component (subunit B) is concerned with binding to a specific receptor on the host cell membrane and transferring the enzyme across the membrane. The enzymatic component is not active until it is released from the native toxin. Isolated A subunits are enzymatically active and lack binding and cell entry capability. Isolated B subunits may bind to target cells (and even block the binding of the native A+B toxin), but they are nontoxic. There are a variety of ways that toxin subunits may be synthesized and arranged: **A-B** or **A-5B** indicates that subunits synthesized separately and associated by noncovalent bonds; **A/B** denotes subunit domains of a single protein that may be separated by proteolytic cleavage; **A + B** indicates separate protein subunits that interact at the target cell surface; **5B** indicates that the binding domain is composed of 5 identical subunits.

Attachment and Entry of Toxins

There are at least two mechanisms of toxin entry into target cells. In one mechanism called **direct entry**, the B subunit of the native toxin (A+B) binds to a specific receptor on the target cell and induces the formation of a pore in the membrane through which the A subunit is transferred into the cell cytoplasm. In an alternative mechanism, the native toxin binds to the target cell and the A+B structure is taken into the cell by the process of **receptor-mediated endocytosis (RME)**. The toxin is internalized in the cell in a membrane-enclosed vesicle called an endosome. H⁺ ions enter the endosome lowering the internal pH which causes the A+B subunits to separate. Somehow, the B subunit affects the release of the A subunit from the endosome so that it will reach its target in the cell cytoplasm. The B subunit remains in the endosome and is recycled to the cell surface. In both cases, a large protein molecule must insert into and cross a membrane lipid bilayer. This activity is reflected in the ability of most A/B native toxins, or their B components, to insert into artificial lipid bilayers, creating ion permeable pathways.

Other Considerations

In keeping with the observation that genetic information for functions not involved in viability of bacteria is frequently located extrachromosomally, the genes encoding toxin production are generally located on plasmids or in lysogenic bacteriophages. Thus the processes of genetic exchange in bacteria, notably conjugation and transduction, can mobilize these genetic elements between strains of bacteria, and therefore may play a role in determining the pathogenic potential of a bacterium.

Why certain bacteria produce such potent toxins is mysterious and is analogous to asking why an organism should produce an antibiotic. The production of a toxin may play a role in adapting a bacterium to a particular niche, but it is not essential to the viability of the organism. Many toxigenic bacteria are free-living in Nature and in associations with humans in a form which is phenotypically identical to the toxigenic strain but lacking the ability to produce the toxin.

There is conclusive evidence for the pathogenic role of diphtheria, tetanus and botulinum toxins, various enterotoxins, staphylococcal toxic shock syndrome toxin, and streptococcal erythrogenic toxin. And there is clear evidence for the pathological involvement of pertussis toxin, anthrax toxin, shiga toxin and the necrotizing toxins of clostridia in host-parasite relationships.

Table 4. SOURCES AND ACTIVITIES OF BACTERIAL TOXINS

NAME OF TOXIN	BACTERIUM INVOLVED	ACTIVITY
Anthrax toxin (EF)	<i>Bacillus anthracis</i>	Edema Factor (EF) is an adenylate cyclase that causes increased levels in intracellular cyclic AMP in phagocytes and formation of ion-permeable pores in membranes (hemolysis)
Adenylate cyclase toxin	<i>Bordetella pertussis</i>	Acts locally to increase levels of cyclic AMP in phagocytes and formation of ion-permeable pores in membranes (hemolysis)

Cholera enterotoxin	<i>Vibrio cholerae</i>	ADP ribosylation of G proteins stimulates adenylate cyclase and increases cAMP in cells of the GI tract, causing secretion of water and electrolytes
<i>E. coli</i> LT toxin	<i>Escherichia coli</i>	Similar to cholera toxin
Shiga toxin	<i>Shigella dysenteriae</i>	Enzymatically cleaves rRNA resulting in inhibition of protein synthesis in susceptible cells
Botulinum toxin	<i>Clostridium botulinum</i>	Zn ⁺⁺ dependent protease that inhibits neurotransmission at neuromuscular synapses resulting in flaccid paralysis
Tetanus toxin	<i>Clostridium tetani</i>	Zn ⁺⁺ dependent protease that inhibits neurotransmission at inhibitory synapses resulting in spastic paralysis
Diphtheria toxin	<i>Corynebacterium diphtheriae</i>	ADP ribosylation of elongation factor 2 leads to inhibition of protein synthesis in target cells
Pertussis toxin	<i>Bordetella pertussis</i>	ADP ribosylation of G proteins blocks inhibition of adenylate cyclase in susceptible cells
Staphylococcus enterotoxins*	<i>Staphylococcus aureus</i>	Massive activation of the immune system, including lymphocytes and macrophages, leads to emesis (vomiting)
Toxic shock syndrome toxin (TSST-1)*	<i>Staphylococcus aureus</i>	Acts on the vascular system causing inflammation, fever and shock
Erythrogenic toxin (scarlet fever toxin)*	<i>Streptococcus pyogenes</i>	Causes localized erythematous reactions

* The "pyrogenic exotoxins" produced by *Staphylococcus aureus* and *Streptococcus pyogenes* have been designated as superantigens. They represent a family of molecules with the ability to elicit massive activation of the immune system. These proteins share the ability to stimulate T cell proliferation by interaction with Class II MHC molecules on APCs and specific V beta chains of the T cell receptor. The important feature of this interaction is the resultant production of IL-1, TNF, and other lymphokines which appear to be the principal mediators of disease processes associated with these toxins.

ENDOTOXINS

Endotoxins are part of the outer cell wall of bacteria. Endotoxins are invariably associated with Gram-negative bacteria as constituents of the outer membrane of the cell wall. Although the term **endotoxin** is occasionally used to refer to any "cell-associated" bacterial toxin, it should be reserved for the lipopolysaccharide complex associated with the outer envelope of Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, and other leading pathogens. Lipopolysaccharide (LPS) participates in a number of outer membrane functions that are essential for bacterial growth and survival, especially within the context of a host-parasite interaction.

The biological activity of endotoxin is associated with the **lipopolysaccharide (LPS)**. Toxicity is associated with the lipid component (**Lipid A**) and immunogenicity (antigenicity) is associated with the polysaccharide components. The cell wall antigens (**O antigens**) of Gram-negative bacteria are components of LPS. LPS activates complement by the alternative (properdin) pathway and may be a part of the pathology of most Gram-negative bacterial infections.

For the most part, endotoxins remain associated with the cell wall until disintegration of the bacteria. In vivo, this results from autolysis, external lysis, and phagocytic digestion of bacterial cells. It is known, however, that small amounts of endotoxin may be released in a soluble form, especially by young cultures.

Compared to the classic exotoxins of bacteria, endotoxins are less potent and less specific in their action, since they do not act enzymatically. Endotoxins are heat stable (boiling for 30 minutes does not destabilize endotoxin), but certain powerful oxidizing agents such as , superoxide, peroxide and hypochlorite degrade them. Endotoxins, although strongly antigenic, cannot be converted to toxoids. A comparison of the properties of bacterial endotoxins compared to classic exotoxins is shown in Table 5.

Table 5. CHARACTERISTICS OF BACTERIAL ENDOTOXINS AND EXOTOXINS

PROPERTY	ENDOTOXIN	EXOTOXIN
CHEMICAL NATURE	Lipopolysaccharide(mw = 10kDa)	Protein (mw = 50-1000kDa)
RELATIONSHIP TO CELL	Part of outer membrane	Extracellular, diffusible
DENATURED BY BOILING	No	Usually
ANTIGENIC	Yes	Yes
FORM TOXOID	No	Yes
POTENCY	Relatively low (>100ug)	Relatively high (1 ug)
SPECIFICITY	Low degree	High degree
ENZYMATIC ACTIVITY	No	Usually
PYROGENICITY	Yes	Occasionally

Lipopolysaccharides are complex amphiphilic molecules with a mw of about 10kDa, that vary widely in chemical composition both between and among bacterial species. In a basic groundplan common to all endotoxins, LPS consists of three components or regions:

(1) Lipid A---- (2) Core polysaccharide---- (3) O polysaccharide

Lipid A is the lipid component of LPS. It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. Usually 6 FA are found. All FA in Lipid A are saturated. Some FA are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The structure of Lipid A is highly conserved among Gram-negative bacteria. Among *Enterobacteriaceae* Lipid A is virtually constant.

The **Core (R) polysaccharide** is attached to the 6 position of one NAG. The R antigen consists of a short chain of sugars. For example: KDO - Hep - Hep - Glu - Gal - Glu - GluNAc.

Two unusual sugars are usually present, heptose and 2-keto-3-deoxyoctonoic acid (KDO), in the core polysaccharide. KDO is unique and invariably present in LPS and so has been an indicator in assays for LPS (endotoxin).

With minor variations, the core polysaccharide is common to all members of a bacterial genus (e.g. *Salmonella*), but it is structurally distinct in other genera of Gram-negative bacteria. *Salmonella*, *Shigella* and *Escherichia* have similar but not identical cores.

The **O polysaccharide** (also referred to as the **O antigen** or **O side chain**) is attached to the core polysaccharide. It consists of repeating oligosaccharide subunits made up of 3-5 sugars. The individual chains vary in length ranging up to 40 repeat units. The O polysaccharide is much longer than the core polysaccharide and it maintains the hydrophilic domain of the LPS molecule. Often, a unique group of sugars, called **dideoxyhexoses**, occurs in the O polysaccharide.

A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in the O polysaccharide. Great variation occurs in the composition of the sugars in the O side chain between species and even strains of Gram-negative bacteria.

LPS and virulence of Gram-negative bacteria

Endotoxins are toxic to most mammals. They are strong antigens but they seldom elicit immune responses which give full protection to the animal against secondary challenge with the endotoxin. They cannot be toxoided. Endotoxins released from multiplying or disintegrating bacteria significantly contribute to the symptoms of Gram-negative bacteremia and septicemia, and therefore represent important pathogenic factors in Gram-negative infections. Regardless of the bacterial source, all endotoxins produce the same range of biological effects in the animal host. The injection of living or killed Gram-negative cells, or purified LPS, into experimental animals causes a wide spectrum of nonspecific **pathophysiological reactions related to inflammation** such as:

fever

changes in white blood cell counts

disseminated intravascular coagulation**tumor necrosis****hypotension****shock****lethality**

The sequence of events follows a regular pattern: 1. latent period; 2. physiological distress (fever, diarrhea, prostration, shock); 3. death. How soon death occurs varies on the dose of the endotoxin, route of administration, and species of animal. Animals vary in their susceptibility to endotoxin.

The role of Lipid A

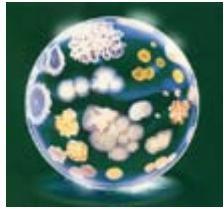
The physiological activities of endotoxins are mediated mainly by the Lipid A component of LPS. Lipid A is the toxic component of LPS, as evidence by the fact that injection of purified Lipid A into an experimental animal will elicit the same response as intact LPS. The primary structure of Lipid A has been elucidated, and Lipid A has been chemically synthesized. Its biological activity appears to depend on a peculiar conformation that is determined by the glucosamine disaccharide, the PO₄ groups, the acyl chains, and also the KDO-containing inner core. Lipid A is known to react at the surfaces of macrophages causing them to release cytokines that mediate the pathophysiological response to endotoxin.

The role of the O polysaccharide

Although nontoxic, the polysaccharide side chain (O antigen) of LPS may act as a determinant of virulence in Gram-negative bacteria. The O polysaccharide is responsible for the property of "smoothness" of bacterial cells, which may contribute to their resistance to phagocytic engulfment. The O polysaccharide is hydrophilic and may allow diffusion or delivery of the toxic lipid in the hydrophilic (*in vivo*) environment. The long side chains of LPS afforded by the O polysaccharide may prevent host complement from depositing on the bacterial cell surface which would bring about bacterial cell lysis. The O polysaccharide may supply a bacterium with its specific ligands (adhesins) for colonization which is essential for expression of virulence. Lastly, the O-polysaccharide is antigenic, and the usual basis for antigenic variation in Gram-negative bacteria rests in differences in their O polysaccharides.

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Bacteria of Medical Importance

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Introduction

Historically, bacteria have been the cause of some of the most deadly diseases and widespread epidemics of human civilization. Although smallpox and malaria, diseases caused by other microbes, may have killed more humans than bacterial diseases, bacterial diseases such as tuberculosis, typhus, plague, diphtheria, typhoid fever, cholera, dysentery, and pneumonia have taken a mighty toll on humanity. Water purification, immunization (vaccination) and modern antibiotic treatment continued to reduce the morbidity and the mortality of bacterial disease in the Twenty-first Century, at least in the developed world where these are acceptable cultural practices. However, many new bacterial pathogens have been recognized in the past 25 years (see Table 1) and many "old" bacterial pathogens, such as *Staphylococcus aureus* and *Mycobacterium tuberculosis*, have emerged with new forms of virulence and new patterns of resistance to antimicrobial agents.

Table 1. Bacterial pathogens and diseases recognized or reemerged since 1977

Bacterium	Disease
<i>Legionella pneumophila</i>	Legionnaires' pneumonia
<i>Listeria monocytogenes</i>	listeriosis
<i>Campylobacter jejuni</i>	gastroenteritis distributed world-wide
<i>Staphylococcus aureus</i>	toxic shock syndrome
<i>E. coli</i> O157:H7	hemorrhagic colitis; hemolytic uremic syndrome
<i>Borrelia burgdorferi</i>	Lyme Disease and complications
<i>Helicobacter pylori</i>	gastric and duodenal ulcers
<i>Ehrlichia chaffeensis</i>	human ehrlichiosis
<i>Clostridium difficile</i>	antibiotic induced diarrhea; pseudomembranous colitis
<i>Vibrio cholerae</i> O139	epidemic cholera
<i>Salmonella enterica</i> Serotype Typhimurium DT 104	salmonellosis
<i>Bartonella henselae</i>	cat scratch fever
<i>Streptococcus pyogenes</i>	necrotizing fasciitis (GAS); streptococcal toxic shock syndrome
<i>Chlamydia pneumoniae</i>	atherosclerosis
<i>Clostridium botulinum</i>	sudden infant death syndrome (SIDS)
<i>Vibrio vulnificus</i>	wound infection, septicemia, gastrointestinal disease
<i>Parachlamydia</i>	pneumonia
<i>Corynebacterium amycolatum</i>	hospital-acquired endocarditis

Most of the bacterial pathogens of humans are classified as **Gram-positive** or **Gram-negative**, some notable exceptions being the mycoplasmas, chlamydiae, spirochetes and the mycobacteria. In this article the major pathogens of humans are organized into natural groups based on bacteriological criteria, rather than on the basis of

affected organ, mode of transmission, or type of disease. This goes with being written by a bacteriologist.

Spirochetes

The 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 prokaryotes 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 *Treponema pallidum* is the agent of [syphilis](#), a sexually transmitted disease, and *Borrelia burgdorferi* causes [Lyme Disease](#), which is transmitted by the bite of the deer tick.



Figure 1. 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. (CDC)

Spirilla and other curved bacteria

Spirilla are Gram-negative bacteria with a helical or spiral shape. Their metabolism is respiratory and never fermentative. Unlike spirochetes, they have a rigid cell wall and are motile by means of ordinary polar flagella. Two important pathogens of humans are found among the spiral forms. *Campylobacter jejuni* is the 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** and there is strong evidence for its involvement in [adenocarcinoma](#).



Figure 2. *Helicobacter pylori* from [Helicobacter pylori Causing Adenocarcinoma](#), by Jon Cutlan

Vibrios

The term **vibrio** refers to a Gram-negative bacterium which has the cell shape of a curved rod or a comma. Members of the genus **Vibrio** are common bacteria in aquatic environments, especially marine environments. They have structural and metabolic properties that overlap with both the enterics and the pseudomonads. Vibrios are facultative (grow in the presence or absence of O₂), like enterics, but they have polar flagella, are oxidase-positive,

and degrade sugars in the same manner as the pseudomonads. In aquatic habitats they overlap with the pseudomonads in their ecology, although pseudomonads favor fresh water and vibrios prefer salt water. Some marine vibrios are bioluminescent (they emit light) and some are symbionts of fish, squid and other marine life. *Vibrio cholerae* causes **epidemic or Asiatic cholera** which, untreated, is one of the most rapidly fatal infectious diseases known. The pathology is related to diarrheal diseases caused by the enteric bacteria, except it is relentless, and a patient can die rapidly from dehydration. The cholera toxin, which is the classic model of a bacterial enterotoxin, is also produced by some strains of *E. coli*.



Figure 3. *Vibrio cholerae*, the agent of Asiatic or epidemic cholera.

The Gram-negative aerobic rods and cocci

The name refers to Gram-negative bacteria phenotypically related to members of the genus *Pseudomonas*. Their metabolism is respiratory and never fermentative. Important human pathogens include *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Bordetella pertussis*, *Haemophilus influenzae*, *Legionella*, *Brucella* and *Francisella*, and a few others. Many bacteria in this physiological group are free-living in soil and water, and they play an important role in decomposition, biodegradation, and the C and N cycles. Also, many bacteria which are pathogens of plants are found in this group, including *Pseudomonas*, *Xanthomonas* and *Agrobacterium*.

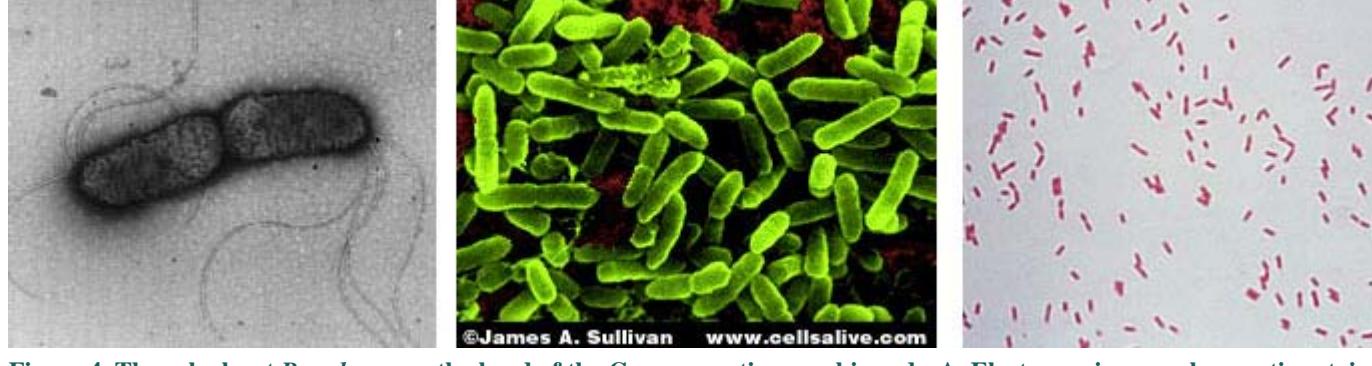


Figure 4. Three looks at *Pseudomonas*, the head of the Gram-negative aerobic rods. A. Electron micrograph, negative stain. B. Scanning electron micrograph. C. Gram stain.

Pseudomonas aeruginosa is the quintessential **opportunistic pathogen** of humans. It is a leading cause of **hospital-acquired infections** (nosocomial infections), and it is difficult to eradicate due to its resistance to most antimicrobial agents. There is probably no tissue that cannot become infected by *Pseudomonas* if the host defenses are weakened, and it is difficult to eradicate due to its resistance to antimicrobial agents. It is usually involved in soft tissue infections, urinary tract infections and pneumonia.

Whooping cough (or **pertussis**) is caused by *Bordetella pertussis*. The disease is particularly serious in infants and young children and has a high mortality rate. Whooping cough is controlled by vaccination with the **acellular pertussis vaccine**, which is usually given in association with diphtheria, tetanus and sometimes *H. influenzae* type b (Hib), as part of the childhood immunization program in the U.S.

Legionaires' pneumonia is caused by *Legionella pneumophila*. This pneumonia, and the bacterium, were not discovered until 1976, when there was an outbreak of disease at a Legionnaire's meeting in Philadelphia. It took several months to find, culture and grow the bacterium. The incident was a wake-up call to public health officials that there were probably a lot of disease-producing bacteria out there that they know nothing about.

Neisseria gonorrhoeae causes the sexually-transmitted disease **gonorrhea**, and ***Neisseria meningitidis*** is the agent of **meningococcal meningitis**. The **Neisseriae** are discussed below with the **Pyogenic Cocc.**

Haemophilus influenzae is also a cause of **meningitis**, but the incidence of the disease has declined rapidly with the use of the Hib vaccine which began in 1994. *Haemophilus* is sometimes involved in infections of the upper respiratory tract, particularly the sinuses.

Brucellosis is a chronic debilitating infection in humans associated with reproductive failure in domestic animals. Person-to-person transmission of brucellae is extremely rare. ***Brucella abortus*** is the species usually involved in human disease. The primary reservoir of the organism is in cattle, although bison are sometimes wrongfully accused.

Enterics

Enteric bacteria are Gram-negative rods with facultative anaerobic metabolism that live in the intestinal tracts of animals in health and disease. 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 ***Vibrios***. 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 (e.g. pseudomonads) cannot ferment glucose. Because they are consistent members of the **normal flora** 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 bacterium 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 (genome) 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 now notorious, strain O157:H7, that keeps turning up in raw hamburger headed for a fast-food restaurants).

Escherichia coli causes **intestinal tract infections** (usually acute and uncomplicated, except in the very young) or uncomplicated **urinary tract infections** and **neonatal meningitis**.

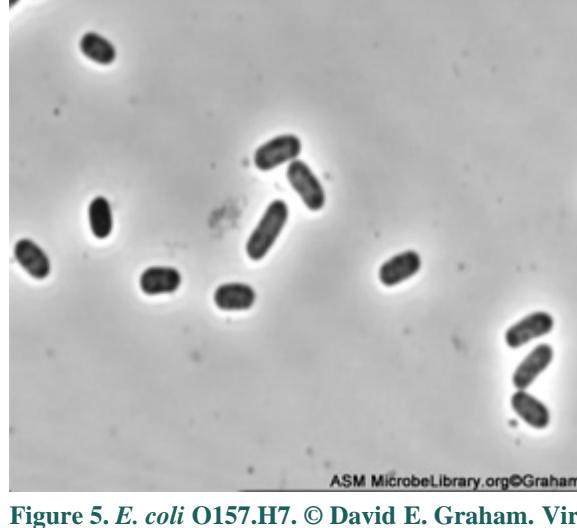


Figure 5. *E. coli* O157:H7. © David E. Graham. Virginia Polytechnic Institute and State University, Blacksburg, Virginia. Image by William Ghiorse, Department of Microbiology, Cornell University, Ithaca, New York. Licensed for use by ASM Microbe Library <http://www.microbelibrary.org/>. This is a phase contrast image of cells immobilized on an agar-coated slide.

The enteric group also includes some other intestinal pathogens of humans such as ***Shigella dysenteriae***, cause of **bacillary dysentery**, and ***Salmonella enteritidis***, cause of food poisoning and **gastroenteritis**. ***Salmonella typhi***, which infects via the intestinal route, causes **typhoid fever**. 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 and ***Yersinia pestis***, which causes **bubonic plague**. Also classified as an enteric is ***Erwinia***, a pathogen of plants that causes fireblight in pear and apple trees and soft rot of carrots and

potatoes.

Pyogenic Cocci

The **pyogenic cocci** are spherical bacteria that 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*. In terms of their phylogeny, physiology and genetics, these genera of bacteria are unrelated to one another. They share a common ecology, however, as parasites of humans.

The Gram-positive cocci are the 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**. The Gram-negative cocci, notably the neisseriae, cause **gonorrhea** and **meningococcal meningitis**.

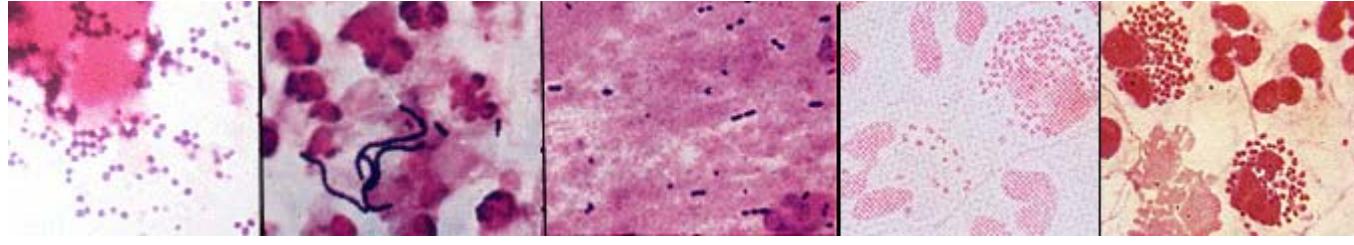


Figure 6. Gallery of pyogenic cocci, Gram stains of clinical specimens (pus), L to R: *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*. The large cells with lobed nuclei are neutrophils. Pus is the outcome of the battle between 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.

S. 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 all known antibiotics 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.

Staphylococcus aureus is a successful bacterial pathogen because it has a very wide range of **virulence determinants** (structural, biochemical or genetic features that allow the bacterium to cause disease), and it 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.

Streptococcus pyogenes, 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 occasionally found as **normal flora** in the upper respiratory tract (<15% of individuals), 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 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 ***Neisseriae*** cause **gonorrhea and meningitis**. ***Neisseriaceae*** is a family of Gram-negative bacteria with characteristics of enterics and 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 man, ***Neisseria gonorrhoeae*** and ***Neisseria meningitidis***.

Neisseria gonorrhoeae is the second leading cause of sexually-transmitted disease in the U.S., causing over 300,000 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 in **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 newborn eye at the time of birth.

Neisseria meningitidis is an important cause of bacterial 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.

Endospore-forming bacteria

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 medically-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.

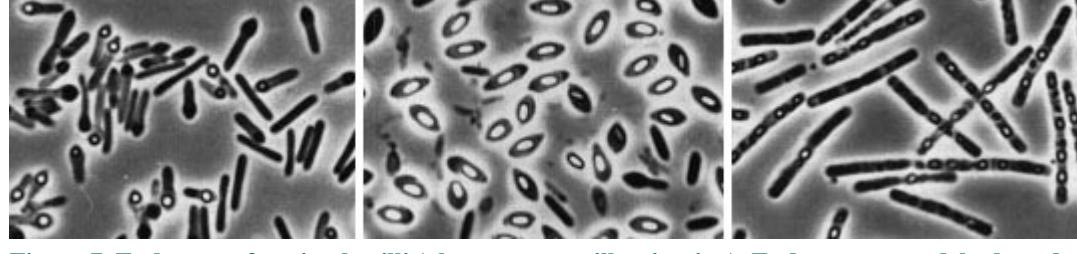


Figure 7. Endospore-forming bacilli (phase contrast illumination). Endospores are dehydrated, refractile cells appearing as points of bright light under phase microscopy. Endospore-forming bacteria are characterized by the location (position) of the endospore in the mother cell (sporangium) before its release. The spore may be central, terminal or subterminal, and the sporangium may or may not be swollen to accommodate the spore.

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*** causes **food poisoning**. ***Clostridium botulinum*** causes **botulism**, a form of **food poisoning**, and ***Clostridium tetani*** is the agent of **tetanus**. ***Clostridium perfringens*** causes **food poisoning**, **anaerobic wound infections** and **gas gangrene**, and ***Clostridium difficile*** causes a severe form of colitis called **pseudomembranous colitis**. Whenever the spore-formers act as pathogens, it is not uncommon or surprising that their spores are somehow involved in transmission or survival of the organism between hosts.

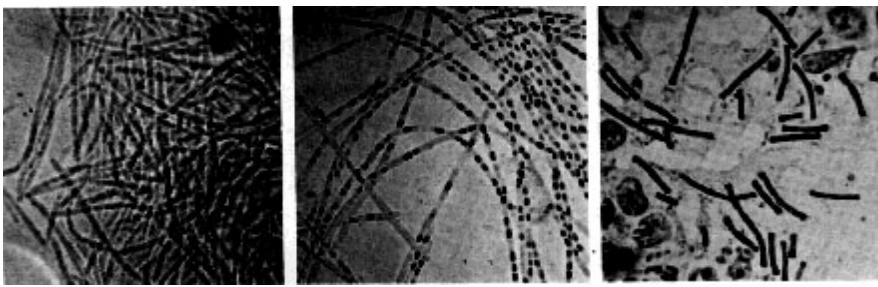


Figure 8. Robert Koch's original photomicrographs of *Bacillus anthracis*. In 1876, Koch established by careful microscopy that the bacterium was always present in the blood of animals that died of anthrax. He took a small amount of blood from such an animal and injected it into a healthy mouse, which 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.

Listeria monocytogenes is a Gram-positive rod-shaped bacterium related to bacillus and Clostridium but it does not form endospores. *Listeria monocytogenes* is the agent of **listeriosis**, a serious infection caused by eating food contaminated with the bacteria. Listeriosis has recently been recognized as an important public health problem in the United States. The disease affects primarily pregnant women, newborns, and adults with weakened immune systems.



Figure 9. *Listeria monocytogenes*. Transmission EM.

Actinomycetes and related bacteria

The **actinomycetes** are not thought of as pathogenic bacteria, but two of their relatives are among the most important pathogens of humans, these being the agents of **tuberculosis** and **diphtheria**. Actinomycetes are a large group of Gram-positive bacteria that usually grow by filament formation, or at least show a tendency towards branching and filament formation. Many of the organisms can form resting structures called spores, but they are not the same as endospores. Branched forms superficially resemble molds and are a striking example of convergent evolution of a prokaryote and a eukaryote together in the soil habitat. Actinomycetes such as *Streptomyces* have a world-wide distribution in soils. They are important in aerobic decomposition of organic compounds and have an important role in biodegradation and the carbon cycle. Actinomycetes are the main producers of antibiotics in industrial settings, being the source of most tetracyclines, macrolides (e.g. erythromycin), and aminoglycosides (e.g. streptomycin, gentamicin, etc.).

Two genera of bacteria that are related to the actinomycetes, *Corynebacterium* and *Mycobacterium*, contain important pathogens of humans: Otherwise, many nonpathogenic mycobacteria and corynebacteria live in normal associations with animals.

Mycobacterium tuberculosis is the etiologic agent of **tuberculosis** (TB) in humans. **Tuberculosis** is the leading cause of death in the world from a single infectious disease. *Mycobacterium tuberculosis* infects 1.7 billion people/year which is equal to 33% of the entire world population. The bacterium is responsible for over 3 million deaths/year. After a century of decline in the United States, tuberculosis is increasing, and multiple drug-resistant strains have emerged. This increase in cases is attributable to changes in the social structure in cities, the HIV epidemic, and patient failure to comply with treatment programs. A related organism, *Mycobacterium leprae*, causes **leprosy**.

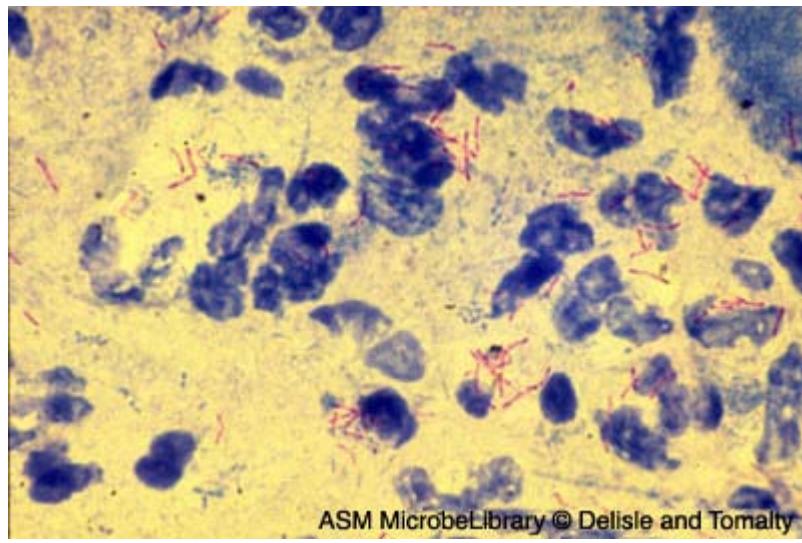


Figure 10. *Mycobacterium tuberculosis* Acid-fast stain. 1000X magnification. © Gloria J. Delisle and Lewis Tomalty, Queens University, Kingston, Ontario, Canada. Licensed for use by ASM Microbe Library <http://www.microbelibrary.org/>. These bacteria were observed in a sputum sample from a patient with active tuberculosis.

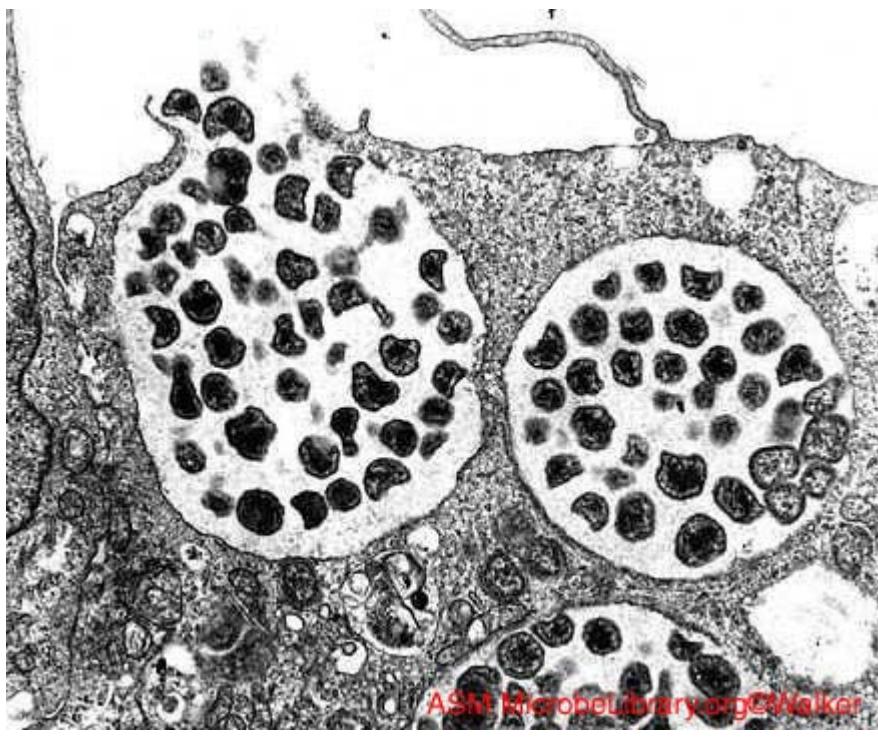
The genus ***Corynebacterium*** consists of a diverse group of bacteria including animal and plant pathogens, as well as saprophytes. Some corynebacteria are part of the normal flora of humans, finding a suitable niche in virtually every anatomic site. The best known and most widely studied species is ***Corynbacterium diphtheriae***, the causal agent of **diphtheria**. The study of ***Corynebacterium diphtheriae*** traces closely the development of medical microbiology, immunology and molecular biology. Many contributions to these fields, as well as to our understanding of host-bacterial interactions, have been made studying diphtheria and the diphtheria toxin.

Rickettsias and chlamydiae are two unrelated groups of bacteria that are **obligate intracellular parasites** of eukaryotic cells. **Rickettsias** cannot grow outside of a host cell because they have leaky membranes and are unable to obtain nutrients in an extracellular habitat. **Chlamydiae** are unable to produce ATP in amounts required to sustain metabolism outside of a host cell and are, in a sense, energy-parasites.

Rickettsias occur in nature in the gut lining of arthropods (ticks, fleas, lice, etc.). They are transmitted to vertebrates by an arthropod bite and produce diseases such as **typhus fever**, **Rocky Mountain Spotted Fever**, **Q fever** and **ehrlichiosis**.

Chlamydiae are tiny bacteria that infect birds and mammals. They may colonize and infect tissues of the eye and urogenital tract in humans. ***Chlamydia trachomatis*** causes several important diseases in humans: **chlamydia**, the most prevalent sexually transmitted disease in the U.S., **trachoma**, a leading cause of blindness worldwide, and **lymphogranuloma venereum**.

Chlamydia pneumoniae is a cause of pneumonia and has been recently linked to atherosclerosis.



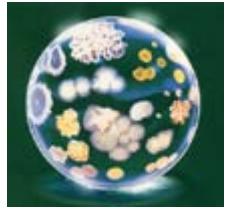
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Figure 11. *Ehrlichia chaffeensis* © Vsevolod Popov, J. Steven Dumler, and David H. Walker. University of Texas Medical Branch at Galveston. Licensed for use by ASM Microbe Library <http://www.microbelibrary.org/>. *Ehrlichiae* are obligate intracellular parasites related to the rickettsiae that are tick-borne pathogens of dogs and humans. In humans, they cause human granulocytic ehrlichiosis (HGE) and human monocytic ehrlichiosis (HME). In this electron micrograph, dense-core cells of *E. chaffeensis* are seen exiting the host cell following rupture of the cytoplasmic membrane. The ehrlichiae will now go on to infect additional host cells or they may be ingested by a feeding tick, and spread to another animal.

Mycoplasmas are a group of bacteria that lack a cell wall. The cells are bounded by a single triple-layered membrane. They may be free-living in soil and sewage, parasitic inhabitants of the mouth and urinary tract of humans, or pathogens in animals and plants. In humans, *Mycoplasma pneumoniae* causes **primary atypical pneumonia**, also called **walking pneumonia**.

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The Constitutive Defenses of the Host against Microbial Pathogens

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Introduction: Host Defense Mechanisms

Although humans are in continuous associations with microorganisms, and some readily colonize the body surfaces (see [The Bacterial Flora of Humans](#)), it is relatively rare that these microorganisms cause damage to their host. In part, this is due to the effectiveness of the host defense mechanisms, which restrict invasion by normal flora (some of which may be potential pathogens), and which defend against non-indigenous microorganisms that are overt pathogens.

Just as the outcome of an interaction between the host and a member of the normal flora always depends on specific properties inherent to both the host and the microbe, so does the outcome of an interaction between the host and a parasite. Sometimes the host tolerates **colonization** by a parasite but restricts it to regions of the body where it can do no harm (e.g. *Staphylococcus aureus* on the nasal membranes or *Streptococcus pneumoniae* in the upper respiratory tract). If the parasite **invades** (i.e., breaches an anatomical barrier or progress beyond the point of colonization), an **infection** is said to have occurred. If, as a result of infection, pathological harm to the host becomes evident, this is called an **infectious disease**. An infectious disease is a consequence of a microbial parasite causing such a degree of harm to its host that it results in a pathological process.

The healthy animal defends itself against pathogens different stages. The host defenses may be of such a degree that infection can be prevented entirely. Or, if infection does occur, the defenses may stop the process before disease is apparent. At other times, the defenses that are necessary to defeat a parasite may not be effective until infectious disease is well into progress.

Typically the host **defense mechanisms are divided into two groups**:

1. **Constitutive Defenses:** Defenses common to all healthy animals. These defenses provide general protection against invasion by normal flora, or colonization, infection, and infectious disease caused by pathogens. The constitutive defenses have also been referred to as "natural" or "innate" resistance, since they are inherent to a specific host, but these terms are better reserved for certain types of constitutive defense (see below).
2. **Inducible Defenses:** Defense mechanisms that must be induced or turned on by host exposure to a pathogen (as during an infection). Unlike the constitutive defenses, they are not immediately ready to come into play until after the host is appropriately exposed to the parasite. The inducible defenses involve the **immune responses** to a pathogen causing an infection.

The inducible defenses are generally quite specifically directed against an invading pathogen. The constitutive defenses are not so specific, and are directed toward general strategic defense.

Constitutive Defenses of the Host

The **constitutive defenses** of the host can be arranged in the following categories:

Differences in susceptibility to certain pathogens

Anatomical defenses

Microbial antagonism

Tissue bactericides, including complement

Inflammation (ability to undergo an inflammatory response)

Phagocytosis

Each of these topics is discussed in the sections below.

Differences in Susceptibility of Animal Hosts to Microbial Pathogens

This type of resistance is also called **innate** and natural **resistance**. There are two aspects innate resistance: (1) natural (genetic) resistance among all members of a species, called **species resistance** and (2) **individual resistance** within the same animal species

Species resistance

Certain animals are naturally resistant or nonsusceptible to certain pathogens. Certain pathogens infect only humans, not lower animals, e.g. syphilis, gonorrhea, measles, poliomyelitis. On the other hand, certain pathogens (e.g. canine distemper virus) do not infect humans. *Shigella* infects humans and baboons but not chimpanzees. Little information is available to explain these absolute differences in susceptibility to a pathogen but it could be due to:

Absence of specific tissue or cellular receptors for attachment (colonization) by the pathogen. For example, different strains of enterotoxigenic *E. coli*, defined by different fimbrial antigens, colonize human infants, calves and piglets, by recognizing species-specific carbohydrate receptors on enterocytes in the gastrointestinal tract.

Temperature of the host and ability of pathogen to grow. For example, birds do not normally become infected with mammalian strains of *Mycobacterium tuberculosis* because these strains cannot grow at the high body temperature of birds. The anthrax bacillus (*Bacillus anthracis*) will not grow in the cold-blooded frog (unless the frog is maintained at 37°).

Lack of the exact nutritional requirements to support the growth of the pathogen. Naturally-requiring purine-dependent strains of *Salmonella typhi* grow only in hosts supplying purines. Mice and rats lack this growth factor and pur- strains are avirulent. By injecting purines into these animals, such that the growth factor requirement for the bacterium is satisfied, the organisms prove virulent.

Lack of a target site for a microbial toxin. Most toxins produced by microbial cells exert their toxic activity only after binding to susceptible cells or tissues in an animal. Certain animals may lack an appropriate target cell or specific type of cell receptor for the toxin to bind to, and may therefore be nonsusceptible to the activity of the toxin. For example, injection of diphtheria toxin fails to kill the rat. The unchanged toxin is excreted in the urine. Inject a sample of the urine (or pure diphtheria toxin) into the guinea pig, and it dies of typical lesions caused by diphtheria toxin.

Individual resistance

There are many reasons why individuals of the same animal species may exhibit greater or lesser susceptibility to the same infective agent.

Age: usually this relates to the development and status of the immune system which varies with age. May also be associated with changes in normal flora coincidental to developmental changes in the animal.

Sex: usually linked to the presence and/or development of the sex organs. For example, mastitis and infectious diseases leading to abortion will obviously occur only in the female; orchitis would occur only in males). Could also be due to anatomical structure related to sex (bladder infections are 14-times more common in females than males), and possibly the effects of sex hormones on infections.

Stress. Stress is a complex of different factors and apparently has a real influence on health. Undue exertion, shock, change in environment, climatic change, nervous or muscular fatigue, etc. are factors known to contribute to increases in susceptibility to infection. The best explanation is that in time of stress the output of cortisone from the adrenal cortex is increased. This suppresses the inflammatory processes of the host and the overall effect may be harmful. There are also a number of relationships between stress-related hormones and the functioning of the immune defenses.

Diet, malnutrition. Infections may be linked with vitamin and protein deficiencies and this might explain partly why many infectious diseases are more prevalent and infant mortality rates are highest in parts of the world where malnourishment is a problem. Also, overfed and obese animals are more susceptible to infection. Diets high in sucrose predispose individuals to dental caries.

Intercurrent disease or trauma. The normal defenses of an animal are impaired by organic diseases such as leukemia, Hodgkin's disease, diabetes, AIDS, etc. Frequently, inflammatory or immune responses are delayed or suppressed. Colds or influenza may predispose an individual to pneumonia. Smoking tobacco predisposes to infections of the respiratory tract. Burned tissue is readily infected by *Pseudomonas aeruginosa*.

Therapy against other diseases. Modern therapeutic procedures used in some diseases can render an individual more susceptible to infection. Under these conditions, not only pathogens but organisms of the normal flora and nonpathogens in the host's environment may be able to initiate infection. Examples of therapeutic procedures that reduce the efficiency of the host's defenses are treatment with corticosteroids, cytotoxic drugs, antibiotics, or irradiation.

Anatomical Defenses

The structural integrity of the body surfaces, i.e., the skin and mucous membranes, forms an effective barrier to initial lodgement or penetration by microorganisms. The skin is a very effective barrier to bacteria so that no bacterium by itself is known to be able to penetrate unbroken skin. Of course, a puncture, cut or scrape in the skin could introduce infectious bacteria. The mucous membranes are more vulnerable to penetration by infectious bacteria but still pose a formidable barrier of mucus and antimicrobial substances. Nonetheless, most infectious agents impinge on the skin or mucous membranes of the oral cavity, respiratory tract, GI tract or urogenital tract, and from these sites most infections occur.

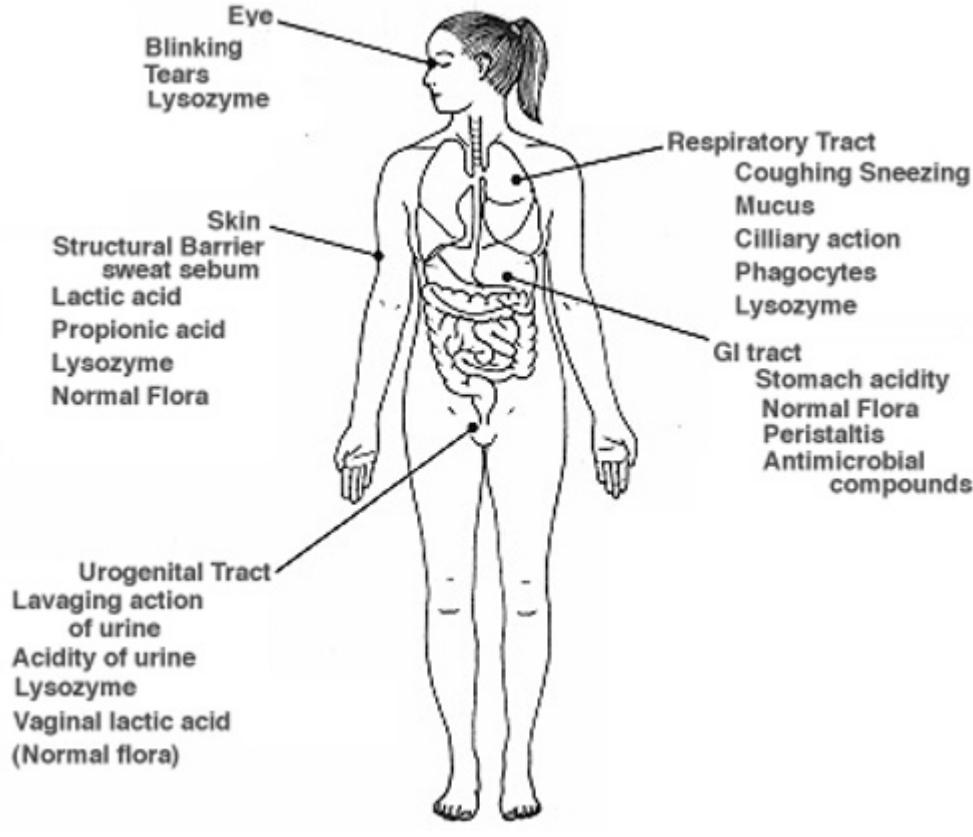


Figure 1. Anatomical defenses associated with tissue surfaces

Skin. The intact surface of the healthy epidermis seems to be rarely if ever penetrated by bacteria. If the integrity of the epidermis is broken (by the bite of an insect, needle stick, abrasion, cut, etc.) invasive microbes may enter. The normal flora of the skin, which metabolize substances secreted onto the skin, produce end products (e.g. fatty acids) that discourage the colonization of skin by potential pathogens. Perspiration contains lysozyme and other antimicrobial substances.

Mucous membranes. Many are heavily colonized with bacteria in whose moist secretions they survive. These normal flora are restricted from entry and usually occupy any attachment sites that might otherwise be used by pathogens. The normal flora established on mucous membranes may antagonize non-indigenous species by other means, as well. Typically, mucus contains a number of types of anti-microbial compounds, including lysozyme and secretory antibodies (IgA). Sometimes phagocytes patrol mucosal surfaces (e.g. in the lower respiratory tract). Nonetheless, some pathogens are able to penetrate the mucous membranes, and this is probably the major site from which pathogens invade. Probably, damage to the epithelial cells caused by toxic products of these bacteria plays a role.

Respiratory tract. Fine hairs and baffles of the nares (nasal membranes) entrap bacteria which are inhaled. Those which pass may stick to mucosal surfaces of the trachea or be swept upward by the ciliated epithelium of the lower respiratory tract. Coughing and sneezing also eliminate bacteria. The lower respiratory tract (lung) is well protected by mucus, lysozyme, secretory antibody, and phagocytosis.

Mouth, stomach and intestinal tract. Microorganisms entering by the oral route, more than any other, have to compete with the well-adapted normal flora of the mouth and intestine. Most organisms that are swallowed are destroyed by acid and various secretions of the stomach. Alkaline pH of the lower intestine can discourage other organisms. The peristaltic action of the intestine ultimately flushes out organisms which have not succeeded in colonization. Bile salts and lysozyme are present, which kill or inhibit many types of bacteria.

Urogenital Tract. The flushing mechanisms of sterile urine, and the acidity of urine, maintain the bladder and most of the urethra free of microorganisms. The vaginal epithelium of the female maintains a high population of Doderlein's bacillus (*Lactobacillus acidophilus*) whose acidic end products of metabolism (lactic acid) prevent colonization by most other types of microorganisms including potentially-pathogenic yeast (*Candida albicans*).

Eyes (Conjunctiva). The conjunctiva of the eye is remarkably free of most microorganisms. Blinking mechanically removes microbes, the lavaging action of tears washes the surface of the eye, and lachrymal secretions (tears) contain relatively large amounts of lysozyme.

Microbial Antagonism

This refers to the protection of the surfaces afforded by an intact normal flora in a healthy animal, and it has already been discussed in several contexts. There are **three main ways that the normal flora protect the surfaces where they are colonized:**

Competition with non-indigenous species for binding (colonization) sites. The normal flora are highly-adapted to the tissues of their host. That is why they are there!

Specific antagonism against non-indigenous species. Members of the normal flora may produce highly specific proteins called bacteriocins which kill or inhibit other (usually closely-related) species of bacteria.

Nonspecific antagonism against non-indigenous species. The normal flora produce a variety of metabolites and end products that inhibit other microorganisms. These include fatty acids (lactate, propionate, etc.) and peroxides.

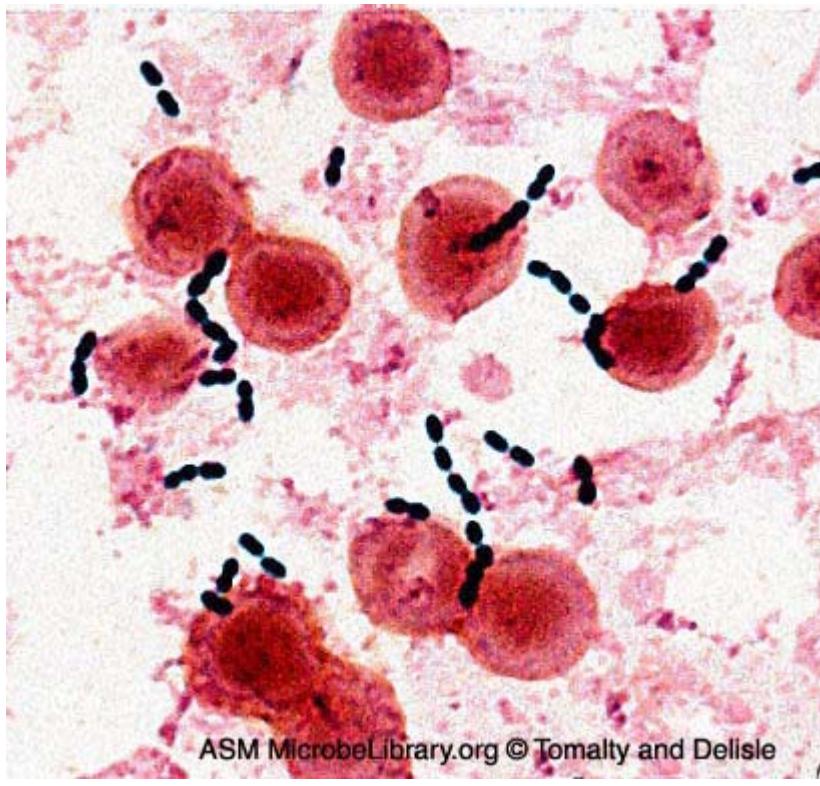


Figure 2. *Enterococcus faecalis*, also classified as *Streptococcus faecalis*. Occasionally there is invasion of the host by the normal flora, as evidenced by this blood culture. *Enterococcus faecalis*, blood culture. © Gloria J. Delisle and Lewis Tomalty, Queens University Kingston, Ontario, Canada. Licensed for use by ASM Microbe Library <http://www.microbelibrary.org/>.

Antimicrobial Substances in Host Tissues

The body fluids and organized tissues of animals naturally contain a variety of antimicrobial agent that kill or inhibit the growth of microbes. The sources and activities of a variety of host antimicrobial substances are summarized in Table 1.

TABLE 1. ANTIMICROBIAL SUBSTANCES OF HOST ORIGIN PRESENT IN BODY FLUIDS AND ORGANIZED TISSUES

Substance	Common Sources	Chemical Composition	Activity
Lysozyme	Serum, saliva, sweat, tears	Protein	Bacterial cell lysis
Complement	Serum	Protein-carbohydrate lipoprotein complex	Cell death or lysis of bacteria; participates in inflammation
Basic proteins and polypeptides (histones, β -lysins and other cationic proteins, tissue polypeptides)	Serum or organized tissues	Proteins or basic peptides	Disruption of bacterial plasma membrane
Lactoferrin and transferrin	Body secretions, serum, organized tissue spaces	Glycoprotein	Inhibit microbial growth by binding (withholding) iron
Peroxidase	Saliva, tissues, cells (neutrophils)	Protein	Act with peroxide to cause lethal oxidations of cells
Fibronectin	Serum and mucosal surfaces	Glycoprotein	Clearance of bacteria (opsonization)
Interferons	Virus-infected cells, lymphocytes	Protein	Resistance to virus infections

Interleukins	Macrophages, lymphocytes	Protein	Cause fever; promote activation of immune system
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Complement

Complement can be considered as part of the constitutive host defense mechanisms (it is present at constitutive levels) because of its role in inflammation and phagocytosis. However, the antimicrobial activities of complement can be activated completely by reactions between antigens and antibodies and, therefore, it may play a role in the inducible (immune) defenses, as well.

Complement is an enzymatic system of serum proteins made up of 9 major components (C1 - C9) that are sequentially activated in many Ag - Ab reactions resulting in disruption of membranes. Therefore, complement (C') may be involved in the lysis of certain bacteria, some viruses, and other microorganisms. In addition, some C' components play a part in phagocytic chemotaxis, opsonization and the inflammatory response.

Complement is activated in the **classical pathway** by reactions between antibodies and antigens on the surface of a microbe. Some Immunoglobulins (i.e., IgG and IgM) can "fix complement" because they have a complement binding site on the Fc portion of the molecule. The reaction between IgG and Ag activates the complement and initiates a "cascade reaction" on the surface of the microbe that results in the principal effects of complement which are:

1. Generation of inflammatory factors, C3a and C5a, which focus antimicrobial serum factors and leukocytes into the site of infection.

2. Attraction of phagocytes. Chemotactic factors C3a and C5a attract phagocytes to the site.

3. Enhancement of phagocytic engulfment. C3b component on Ag - Ab complex attaches to C3b receptors on phagocytes and promotes opsonization of Ab-coated cells. C3b-opsonization is important when Ab is IgM because phagocytes have receptors for Fc of IgM only when it is associated with C3b.

4. Lysis of bacterial cells (lysozyme-mediated) or virus-infected cells. When C8 and C9 are bound to the complex, a phospholipase is formed that destroys the membrane of Ag-bearing host cells (e.g. virus-infected cells) or the outer membrane of Gram-negative bacteria. Lysozyme gains access to peptidoglycan and completes destruction of the bacterial cell.

In addition to the classical pathway"of complement activation an **alternative pathway** (sometimes called the "properdin pathway") of complement activation exists which is independent of immunoglobulins. Insoluble polysaccharides (including bacterial LPS, peptidoglycan and teichoic acids) can activate complement. This allows antibody-independent activation of the complement cascade that may be important in initial (pre-antibody) defense against various types of infections caused by bacteria.

Inflammation

Of all the defense mechanisms in the animal host, the **inflammatory response** may be the most important for dealing with microbial infection. Inflammation is necessary for the proper functioning of all the host defenses, including the immune defenses, because it focuses all circulating antimicrobial factors on the site of infection. These include phagocytes, lymphocytes, antibodies, complement and other antimicrobial components of plasma. However, inflammation is also an important aspect of bacterial pathogenesis since the inflammatory response induced by a microbe can result in considerable damage to the host and, therefore, be part of the pathology of microbial disease.

Inflammation is a tissue reaction to infection or injury, the **characteristic symptoms** of which are **redness, swelling, heat and pain**. These are sometimes called the cardinal signs of inflammation. The redness is due to increased blood flow to the area of injury. The swelling (edema) is due to increased extravascular fluid and phagocyte infiltration to the damaged area. The heat is due to the increased blood flow and the action of pyrogens

(fever-inducing agents). The pain is caused by local tissue destruction and irritation of sensory nerve receptors.

Inflammation can be induced by certain **immunological reactions**, **tissue damage**, or the **entry of an injurious agent** (microbial or nonmicrobial). Certain bacterial cells and/or their products (e.g. structural components or toxins) can induce an inflammatory response. Inflammation increases the blood supply and temperature in the inflamed tissues, which favors maximal metabolic activity of the leukocytes, and lowers the pH slightly, which tends to inhibit the multiplication of many microorganisms.

The events involved in the induction and maintenance of the inflammatory response are summarized below.

(1) The inflammatory response is triggered by pathogen invasion or tissue injury. Injured and dying cells release cytoplasmic constituents which lower the pH in the surrounding extracellular environment.

(2) The increased acidity activates an extracellular enzyme **kallikrein** which in turn activates **bradykinin**.

(3a) Bradykinin binds to receptors on the capillary walls opening junctions between cells to allow leakage of plasma components collectively referred to as the **inflammatory exudate**.

Increased capillary permeability allows leukocytes to pass from the vessels into tissues (this process is called **diapedesis**). The first to appear, and the most dominant, are neutrophils, which are actively phagocytic. The other components of the inflammatory exudate and their functions are described in Table 2 below).

(3b) Bradykinin also binds to mast cells of the connective tissue that are associated with the small vessels of most tissues. This initiates other events that are associated with the process of inflammation.

Initially there is a rapid influx of Ca++, intracellular cAMP levels drop, and mediator-rich lysosomal granules migrate to the cell surface, fuse with the cell membrane, and discharge their contents (preformed mediators of inflammation such as **histamine**, **heparin**, etc.) to the exterior by exocytosis

The change in mast cell permeability activates an enzyme, **phospholipase A2** to synthesize a substance called **arachidonic acid**. This compound can be acted upon subsequently by the **cyclooxygenase pathways** or **lipooxygenase** pathways of the mast cell leading to new synthesis of **prostaglandins**, **leukotrienes**, and other mediators of inflammation. These substances contribute to the inflammatory exudate.

TABLE 2. FUNCTION OF COMPONENTS AND CELLS IN THE INFLAMMATORY EXUDATE

Component	Function
Bradykinin, histamine, leukotrienes, serotonin, prostaglandins	Inflammatory Agents (IA) which act on the vascular system to produce increased blood flow and permeability
Fibrin: (formed from fibrinogen in plasma)	coagulates and may localize an invading pathogen
Lysozyme	causes lysis of bacterial cell walls
Complement	various activities increase the inflammatory response and lead to increased phagocytosis and complement-mediated lysis of cells
Antibodies (in immune individuals)	block colonization by pathogens; neutralize microbial toxins or viruses; opsonize pathogens making them more susceptible to phagocytosis; activate complement
Pyrogens, including endogenous pyrogen (Interleukin 1)	cause fever acting on the thermo-regulatory control centers in the hypothalamus. (Interleukin-1, which is produced by macrophages, also promotes activation and mitosis of B-cells and T-cells)
Neutrophils	migrate to focus of infection and ingest and destroy foreign agents by phagocytosis
Macrophages	engulf and destroy infective agents, process antigenic components and convey them to lymphocytes
Immunocompetent lymphocytes (B-cells and T-cells)	for direct participation in immunological responses (AMI and CMI)

The overall effect of an inflammatory reaction is to recruit various cells and components to the actual site of

microbial invasion. Many of these cells and plasma components have a direct role in defense against the intruding microorganism. These include **neutrophils** (phagocytes which engulf and destroy the microbes); **macrophages** and **lymphocytes** which are the cells necessary to initiate immunological responses against the pathogen; pre-existing **antibodies** which can neutralize microbial pathogens or their toxins; and plasma components such as **lysozyme**, **complement** and **fibrin**, which have a variety of antimicrobial activities.

Phagocytic Defenses

When invading parasites penetrate the tissues the inflammatory response, previously described, is immediately brought into play. Part of this response leads to the recruitment of phagocytes to the site of inflammation.

Phagocytes are a class of cells which are capable of ingestion (engulfment) and destruction of microorganisms that are responsible for inciting the inflammatory response. First to accumulate around the invaders and initiate the phagocytic process are **neutrophils**. Later, local and blood-borne **macrophages** also migrate to the tissue site and initiate phagocytosis. Neutrophils (also known as polymorphonuclear leucocytes or PMNs) and macrophages are sometimes referred to as **professional phagocytes** for their roles in this process.

Properties of Neutrophils

Neutrophils have their origin in multi-potential **stem cells** in the bone marrow. They differentiate in the marrow and are released in a mature form, containing a full complement of bactericidal agents. They are short-lived cells which constitute 30-70% of the circulating white blood cells (leukocytes).

During differentiation in the marrow (2-3 days) the nucleus of the cell becomes multilobed (hence the name **polymorphonuclear leukocyte**), cell division ceases, and mitochondria and endoplasmic reticulum disappear from the cytoplasm. At the same time the cell becomes motile and actively phagocytic. Cytoplasmic granules are formed from the Golgi apparatus. These granules are called **lysosomes** and contain the various bactericidal and digestive enzymes which can destroy bacterial cells after engulfment. The contents of lysosomal granules include lysozyme, cationic proteins, acid hydrolases, proteases, peroxidase and lactoferrin. Neutrophils also contain large store of glycogen; since they derive most of their metabolic energy from glycolysis, they can function efficiently in anaerobic environments.

Some additional properties of neutrophils are:

- Only half the neutrophils in human circulation are detectable in the blood; the rest adhere to vessel walls.
- For every circulating neutrophil, approximately 100 near mature cells are held in reserve in the bone marrow pool.
- Once a neutrophil enters the tissues, intestinal tract or respiratory tract, it never returns to the circulation.

Properties of Macrophages

Macrophages (also called **mononuclear phagocytes**) also arise from bone marrow stem cells which give rise to promonocytes which develop into **monocytes** that are released into the blood stream. Monocytes make up 3-7% of the circulating white blood cells. The monocyte is actively phagocytic and bactericidal. Within 2 days or so, the blood stream monocytes (sometimes called wandering macrophages) emigrate into the tissues where they settle down, enlarge and become fixed macrophages (tissue histiocytes), which also have phagocytic potential. Macrophages are more active in phagocytosis than monocytes and develop many more granules containing hydrolytic enzymes. New macrophages can develop by cell division under inflammatory stimuli, but most macrophages are matured blood monocytes.

The total pool of macrophages is referred to as the **system of mononuclear phagocytes**. The system is scattered throughout connective tissue, basement membranes of small blood vessels, liver sinusoids, the spleen, lung, bone marrow and lymph nodes. Monocytes from the blood migrate into virtually every organ in the body where they mature into fixed macrophages. In the lymph nodes, they function as scavengers to remove foreign material from the circulation.

Compared to neutrophils, macrophages are long-lived cells. As phagocytes, neutrophils play a more important role

in the acute stages of an infection, while macrophages are principally involved in chronic types of infections. Neutrophils circulate in the blood stream, and during an acute inflammatory response they migrate through the endothelial cell junctions as part of the inflammatory exudate. They migrate to the focus of the infection and ingest or phagocytose the foreign agents, Neutrophils which have become engorged with bacteria usually die and largely make up the material of pus. Macrophages, which are also attracted to the area during an inflammatory response, are slower to arrive and become increasingly involved in chronic infections. They, too, are actively phagocytic and will engulf and destroy foreign particles such as bacteria. However, macrophages have another indispensable function in host defense: they "process" the antigenic components of infective agents and present them to lymphocytes, a process that is usually required for the initiation of the immune responses of the host. Macrophages are among an elite corps of **antigen-presenting cells** or APC's.

The Phagocytic Process

Phagocytosis and destruction of engulfed bacteria involves the following sequence of events:

1. **Delivery** of phagocytic cells to the site of infection
2. **Phagocytic adherence** to the target
3. **Ingestion** or engulfment of the target particle
4. **Phagolysosome** formation
5. **Intracellular killing**
6. **Intracellular digestion** (and egestion, in the case of macrophages)

These steps involved in the phagocytic process in macrophages are illustrated below.

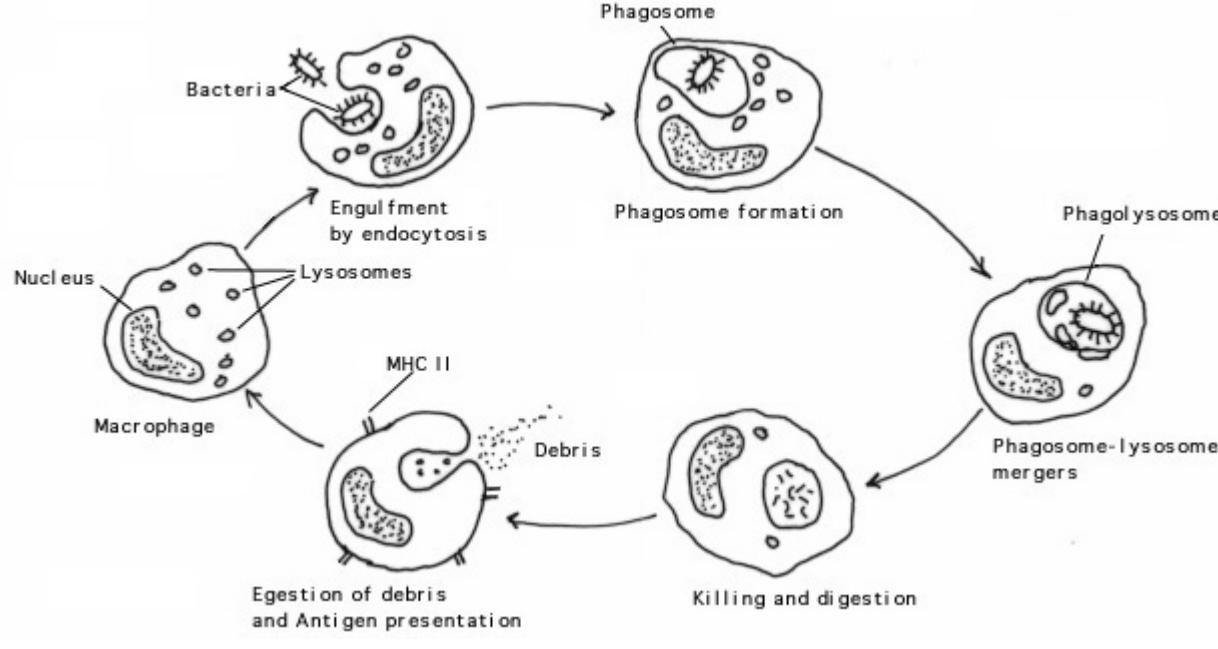


Figure 3. Phagocytosis by a Macrophage. A bacterium, which may or may not be opsonized, is engulfed by the process of endocytosis. The bacterium is ingested in a membranous vesicle called the phagosome. Digestive granules (lysosomes) merge with phagosome, release their contents, and form a structure called the phagolysosome. The killing and digestion of the bacterial cell takes place in the phagolysosome. The macrophage egests debris while processing the antigenic components of the bacterium, which it returns to its surface in association with MHC II for antigen presentation to TH cells.

Delivery of phagocytic cells to the site of infection

The **delivery** of phagocytic cells, monocytes or neutrophils, to the site of microbial infection involves two processes:

Diapedesis: the migration of cells across vascular walls which is initiated by the mediators of inflammation (kinins, histamine, prostaglandins, etc.)

Chemotaxis. Phagocytes are motile by ameboid action. Chemotaxis is movement of the cells in response to a chemical stimulus. The eventual concentration of phagocytes at a site of injury results from chemotactic response by the phagocytes which is analogous to bacterial chemotaxis. A number of chemotactic factors (attractants) have been identified, both for neutrophils and monocytes. These include bacterial products, cell and tissue debris, and components of the inflammatory exudate such as peptides derived from complement.

Phagocytic adherence

Phagocytosis is initiated by adherence of a particle to the surface of the plasma membrane of a phagocyte. This step usually involves several types of surface receptors on the phagocyte membrane. Three major receptors on phagocytes recognize the Fc portion of IgG: one is for monomeric IgG and the others are for antigen-crosslinked IgGs. Another receptor binds a complement factor C3b. Other phagocyte receptors bind fibronectin and mannose-terminated oligosaccharides. Under certain circumstances of infection, bacteria or viruses may become coated or otherwise display on their surfaces one or another of these substances (i.e., IgG, C3b, fibronectin or mannose). Such microbes are said to be **opsonized** and such substances as IgG or complement C3b bound to the surface of microbes are called **opsonins**. (Opsonin comes from a Greek word meaning "sauce" or "seasoning": they make the bacterium or virus more palatable and more easily ingested by the phagocyte.) Opsonins provide extrinsic ligands for specific receptors on the phagocyte membrane, which dramatically increases the rate of adherence and ingestion of the pathogen. Opsonized bacteria can be cleared from the blood by phagocytes; many types of non opsonized bacteria cannot be cleared.

Less firm attachments of a phagocyte to a particle can take place in the absence of opsonization. This can be thought of as **nonspecific attachment** which might be due to net surface charge on the phagocyte or particle and/or hydrophobicity of the particle.

Also, a phenomenon called **surface phagocytosis** exists: a phagocyte can simply trap an organism against a surface and initiate ingestion. Surface phagocytosis may be an important pre-antibody defense mechanism which may determine whether an infection will become a disease and how severe the disease will become.

Ingestion

After attachment of the phagocyte to its target, some sort of signal generation, which is poorly understood, results in physical or chemical changes in the cell that triggers ingestion. Ingestion is an engulfment process that involves infolding or invagination of the cell membrane enclosing the particle and ultimately releasing it into the cytoplasm of the cell within a membrane vesicle. The end result of ingestion is entry of the particle enclosed in a vesicle derived from the plasma membrane of the cell. This structure is called the **phagosome**.

Formation of the phagolysosome

The phagosome migrates into the cytoplasm and collides with lysosomal granules which explosively discharge their contents into the membrane-enclosed vesicle (phagosome). Membranes of the phagosome and lysosome actually fuse resulting in a digestive vacuole called the **phagolysosome**. Other lysosomes will fuse with the phagolysosome. It is within the phagolysosome that killing and digestion of the engulfed microbe takes place. Some of the microbial constituents of the lysosomes of neutrophils and macrophages include lysozyme, cationic proteins, various proteases and hydrolyases and peroxidases. The killing processes are confined to the membranous organelles of the phagocytes (the phagolysosome) such that none of the toxic substances and lethal activities of the phagocytes are turned against themselves.

Intracellular killing of organisms

After phagolysosome formation the first detectable effect on bacterial physiology, occurring within a few minutes after engulfment, is loss of viability (ability to reproduce). The exact mechanism is unknown. Inhibition of macromolecular synthesis occurs later. By 10 to 30 minutes after ingestion many pathogenic and nonpathogenic bacteria are killed followed by lysis and digestion of the bacteria by lysosomal enzymes. The microbial activities

of phagocytes are complex and multifarious. Metabolic products, as well as lysosomal constituents, are responsible. These activities differ to some extent in neutrophils, monocytes and macrophages.

The microbicidal activities of phagocytes are usually divided into **oxygen-dependent** and **oxygen-independent** events

Oxygen-independent activity

Lysosomal granules contain a variety of extremely basic proteins that strongly inhibit bacteria, yeasts and even some viruses. A few molecules of any one of these cationic proteins appear able to inactivate a bacterial cell by damage to their permeability barriers, but their exact modes of action are not known. The lysosomal granules of neutrophils contain lactoferrin, an extremely powerful iron-chelating agent, which withholds potential iron needed for bacterial growth. The pH of the phagolysosome may be as low as 4.0 due to accumulation of lactic acid, which is sufficiently acidic to prevent the growth of most pathogens. This acidic environment apparently optimizes the activity of many degradative lysosomal enzymes including lysozyme, glycosylases, phospholipases, and nucleases.

Oxygen-dependent activity

Liganding of Fc receptors (on neutrophils, monocytes or macrophages) and mannose receptors (on macrophages) increases their O_2 uptake, called the **respiratory burst**. These receptors activate a membrane-bound **NADPH oxidase** that reduces O_2 to O_2^- (superoxide). Superoxide can be reduced to OH^- (hydroxyl radical) or dismutated to H_2O_2 (hydrogen peroxide) by superoxide dismutase. O_2^- , OH^- , and H_2O_2 are activated oxygen species that are potent oxidizing agents in biological systems which adversely affect a number of cellular structures including membranes and nucleic acids. Furthermore, at least in the case of neutrophils, these reactive oxygen intermediates can act in concert with a lysosomal enzyme called **myeloperoxidase** to function as the myeloperoxidase system, or MPO.

Myeloperoxidase is one of the lysosomal enzymes of neutrophils which is released into the phagocytic vacuole during fusion to form the phagolysosome. Myeloperoxidase uses H_2O_2 generated during the respiratory burst to catalyze halogenation (mainly chlorination) of phagocytosed microbes. Such halogenations are a potent mechanism for killing cells.

When the NADPH oxidase and myeloperoxidase systems are operating in concert, a series of reactions leading to lethal oxygenation and halogenation of engulfed microbes occurs.

Intracellular digestion

Dead microbes are rapidly degraded in phagolysosomes to low molecular-weight components. Various hydrolytic enzymes are involved including lysozyme, proteases, lipases, nucleases, and glycosylases. Neutrophils die and lyse after extended phagocytosis, killing, and digestion of bacterial cells. This makes up the characteristic properties of pus.

Macrophages egest digested debris and allow insertion of microbial antigenic components into the plasma membrane for presentation to lymphocytes in the immunological response.

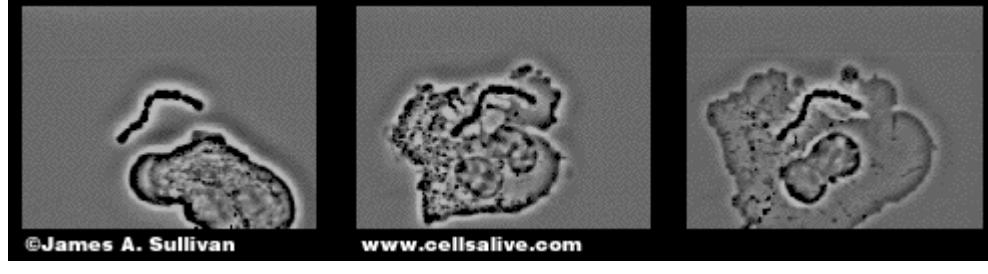


Figure 4. Phagocytosis of *Streptococcus pyogenes* by a macrophage. [CELLS alive!](#)

Bacterial Defense Against Phagocytosis

Pathogenic bacteria have a variety of **defenses against phagocytes**. In fact, most successful pathogens have some mechanism(s) to contend with the phagocytic defenses of the host. These mechanisms will be discussed in detail later as part of the determinants of virulence of pathogens. However, in general, pathogens may resist phagocytosis by:

Evading phagocytes by growing in regions of the body which are not accessible to them

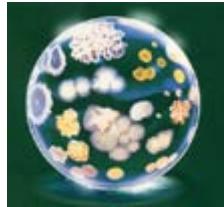
Avoiding engulfment by phagocytes after contact

Being able to **kill phagocytes** either before or after engulfment

Being able to **survive inside of phagocytes** (or other types of cells) and to persist as intracellular parasites

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Immune Defense against Microbial Pathogens

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Inducible defenses

The constitutive defenses, by themselves, may not be sufficient to protect the host against pathogens. However, even if pathogens evade or overcome the relatively nonspecific constitutive defenses, they may yet be detected and attacked by the more specific inducible defenses, once they have developed. The **inducible defenses** are so-called because they are induced upon primary exposure to a pathogen or one of its products. The inducible defenses are a function of the **immunological system** and the **immune responses**. They must be triggered in a host and initially take time to develop. The type of resistance thus developed in the host is called **acquired immunity**.

Acquired immunity

Acquired immunity may be divided into two types based on how it is acquired by the host.

In the case of **active immunity**, the host undergoes an immunological response and produces the cells and factors responsible for the immunity, i.e., the host produces its own antibodies and/or immuno-reactive lymphocytes. Active immunity can persist a long time in the host, up to many years in humans.

Passive immunity is acquisition by a host of immune factors which were produced in another animal, i.e., the host receives antibodies and/or immuno-reactive lymphocytes originally produced during an active response in another animal. Passive immunity is typically short-lived and usually persists only a few weeks or months.

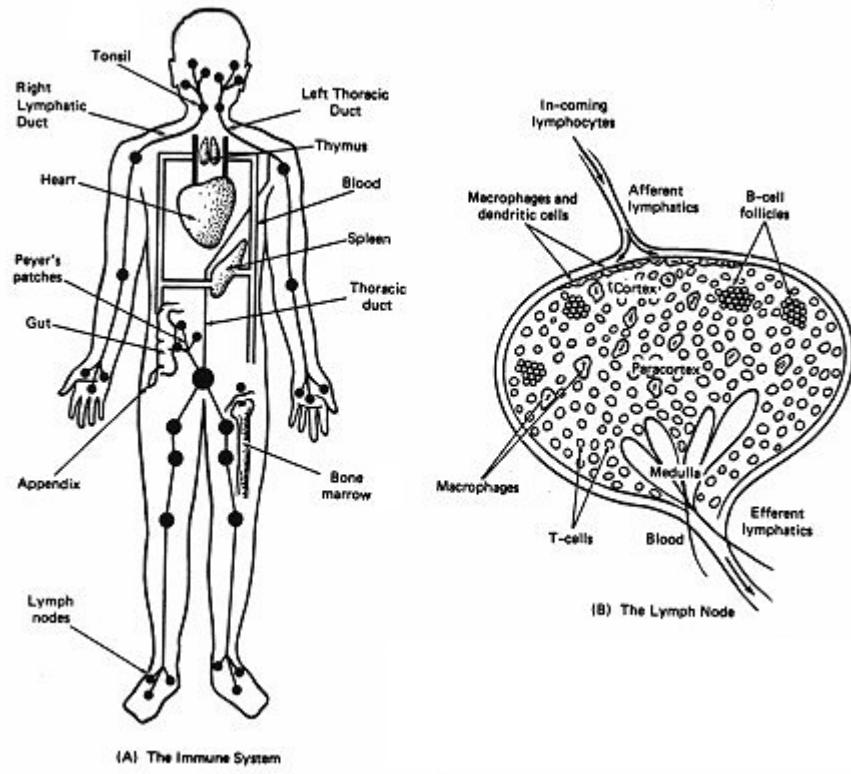
In either case of active or passive immunity, resistance may be acquired by **natural** means or by **artificial** means (i.e., vaccination and immunization procedures). Some familiar examples of active and passive immunity are given in the table below.

Table 1. Examples of Active and Passive Immunity

Type of Immunity	How Acquired by Host	Examples
Active Immunity	As a result of exposure to an infectious agent or one of its products (antigens)	Antibodies are produced by the host in response to the infectious agent itself (e.g. recovery from the disease), or in response to artificial immunization (vaccination) with some product derived from the infectious agent (e.g. toxoid, killed cells, structural components of cells, inactivated virus, etc.).
Passive Immunity	As a result of the acquisition of antibodies which have been produced in another animal (by active means) or derived from cells grown in tissue culture (monoclonal antibodies)	Injection of immune serum from an individual previously immunized or recovered from disease, e.g. hepatitis; Injection of serum from an animal hyperimmunized with tetanus toxoid; Placental transfer of antibodies from mother to fetus; Transfer of antibodies from mother to infant in milk by nursing.

The immune system

The **immunological system** is comprised of the **lymphoid tissues and organs of the body**. Lymphoid tissues are widely distributed : they are concentrated in bone marrow, lymph nodes, spleen, liver, thymus, and Peyer's patches scattered in linings of the GI tract. The lymphoid system is encompassed by the **system of mononuclear phagocytes**. Lymphocytes are the predominant cells, but macrophages and plasma cells are present also. Lymphocytes are cells which circulate, alternating between the circulatory blood stream and the lymphatic channels. The distribution of lymphatic tissues that make up the immune system in humans is illustrated in the figure below.



(A) The Immune System

Figure 1. Anatomy of the Immune System. (A): The major components of the immune system are lymph nodes connected by lymph ducts, Peyer's patches (masses of lymphocytes in the lower gastrointestinal tract), thymus, spleen, and bone marrow. **(B):** A lymph node. Afferent lymph ducts bring lymph-containing antigens into the lymph node. Macrophages, B cells or dendritic cells in the cortical region make contact with the antigen and process it for presentation to immunocompetent B cells and T cells, thereby initiating an immune response. As a result, B cells are stimulated to develop into antibody-secreting plasma cells, and T-cells are stimulated to develop into effector T cells of various classes. Antibodies leave the lymph node by the efferent ducts that empty into the blood stream. Lymphocytes can also leave the node by the efferent duct and travel to other sites in the lymphatic system or enter into the blood circulation. A single lymphocyte completes a circuit through the circulating blood and lymphatic systems once every 24 hours.

The immunological system is able to recognize foreign substances (antigens) which stimulate the system to produce **antibody-mediated immunity (AMI)**, **cell-mediated immunity (CMI)**, or both. **AMI** and **CMI** are the two great arms of the immune system that are discussed in more detail below.

An **antigen (Ag)** is a substance, usually macromolecular, that induces an immunological response. Because of its complex macromolecular structure, a single microorganism consists of multiple antigens (e.g. surface structures such as cell wall components, fimbriae, flagella, etc., or extracellular proteins, such as toxins or enzymes produced by the microorganism). The coat proteins and some of the envelope proteins of animal viruses are also usually antigenic. The host is able to respond specifically to each and every antigen to come into contact with the components of the immunological system.

The immune response

Immunological responses are associated with **macrophages** and two subpopulations of lymphocytes which are derived from primitive bone marrow cells. All of the cells involved in the immunological responses are derived from bone marrow **stem cells** which have differentiated under the influence of various tissues and stimuli. Macrophages develop from monocytes previously released from the bone marrow into the blood circulation.

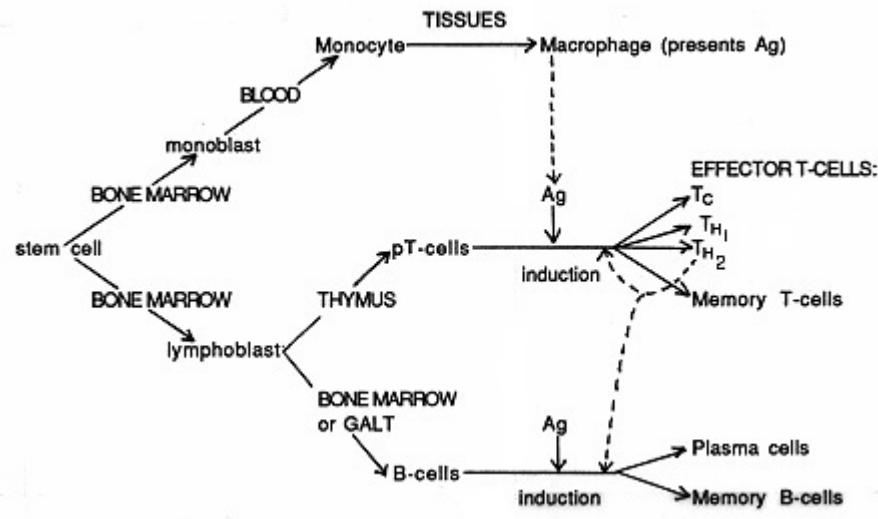
Lymphocytes responsible for AMI are processed by lymphoid tissue in the bone marrow and develop there into **B lymphocytes** or **B cells**. Lymphocytes responsible for CMI are processed by the thymus gland and mature into **T lymphocytes** or **T cells**.

Under antigenic stimulus, B-lymphocytes become transformed into antibody-secreting **plasma cells**. The plasma cells synthesize large amounts of **immunoglobulins (antibodies)** which will react stereochemically with the stimulating antigen.

Under antigenic stimulus, pre T-lymphocytes differentiate into several classes of **effector T cells** which are committed to various activities upon recognition of the specific antigen that induced their formation. T cells have many activities relevant to immunity including (1) mediation of the B-cell response to antigen; (2) ability to recognize and destroy cells bearing foreign Ag on their surface; and (3) production of a variety of diffusible compounds called cytokines and/or lymphokines, which include substances that are activators of macrophages, mediators of inflammation, chemotactic attractants, lymphocyte mitogens, and interferon. **Cytokines** and **lymphokines** are molecules (peptides, proteins) produced by cells as a means of intercellular communication. Generally, they are secreted by a cell to stimulate the activity of another cell.

The overall aspects of the induction of the immune responses (AMI and CMI) are shown in the following schematic diagram.

Figure 2. Schematic Diagram of the Development of the Immune Responses



Three important features of the immunological system relevant to host defense and/or "immunity" to pathogenic microorganisms are:

- 1. Specificity.** An antibody or reactive T cell will react specifically with the antigen that induced its formation; it will not react with other antigens. Generally, this specificity is of the same order as that of enzyme-substrate specificity or receptor-ligand specificity. However, cross-reactivity is possible. The specificity of the immune response is explained on the basis of the clonal selection hypothesis: during the primary immune response, a specific antigen selects a pre-existing clone of specific lymphocytes and stimulates exclusively its activation, proliferation and differentiation.
- 2. Memory.** The immunological system has a "memory". Once the immunological response has reacted to produce a specific type of antibody or reactive T cell, it is capable of producing more of the antibody or activated T cell more rapidly and in larger amounts. This is sometimes referred to as a **secondary, or memory response**.
- 3. Tolerance.** An animal generally does not undergo an immunological response to its own (potentially-antigenic) components. The animal is said to be **tolerant**, or unable to react to its own potentially-antigenic components. This ensures that under normal conditions, an immune response to "self" antigens (called an **autoimmune response**) does **not** occur. Autoimmune responses are potentially harmful to the host. Tolerance is brought about in a number of ways, but basically the immunological system is able to distinguish "self" components from "non-self" (foreign)

antigens; it will respond to "non-self" but not to "self". Sometimes in an animal, tolerance can be "broken", which may result in an **autoimmune disease**.

The two types immunity: AMI and CMI

Antibody-mediated immunity (AMI) is the type of immunity that is mediated by soluble host proteins called **antibodies** or **immunoglobulins**. Because it is largely due to the presence of circulating antibody molecules in the serum, is also called **circulating immunity** or **humoral immunity**. **Antibodies (Ab)** are proteins (globulins) produced in response to an encounter with an antigen. There are several classes or types of antibodies (and subclasses of the types), but all of the classes of antibodies that are produced in response to a specific antigen react stereochemically with that antigen and not with other (different) antigens. The host has the genetic capacity to produce specific antibodies to thousands of different antigens, but does not do so until there is an appropriate (specific) antigenic stimulus. Due to clonal selection, the host produces only the homologous antibodies that will react with that antigen. These antibodies are found in the blood (plasma) and lymph and in many extravascular tissues. They have a various roles in host defense against microbial and viral pathogens as discussed below.

Cell-mediated immunity (CMI) is the type of immunity that is mediated by specific subpopulations of T-lymphocytes called **effector T cells**. In non immune animals precursor T cells (pT cells) exist as "resting T cells". They bear receptors for specific antigens. Stimulation with Ag results in their activation. The cells enlarge, enter into a mitotic cycle, reproduce and develop into effector T cells whose activities are responsible for this type of immunity. They also develop into clones of identical reactive T cells called memory T cells.

The biological activities of the antibody-mediated and cell-mediated immune responses are different and vary from one type of infection to another. The AMI response involves interaction of B lymphocytes with antigen and their differentiation into antibody-secreting plasma cells. The secreted antibody binds to the antigen and in some way leads to its neutralization or elimination from the body. The CMI response involves several subpopulations of T lymphocytes that recognize antigens on the surfaces of cells. **TH cells** respond to antigen with the production of lymphokines. The distinction between **TH1** and **TH2** is based on their lymphokine profiles. TH2 cells have previously been referred to as **T helper cells** because they provide lymphokines (e.g. IL-2 and IL-4) which activate T cells and B cells at the start of the immune response. TH1 cells were formerly known as **delayed type hypersensitivity cells (TDTH)** because of their role in this allergic process. **TC cells** or **cytotoxic T lymphocytes (CTLs)** are able to kill cells that are showing a new or foreign antigen on their surface (as virus-infected cells, or tumor cells, or transplanted tissue cells).

Membrane receptors on B cells and T cells

The nature of the membrane receptors for antigen on B cells and T cells is fairly well understood. Each B cell has approximately 10^5 membrane-bound antibody molecules (IgD or IgM) which correspond in specificity to the antibody that the cell is programmed to produce. Each T cell has about 10^5 molecules of a specific antigen-binding **T cell receptor (TCR)** exposed on its surface. The TCR is similar, but not identical, to antibody. In addition, T cell subsets bear some distinguishing surface markers, notably **CD4** or **CD8**. T cells bearing CD4 always recognize antigens in association with **class II major histocompatibility complex (class II MHC)** proteins on the surfaces of other cells. $CD4^+$ T lymphocytes generally function as T helper cells. T cells bearing CD8 ($CD8^+$) always recognize antigen in association with **class I MHC** proteins and typically function as **cytotoxic T cells**. The important markers, actions and interactions of **T cells**, **B cells** and **Antigen Presenting Cells (APC)** are illustrated below.

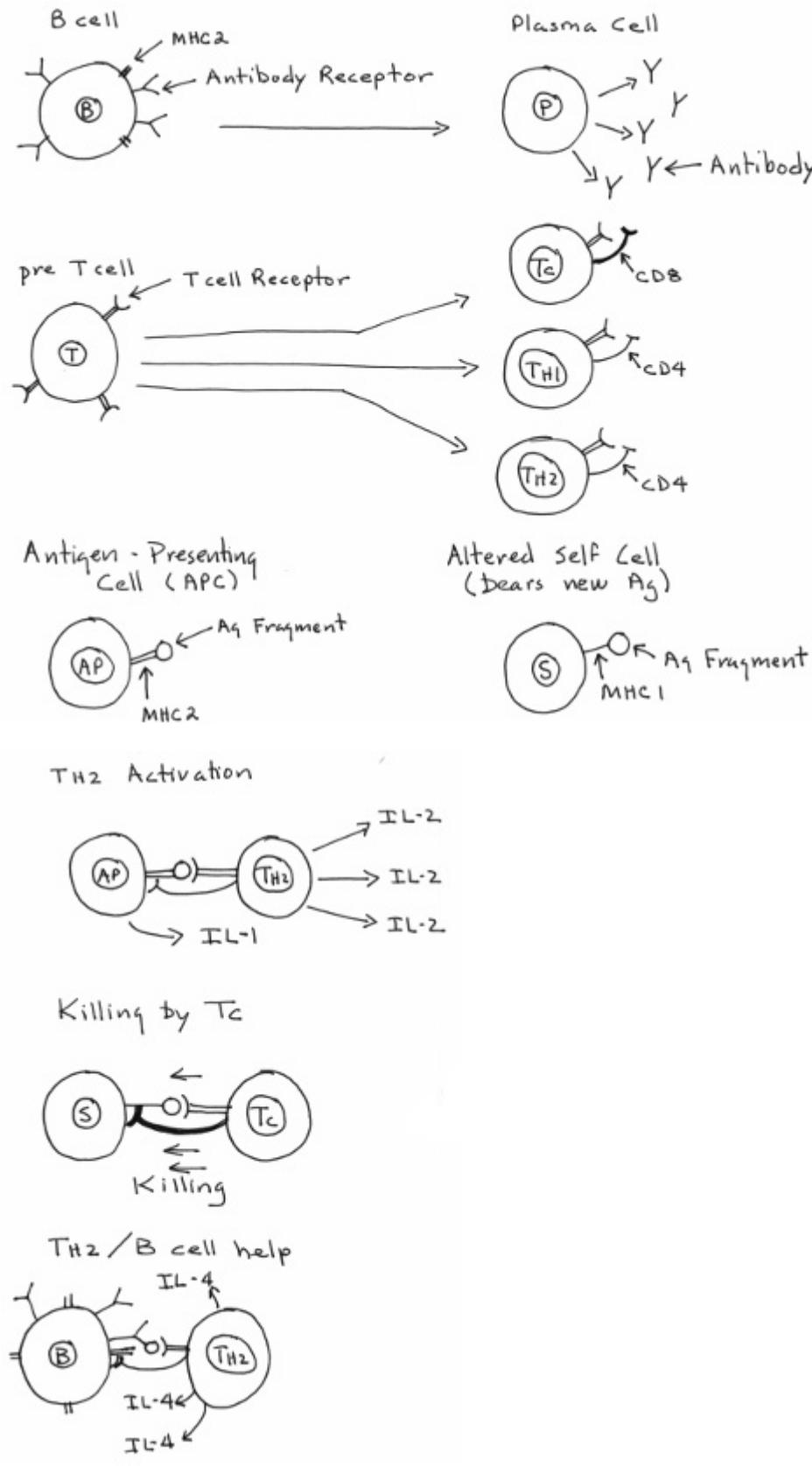


Figure 3. Receptor interactions between B cells, T cells and Antigen Presenting Cells (APC)

Induction of primary immune responses

Induction of a **primary immune response** begins when an **antigen** penetrates epithelial surfaces. It will eventually come into contact with **macrophages** or certain other classes of **Antigen Presenting cells (APCs)**, which include **B cells**, monocytes, dendritic cells, Langerhans cells and endothelial cells. Antigens, such as bacterial cells, are internalized by endocytosis and "processed" by the APC, then "presented" to immunocompetent lymphocytes to initiate the early steps of the immunological response. Processing by a macrophage (for example) results in

attaching antigenic materials to the surface of the membrane in association with **MHC II** molecules on the surface of the cell. The antigen-class II MHC complex is presented to a **T-helper (TH2)** cell which is able to recognize processed antigen associated with a class II MHC molecule on the membrane of the macrophage. This interaction, together with stimulation by **Interleukin 1 (IL-1)**, produced by the macrophage, will activate the TH2 cell. Activation of the TH2 cell causes that cell to begin to produce **Interleukin 2 (IL-2)**, and to express a membrane receptor for IL-2. The secreted IL-2 autostimulates proliferation of the TH2 cells. Stimulated TH2 cells produce a variety of lymphokines including **IL-2, IL-4, IL-6**, and **gamma Interferon** which mediate various aspects of the immune response. For example, IL-2 binds to IL-2 receptors on other T cells (which have bound the Ag) and stimulates their proliferation, while IL-4 causes B cells to proliferate and differentiate into antibody-secreting **plasma cells** and **memory B cells**. IL-4 activates only B cells in the vicinity which themselves have bound the antigen, and not others, so as to sustain the specificity of the immune response.

As previously mentioned, **B cells themselves behave as APCs**. Cross-linked antigens bound to antibody receptors on the surface of a B cell cause internalization of some of the antigen and expression on the B cell membrane together with MHC II molecules. The TH2 cell recognizes the antigen together with the Class II MHC molecules, and secretes the various lymphokines that activate the B cells to become antibody-secreting plasma cells and memory B cells. Even if the antigen cannot cross-link the receptor, it may be endocytosed by the B cell, processed, and returned to the surface in association with MHC II where it can be recognized by specific TH2 cells which will become activated to initiate B cell differentiation and proliferation. In any case, the **overall B-cell response leads to antibody-mediated immunity (AMI)**.

The **antigen receptors on B cell surfaces** are thought to be the specific types of antibodies that they are genetically-programmed to produce. Hence, there are thousands of **sub-populations of B cells** distinguished only by their ability to produce a unique (reactive) type of antibody molecule. A B cell can also react with a homologous antigen on the surface of the macrophage, or with soluble antigens. When a B-cell is bound to Ag, and simultaneously is stimulated by IL-4 produced by a nearby TH2 cell, the B cell is stimulated to grow and divide to form a clone of identical B cells, each capable of producing identical antibody molecules. The activated B cells further differentiate into **plasma cells** which synthesize and secrete large amounts of antibody, and into a special form of B cells called memory B cells. The antibodies produced and secreted by the plasma cells will react specifically with the homologous antigen that induced their formation. Many of these reactions lead to host defense and to prevention of reinfection by pathogens. **Memory cells** play a role in secondary immune responses.

Plasma cells are relatively short-lived (about one week) but produce large amounts of antibody during this period. Memory cells, on the other hand, are relatively long-lived and upon subsequent exposure to Ag they become quickly transformed into Ab-producing plasma cells.

Generation of cell mediated immunity (CMI) begins when (for example) a **TC cell** recognizes a processed **antigen** associated with **MHC I** on the membrane of a cell (usually an altered self cell, but possibly a transplanted tissue cell or a eukaryotic parasite). Under stimulation by **IL-2** produced by **TH2 cells** the TC cell becomes activated to become a **cytotoxic T lymphocyte (CTL)** capable of lysing the cell which is showing the new (foreign) antigen on its surface, a primary manifestation of CMI.

The interaction between an antigen-presenting macrophage and a TH cell stimulates the macrophage to produce and secrete a cytokine called **Interleukin-1 (IL-1)** that acts locally on the TH cell. The IL-1 stimulates the TH-cell to differentiate and produce its own cytokines (which in this case might be called **lymphokines** because they arise from a lymphocyte). These lymphokines have various functions. Interleukin-4 has an immediate effect on nearby B-cells. Interleukin-2 has an immediate effect on T cells as described above.

Time is required before a primary immune response is effective as a host defense. Antigens have to be recognized, taken up, digested, processed, and presented by APCs; a few select TH cells must react with Ag and respond; preexisting B or T lymphocytes must encounter the Ag and proliferate and differentiate into effector cells (plasma cells or CTLs). In the case of AMI, antibody level has to build up to an effective physiological concentration to render its host resistant. It may take several days or weeks to reach a level of effective immunity, even though this immunity may persist for many months, or years, or even a lifetime, due to the presence of the antibodies. In natural infections, the inoculum is small, and even though the antigenic stimulus increases during microbial replication, only small amounts of antibody are formed within the first few days, and circulating antibody is not detectable until about a week after infection.

Induction of a secondary immune response

On re-exposure to microbial antigens (secondary exposure to antigen), there is an accelerated immunological response, the secondary or memory response. Larger amounts of antibodies are formed in only 1-2 days. This is due to the activities of specific memory B cells or memory T cells which were formed during the primary immune response. These memory cells, when stimulated by homologous Ag, "remember" having previously seen the Ag, and are able to rapidly divide and differentiate into effector cells. Stimulating memory cells to rapidly produce very high (effective) levels of persistent circulating antibodies is the basis for giving "booster"-type vaccinations to humans and pets.

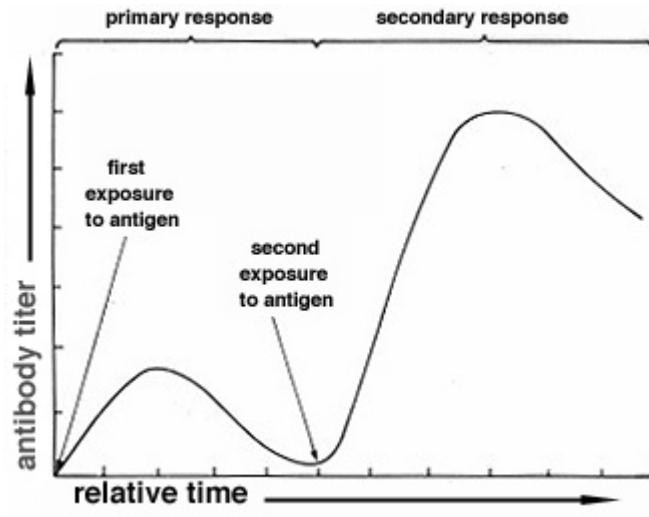


Figure 4. Primary and Secondary Immune Responses. Following the first exposure to an antigen the immune response (as evidenced by following the concentration of specific antibody in the serum) develops gradually over a period of days, reaches a low plateau within 2-3 weeks, and usually begins to decline in a relatively short period of time. When the antigen is encountered a second time, a secondary (memory) response causes a rapid rise in the concentration of antibody, reaching a much higher level in the serum, which may persist for a relatively long period of time. This is not to say that a protective level of antibody may not be reached by primary exposure alone, but usually to ensure a high level of protective antibody that persists over a long period of time, it is necessary to have repeated antigenic stimulation of the immune system.

Antibody-mediated Immunity

Antibodies are proteins produced by lymphocytes that can specifically bind a wide variety of protein and polysaccharide antigens and elicit a response that is significant in antimicrobial defense. In conjunction with the complement system, antibodies are the mediators of humoral (circulating) immunity, and their presence on mucosal surfaces provides resistance to many infectious agents. Antibodies are essential for the prevention and/or cure of many types of bacterial and viral infections.

As mediators of immunity, it was discovered at the turn of the century that antibodies were contained within the serum fraction of blood. It was demonstrated in 1939 that antibodies were specifically located in the gamma fraction of electrophoresed serum, thus the term **gammaglobulin** was coined for serum containing antibodies. Antibodies themselves, were called **immunoglobulins**.

The Classes of Antibodies

There are a number of types of antibodies or immunoglobulins that react stereochemically and specifically with an antigen that induced their formation. Each of these classes of immunoglobulins (abbreviated Ig) is produced by a specific clone of plasma cells. Five immunoglobulin classes are defined on the basis of their heavy chain composition, named IgG, IgM, IgA, IgE, and IgD. IgG and IgA are further divided into subclasses.

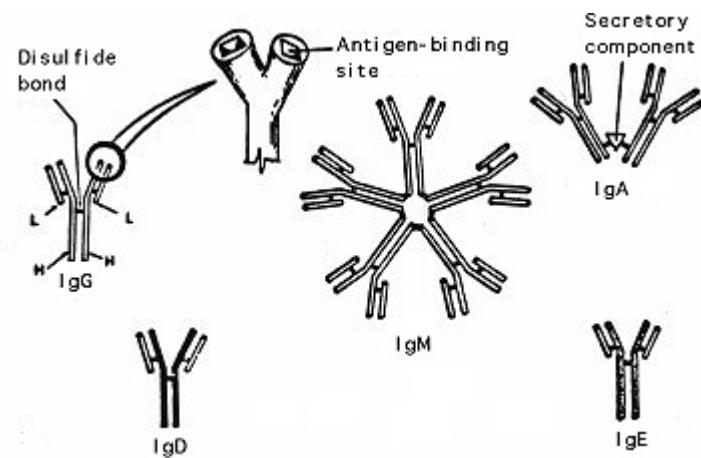


Figure 5. Schematic representation of the various Classes of Immunoglobulins

The classes of immunoglobulins have different physical and chemical characteristics and they exhibit unique biological properties. Their synthesis occurs at different stages and rates during an immune response and/or during the course of an infection. Their importance and functions in host resistance (immunity) are different.

IgG. Immunoglobulin G is the predominant Ig in the serum; it makes up about 80% of the total antibody found in an animal at any given time, being 75% of the total serum antibody. It can diffuse out of the blood stream into the extravascular spaces and it is the most common Ig found there. Its concentration in tissue fluids is increased during inflammation. It is particularly effective at the neutralization of bacterial extracellular toxins and viruses. It also has opsonizing ability and complement-fixing ability. It is IgG that crosses the placental barrier, and thereby provides passive immunity to the fetus and infant for the first six months of life.

IgG is the model for understanding the structure and function of antibody molecules, and it is fitting to examine its biochemical properties before discussion of the properties of all of the other types of immunoglobulins.

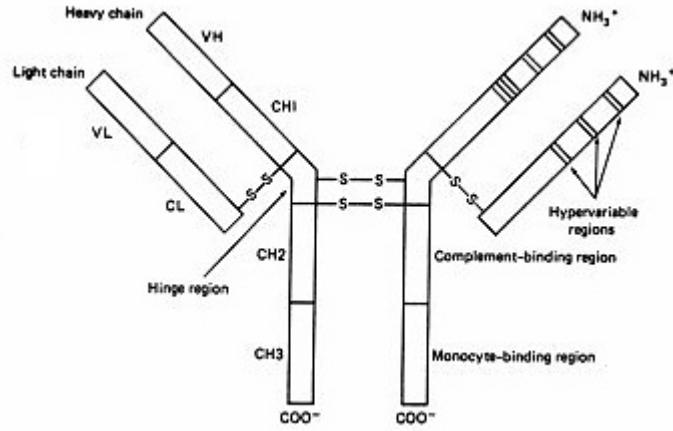


Figure 6. Model of an Immunoglobulin: the Structure of IgG

IgG is a protein with a molecular weight of about 150,000 daltons. The protein consists of **two identical heavy (H) chains** (each with a mw of about 50kd) and **two identical light (L) chains** (mw about 25kd). Each L chain is connected to a H chain and the two H-chains are connected to one another by disulfide bridges. The molecule is drawn to look like a Y. The stem of the Y is called the **Fc region** and it consists mainly of two halves of the identical H chains. Each of the "arms" of the Y contains one complete L-chain and half of one of the H-chains. The Y stem stands on the carboxy termini of the H chains; the tips of the arms contain the amino termini of the H and L-chains. Each arm is sometimes referred to as the **Fab region** of the molecule. The Fab region is the antigen binding fragment of the antibody molecule. A specific region of the antigen (called the antigenic determinant) will react stereochemically with the **antigen-binding region** at the amino terminus of each Fab. Hence, the IgG molecule, which has two antigen binding fragments [(Fab)2] is said to be divalent: it can bind to two Ag molecules. The polypeptide composition of the Fc region of all IgG1 antibody molecules is relatively constant regardless of antibody specificity; however, the Fab regions always differ in their exact amino acid sequences.

depending upon their antigenic specificity. Even though the antigen does not react with the Fc region of the IgG1 molecule, this should not be taken to mean that the Fc region has no importance or biological activity. On the contrary, specific amino acid regions of the Fc portion of the molecule are recognized by receptors on phagocytes and certain other cells, and the Fc domain contains a peptide region that will bind to and activate complement, which is often required for the manifestation of AMI.

Understanding the structure and properties of IgG is useful to discussion of its function in host defense. Since the IgG molecule is divalent, it can cross-link Ag molecules, which may lead to precipitation or agglutination of antigens; if IgG is bound to Ag on a microbial cell or surface, its Fc region may provide an extrinsic ligand which will be recognized by specific receptors on phagocytes. Such microbial cells or viruses coated with IgG molecules are said to be opsonized for phagocytosis. Opsonized pathogens are taken up and destroyed much more readily by phagocytes than their non-opsonized counterparts. IgG, as well as IgM and IgA, will neutralize the activity of toxins, including bacterial exotoxins. Furthermore, cross-linked IgG molecules on the surface of a cell can bind and activate complement from the serum and set off a cascade of reactions that can lead to destruction of the cell (antigen). It is probably due to its relatively small size and its persistence in the serum of a mother, that IgG is shared with the fetus in utero, and the infant is born with the full complement of mother's IgG antibodies.

IgM is the first immunoglobulin to be synthesized by infants and the first to appear in the blood stream during the course of an infection. Mainly, it is confined to the bloodstream giving the host protection against blood-borne pathogens. IgM makes up about 10% serum immunoglobulins. IgM is arranged to resemble a pentamer of five immunoglobulin molecules (mw = 900kd) tethered together at by their Fc domains. In addition to covalent linkages between the monomeric subunits, the pentamer is stabilized by a 15kd polypeptide called the J chain. IgM, therefore, has a theoretical "valence" of 10 (i.e., it has exposed 10 Fab domains). Probably, the most important role of IgM is its ability to function early in the immune responses against blood-borne pathogens. As might be expected, IgM is very efficient at agglutinating particulate antigens. Also, IgM binds complement strongly and such IgM antibodies bound to a microbial surface act as opsonins, rendering the microbe more susceptible to phagocytosis. In the presence of complement and IgM whole microbial cells may be killed and lysed. IgM also appears on the surfaces of mature B cells as a transmembranous monomer where it functions as an antigen receptor, capable of activating B cells when bound to antigen.

IgA exists as a 160kd monomer in serum and as a 400kd dimer in secretions. As in the case of IgM, polymerization (dimerization) is via a J-chain. There are two subclasses based on different heavy chains, IgA1 and IgA2. IgA1 is produced in bone marrow and makes up most of the serum IgA. Both IgA1 and IgA2 are synthesized in GALT (gut associated lymphoid tissues) to be secreted onto the mucosal surfaces. Since IgA may be synthesized locally and secreted in the seromucous secretions of the body, it is sometimes referred to as **secretory antibody** or sIgA. Quantitatively, IgA is synthesized in amounts greater than IgG, but it has a short half life in serum (6 days), and it is lost in secretory products. The concentration of IgA in serum is about 15% of the total antibody. Secretion of dimeric IgA is mediated by a 100kd glycoprotein called the **secretory component**. It is the addition of the secretory piece to the IgA molecules that accounts for their ability to exit the body to mucosal surfaces via the exocrine glands. IgM can be transported similarly and makes up a small proportion of secretory antibodies.

Secretory IgA is the predominant immunoglobulin present in gastrointestinal fluids, nasal secretions, saliva, tears and other mucous secretions of the body. IgA antibodies are important in resistance to infection of the mucosal surfaces of the body, particularly the respiratory, intestinal and urogenital tracts. IgA acts as a protective coating for the mucous surfaces against microbial adherence or initial colonization. IgA can also neutralize toxin activity on mucosal surfaces. Fc receptors for IgA-coated microorganisms found on monocytes and neutrophils derived from the respiratory mucosa, suggest that IgA may have a role in the lung, at least, in opsonization of pathogens.

Secretory IgA is also transferred via the milk, i.e., the colostrum, from a nursing mother to a newborn, which provides passive immunity to many pathogens, especially those that enter by way of the GI tract. The transfer of IgA via the milk lasts about six months in a woman and the infant encounters many infectious agents while thus partially protected. Under these circumstances the infectious agent might multiply, but only to a limited extent, stimulating the infant's own immune response without causing significant disease (e.g. poliovirus). The infant thus acquires active immunity while partially protected by maternal immunity.

IgE is a 190kd immunoglobulin which accounts for 0.002% of the total serum immunoglobulins. It is produced especially by plasma cells below the respiratory and intestinal epithelia. The majority of IgE is bound to tissue cells, especially mast cells. If an infectious agent succeeds in penetrating the IgA barrier, it comes up against the

next line of defense, the MALT (mucosa-associated lymphoid tissues) system which is manned by IgE. IgE is bound very firmly to the Fc receptors (specifically for IgE) on mast cells. Contact with Ag leads to release of mediators of inflammation from the mast cells, which effectively recruits various agents of the immune response including complement, chemotactic factors for phagocytes, T-cells, etc. Although a well-known manifestation of this reaction is a type of **immediate hypersensitivity** reaction called atopic allergy (e.g. hives, asthma, hay fever, etc.), the MALT responses act as a defense mechanism because they amplify the inflammatory response and may facilitate rejection of a pathogen.

IgD is a 175kd molecule that resembles IgG in its monomeric form. IgD antibodies are found for the most part on the surfaces of B lymphocytes. The same cells may also carry IgM antibody. It is thought that IgD and IgM function as mutually-interacting antigen receptors for control of B-cell activation and suppression. Hence, IgD may have an immunoregulatory function. Recall that only specific subclones of B-cells respond to a specific Ag upon stimulation. The specific subclone of B-cells must display an antibody receptor that recognizes specifically the Ag. It would stand to reason that the basis of this specificity involved a B-cell receptor that had the sort of specificity characteristic of antibody molecules.

Functions of Antibodies in Host Defense

The functions of antibodies, and hence the AMI response, in host defense against pathogenic microbes is summarized below.

Opsonization: Antibodies enhance phagocytic engulfment of microbial antigens. IgG and IgM Abs have a combining site for the Ag and a site for cytophilic association with phagocytes. Bacteria and viral particles are ingested with increased efficiency.

Steric hindrance: Antibodies combine with the surfaces of microorganisms and may block or prevent their attachment to susceptible cells or mucosal surfaces. Ab against a viral component can block attachment of the virus to susceptible host cells and thereby reduce infectivity. Secretory IgA can block attachment of pathogens to mucosal surfaces.

Toxin Neutralization: Toxin-neutralizing antibodies (antitoxins) react with a soluble bacterial toxin and block the interaction of the toxin with its specific target cell or substrate.

Agglutination and Precipitation: Antibodies combine with the surfaces of microorganisms or soluble antigens and cause them to agglutinate or precipitate. This reduces the number of separate infectious units and makes them more readily phagocytosed because the clump of particles is larger in size. Also, floccules or aggregates of neutralized toxin may be removed by phagocytes.

Activation of Complement: antibodies combined with the surface of microorganisms or surfaces of Ag activate the complement cascade which has four principal effects related to host defense

1. induction of the inflammatory response/chemotactic
2. attraction of phagocytes to the site of immunological encounter
3. opsonization of cells showing foreign Ag
4. complement-mediated lysis of certain bacteria or viruses

Antibody-dependent cell cytotoxicity (ADCC): IgG can enable certain cells (Natural Killer cells) to recognize and kill opsonized target cells. NK cells are lymphocytes or monocytes, but certain other types of cells including neutrophils also act this way. NK cells attach to opsonized target cells by means of an IgG Fc receptor and kill by an extracellular mechanism after attachment. ADCC will be discussed as part of cell-mediated immunity.

Cell-mediated Immunity

CMI is a type of resistance in which cells of the immune system are directly involved, but antibody production or activity is of minor importance. CMI differs from AMI in that immunity cannot be transferred (passively) from animal to animal by antibodies or serum, but can be transferred by lymphocytes removed from the blood.

The CMI response

During the cell-mediated immune response, various subsets of T lymphocytes are activated and develop into effector T cells. These include **cytotoxic T lymphocytes (CTL's or TC cells)** and T helper cells of the **TH1** and **TH2** subsets. TH1 cells secrete lymphokines that activate macrophages and mediate delayed type hypersensitivity responses. TH2 cells secrete lymphokines that stimulate B cell development and may help activate TC cells to their full cytotoxic capacity.

T cells that generate CMI are present in the lymphoid tissues, blood and lymph. Due to constant recirculation between blood and lymph nodes via lymphatics and back to the blood, one T cell circulates once in about 24 hours. Each carries receptors for the specific Ag with which it can react. T cell recognition of Ag only occurs when the Ag is associated with proteins of the MHC complex. The T cells have receptors (TCR) complementary to the complexed MHC determinant and the antigenic determinant. TH1 cells and TH2 cells recognize Ag in association with MHC II (as displayed by macrophages and other APCs); TC-cells recognize Ag on cells complexed with MHC I (as displayed by altered self cells).

Stepwise Activation of TC cells

During a primary CMI response, antigen is presented to the precursor TC lymphocytes ($CD8^+$) in association with MHC Class I proteins. All nucleated cells express MHC I on their surfaces so virtually any cell in the animal expressing a new ("nonself") Ag on its surface will activate the cytotoxic T lymphocytes. TH2 cells can augment activation of the TC cells, but they probably are not required.

Activation of TH cells

TH-cells ($CD4^+$) reacting with Ag may produce a variety of lymphokines. Notably, Interleukin-2 (IL-2) stimulates T cell activation and IL-4 stimulates B cells. It is now clear that the T-helper cells are composed of distinct subsets that can be distinguished on the basis of their patterns of lymphokine production.

TH1 cells "see" foreign Ag on the surface of APC's in the context of MHC II. Mainly, TH1 cells produce IL-2, gamma IFN and lymphotoxin. This results in macrophage activation and the delayed-type hypersensitivity (DTH) reaction, and in help for TC cell activation.

TH2 cells also see foreign Ag on the surface of APC's in the context of MHC II. Their response is to secrete IL-4, IL-5, IL-6, IL-10 and IL-13 that help activate B cells, provide help for the production of IgE that attaches to mast cells, and promote mast cell and eosinophil activation.

Both types of TH cells develop under most conditions but their ratios and the predominance of certain lymphokines can vary, and this may mediate the pathology and outcome of certain bacterial infections.

The lymphokines produced by TH cells stimulate B cells and pTC cells, inducing them to proliferate and mature into effector cells. Gamma Interferon activates macrophages and Natural Killer (NK) cells to their full cytolytic potential. Lymphotoxins (i.e., tumor necrosis factor or TNF) kill cells at a distance.

Function of cytotoxic T-lymphocytes

TC (cytotoxic) cells can destroy cells bearing new antigens on their surfaces (as might result in a viral infection, a tumor cell, or an infection by a bacterial intracellular parasite). TC cells exert their cytotoxic activity when they are in physical contact with cells bearing new Ag and MHC I protein. Contact between the TC cell and the target cell is required for lysis, although the exact mechanism of lysis is not known. The target cell membrane is damaged at the site of contact (the "kiss of death") leaving a gaping hole about 40 nm in diameter that cannot be repaired. When the TC cell moves away 30-60 seconds later, there is leakage of the cell components, an influx of H_2O , and the target cell swells up and dies. Apparently the TC cell releases some of its cytolytic contents directly into the target cell so that within a few minutes the target cell literally disintegrates. The TC cell can move away and kill

again.

TC cells generally respond to Ag in association with MHC I proteins on the surface of a target cell. If they responded to Ag by itself, they could react with it when it was free in extracellular fluids, and their cytotoxic activity would be triggered off with no purpose. As stated above, almost all host cells, including macrophages, display MHC I. Hence, an effector TC cell can destroy a macrophage which is otherwise carrying out a useful function by presenting Ag to TH lymphocytes as part of the AMI or CMI responses. Usually, the time course of the response is such that TH cells have already developed and have carried out their (helping) function when TC cells begin to become active.

Delayed Type Hypersensitivity

TH1-cells ($CD4^+$) are a subset of T-lymphocytes that recognize Ag in association with Class II (and possibly Class I) MHC proteins. When TH1-cells are presented Ag in association with MHC II by a macrophage, their development is stimulated by macrophage Interleukin-1 (IL-1), and autostimulated by IL-2, which the TH cell produces. They respond by differentiating and producing a variety lymphokines that induce a local inflammatory response, and attract, trap, and activate phagocytes. One aspect of this response is a state of **delayed-type hypersensitivity (DTH)** in the host. This is usually evident in chronic infections wherein CMI is largely involved (e.g. tuberculosis).

Delayed-type hypersensitivity reactions usually present themselves as **allergic reactions**. Such allergic reactions generally require about 24 hours to develop following a secondary exposure to Ag. This time is required for the circulating TH cells (actually memory cells) to encounter the Ag and to begin producing lymphokines, and to attract macrophages and TC cells to the site, for these cells are the real mediators of the allergic reaction. The phagocytic and cytolytic activities of these cells are responsible for the localized tissue destruction which occurs. Poison oak (ivy) rash is a familiar example of delayed hypersensitivity, but the reaction is also evident in several types of chronic or persistent bacterial infections including tuberculosis, leprosy and brucellosis, and in some fungal and protozoal infections.

One of the best known examples of the delayed-type hypersensitivity reaction is the **Mantoux (tuberculin) test** which is utilized to determine current or previous infection by the tubercle bacillus (*Mycobacterium tuberculosis*). A small amount of Ag called the purified protein derivative (PPD), derived from the cell wall of the bacterium, is injected subcutaneously usually just under the skin of the forearm. The test is evaluated after 24-48 hours. A positive test is an allergic response (an "urticarial weal") at the site of the injection, which might look like a swollen reddened area about the size of a quarter. A negative test is no reaction. A positive test does not mean that the individual has an active case of tuberculosis, but that the individual has at least been exposed to the tubercle bacillus or one of its products sufficiently to have undergone a primary immune response. Hence, an individual exhibiting a positive test may have active tuberculosis, may have an unapparent (subclinical) form of the disease, may have previously had the disease, or possibly may have been artificially immunized against the disease.

Two types of cells other than dermal macrophages have been proposed as antigen presenting cells (APCs) to initiate DTH reactions on the skin, epidermal Langerhans cells and venular endothelial cells. In humans, antigen presentation by Langerhans cells (which bear class II MHC), probably initiates sensitization, whereas antigen presentation by endothelial cells probably initiates DTH reactions upon secondary challenge.

Involvement of macrophages in mediation of CMI

During induction of the cell-mediated immune response, macrophages play their usual role in the presentation of Ag to T helper cells and in producing cytokines that are involved in the initiation of immune reactions. In addition, macrophages play a role in the expression of CMI. Many of the lymphokines produced by TH cells are aimed at attraction, entrapment and activation of macrophages at the site of the reaction. One of these lymphokines, **Gamma Interferon**, causes the local macrophage population to develop an increased number of lysosomes and also increased secretion of microbicidal products. Oxygen-dependent killing mechanisms of the macrophage are stimulated, and the macrophage develops increased power to ingest and kill microorganisms. Such lymphokine-stimulated macrophages are referred to as "angry" or activated macrophages.

Compared to normal macrophages, **activated macrophages** exhibit much greater ability to destroy intracellular pathogens. Activated macrophages may play an important role in the recovery from chronic bacterial infections and

in resistance to certain tumors. Activated macrophages may be able to overcome bacterial intracellular parasites which are able to thwart the macrophage killing mechanisms before activation. Macrophage involvement in CMI may be part of the pathology of certain diseases. Where there is difficulty in elimination an intracellular parasite (e.g. the tuberculosis bacillus) the chronic CMI response to local antigens leads to the accumulations of densely-packed macrophages which release fibrinogenic factors and stimulate the formation of granulation and fibrosis. The resulting structure, called a **granuloma**, actually represents an attempt by the host to isolate a persistent infection.

Other Aspects of cell-mediated immunity

Another class of cytotoxic lymphocytes distinct from TC cells may be stimulated during the cell-mediated immune response. These are referred to as **Natural Killer** or **NK cells**. NK cells are found in blood and lymphoid tissues, especially the spleen. They do not bear T cell (or B cell) markers. Like TC cells, they are able to recognize and kill cells that are displaying a new Ag on their surfaces, but unlike TC cells, they do not display TCR and they are not MHC-restricted.

The existence of NK specificity is demonstrated by the phenomenon of "cold target inhibition"; one NK target cell type can inhibit lysis of a different NK target type by competing for effector cells, whereas cells that are not NK targets do not compete. NK cells are present in an animal in the absence of antigenic stimulation, and it is for this reason that they are referred to as "natural" killers. They might also be considered part of the constitutive defenses; however, NK cells become activated in a CMI response by T cell lymphokines, including Interleukin-2 and Gamma Interferon.

Some NK cells are thought to be an immature form of a T-lymphocyte, but various other types of cells including macrophages, neutrophils and eosinophils, display NK activity. Some NK cells have surface receptors (CD16) for the Fc portion of IgG. They bind to target cells by receptors for the Fc portion of antibody that has reacted with antigen on the target cell. This type of CMI is called **antibody-dependent cell-mediated cytotoxicity** or **ADCC**. NK cells may also have receptors for the C3 component of complement, and therefore recognize cells that are coated with C3 as targets. ADCC is thought to be an important defense against a variety of parasitic infections caused by protozoa and helminths.

Summary: cells involved in expression of CMI

Cell mediated immunity (CMI) is carried out by several types of cells including macrophages, TH lymphocytes TC lymphocytes, and NK (natural killer) cells. After an immunological encounter, these cells are activated to produce and/or respond to various classes of lymphokines that are the mediators of CMI. A summary of the types of cells involved in the expression of CMI is provided below.

TC (cytotoxic) Lymphocytes (CTLs): kill cells bearing foreign Ag on surface in association with MHC I. TC cells can kill cells that are harboring intracellular parasites (either bacteria or viruses) as long as the infected cell is displaying a microbial antigen on its surface. TC cells kill tumor cells and account for rejection of transplanted cells. TC cells recognize Ag-MHC I complexes on target cells, contact them, and release the contents of granules directly into the target cell membrane which lyses the cell.

TH Lymphocytes: produce lymphokines that are "helper" factors for development of B-cells into antibody-secreting plasma cells; also produce certain lymphokines which stimulate the differentiation of effector T lymphocytes and the activity of macrophages. TH1 cells recognize Ag on macrophages in association with MHC II and become activated (by IL-1) to produce lymphokines including gamma Interferon that activates macrophages and NK cells. These cells mediate various aspects of the CMI response including delayed type hypersensitivity reactions. TH2 cells recognize Ag in association with MHC II on an APC and then produce interleukins and other substances that stimulate specific B-cell and T-cell proliferation and activity.

Macrophages: are important as Ag-presenting cells that initiate T-cell interactions, development and proliferation. Macrophages are also involved in expression of CMI since they become activated by gamma IFN produced in a CMI response. Activated macrophages have increased phagocytic potential and release soluble substances that cause inflammation and destroy many bacteria and other cells.

Natural Killer (NK) cells: Cytotoxic cells that lyse cells bearing new antigen regardless of their MHC type and even lyse some cells that bear no MHC proteins. Natural Killer cells are defined by their ability to kill cells

displaying a foreign Ag (e.g. tumor cells) regardless of MHC type and regardless of previous sensitization (exposure) to the Ag. Some NK cells are probably derived from TC cells (CTLs), but they do not display T cell markers. NK cells can be activated by IL-2 and gamma IFN. Natural Killers lyse cells in the same manner as CTLs. Some NK cells have receptors for the Fc domain of IgG and so are able to bind to the Fc portion of IgG antibody on the surface of a target cell and release cytolytic components that kill the target cell. This mechanism of killing is referred to as antibody-dependent cell-mediated cytotoxicity (ADCC).

Summary: Lymphokines involved in expression of CMI

Extracellular factors that affect cell proliferation and differentiation have been defined as cytokines. These include the lymphokines, which are proteins produced by T-lymphocytes that have effects on the differentiation, proliferation and activity of various cells involved in the expression of CMI. In general, lymphokines function by (1) focusing circulating leukocytes and lymphocytes into the site of immunological encounter; (2) stimulating the development and proliferation of B-cells and T-cells; (3) stimulating and preparing macrophages for their phagocytic tasks; (4) stimulating natural killer (NK) cells; (5) providing antiviral cover and activity. The names and functions of some of the important lymphokines are described below.

IL-1 (Interleukin-1): Initially called lymphocyte activation factor. Mainly a product of macrophages, IL-1 has a variety of effects on various types of cells. It acts as a growth regulator of T-cells and B-cells, and it induces other cells such as hepatocytes to produce proteins relevant to host defense. IL-1 forms a chemotactic gradient for neutrophils and serves as an endogenous pyrogen which produces fever. Thus, IL-1 plays an important role in both the immune responses and in the inflammatory response.

IL-2 (Interleukin-2): stimulates the proliferation of T-cells and activates NK (natural killer) cells.

IL-3 (Interleukin-3): regulates the proliferation of stem cells and the differentiation of mast cells.

IL-4 (Interleukin-4): causes B cell proliferation and enhanced antibody synthesis.

IL-6 (Interleukin-6): (same as beta Interferon) has effects on B cell differentiation and on antibody production and on T cell activation, growth, and differentiation. Probably has a major role in the mediation of the inflammatory and immune responses initiated by infection or injury.

IL-8 (Interleukin-8): chemotactic attractant for neutrophils.

IL-13 (Interleukin-13): shares many of the properties of IL-4, and is a potent regulator of inflammatory and immune responses.

Interferons: Gamma-Interferon (gamma IFN) is produced by T cells and may be considered a lymphokine. It is sometimes called "immune interferon" (alpha-Interferon is "leukocyte interferon"; beta-Interferon is "fibroblast interferon"). Gamma-interferon has several antiviral effects including inhibition of viral protein synthesis in infected cells. It also activates macrophages and NK cells, and stimulates IL-1, IL-2, and antibody production.

Lymphotoxins: (Tumor Necrosis Factor-Beta): (TNF-beta is produced by T cells; TNF-alpha is produced by T cells, as well as other types of cells.) TNF kills cells, including tumor cells (at a distance).

Colony Stimulating Factor (CSF): several, including GMCSF, cause phagocytic white cells of all types to differentiate and divide.

Contrasting Roles of the AMI and CMI Responses in Host Defense

AMI and CMI responses are generated during almost all infections, but the relative magnitude and importance of each type of response shows great variation in different hosts and with different infectious agents.

In some types of infections antibody plays a major role in immunity or recovery. For example, viruses producing systemic disease with a viremia stage (viruses free in the blood as they spread from infected to uninfected cells), such as poliomyelitis or yellow fever, can be controlled ("neutralized") by circulating antibody. Pathogenic bacteria

that multiply outside of cells (nearly all bacteria) at sights accessible to antibody, phagocytes and complement, can be stopped by the forces of AMI. Diseases caused by circulating bacterial toxins (e.g. diphtheria and tetanus) are controlled by circulating antibodies that neutralize toxins. Circulating antibodies (and perhaps secretory IgA, as well) present in immune animals can prevent reinfection by pathogens.

In other types of infections CMI is of supreme importance in recovery. These tend to be infections where the microbe grows or multiplies intracellularly. Bacterial infections of this nature include tuberculosis, brucellosis and syphilis. Recovery is associated with development of a pronounced CMI response, even though it is CMI that contributes to the pathology of the disease.

The clearest picture of the importance of CMI in recovery from disease is seen in certain viral infections (e.g. herpes, pox viruses and measles virus infections) Viruses are always intracellular parasites and may only rarely expose themselves to the extracellular forces of AMI. Antibodies could neutralize free virus particles liberated from cells but often have little influence on infected cells. The best strategic defense against virus-infected cells seems to be to kill the infected cell when the virus may be in a replicative (noninfectious) form. Many viruses, as they mature, cause foreign (viral) antigens to appear on the infected cell surface. These cells are recognized by the host's CMI defenses and they become target cells for cytotoxicity. The infected cell can be destroyed before virus is liberated.

The CMI response also plays a role in destruction of tumor cells and in rejection of tissue transplants in animals. A major problem in transplantation of tissues from one individual to another is rejection which is often based on CMI response to "foreign" cells (not a perfect match antigenically). Since tumor cells contain specific antigens not seen on normal cells they also may be recognized as foreign and destroyed by the forces of CMI. If tumor cells develop on a regular basis in animals, it may be the forces of CMI that eliminate them or hold them in check. The increase in the incidence of many types of cancer (tumors) in humans with advancement of age may be correlated with a decline in the peak efficiency of the immune system that occurs about 25 years of age.

In summary, antibody-mediated immunity (AMI) is probably most useful as an immune defense because of its ability to neutralize or destroy extracellular pathogens and to prevent occurrence of reinfection. Cell-mediated immunity (CMI) plays the major role in immune defense against infections caused by intracellular parasites, infections caused by viruses (either virulent or oncogenic), rejection of transplanted tissues or cells, and in the destruction of tumor cells. The contrasting roles of AMI and CMI as inducible host defenses are presented in the following table.

Table 2. Relative Importance of Immune Defenses in Various Types of Infections

Type of Infectious Agent	Immune Defense	Mechanisms	Examples
MULTIPLIES INSIDE TISSUE CELLS	Prevent entry	AMI: IgG, IgA, IgM	viruses, Rickettsia
	Kill infected cell	CMI: TC, NK, ADCC	
MULTIPLIES INSIDE PHAGOCYTES	Activate phagocytes	CMI: lymphokines	viruses, <i>Mycobacterium tuberculosis</i>
	Kill infected phagocytes	CMI: TC, NK, ADCC	
MULTIPLIES OUTSIDE CELLS	Kill microbe extracellularly	AMI: Complement mediated lysis	most bacteria
	Opsonized phagocytosis and lysis	AMI: IgG, IgM	
MULTIPLIES OUTSIDE CELLS BUT ATTACHMENT	Neutralize toxins	AMI: IgG, IgM	
	Prevent attachment	AMI: IgA	streptococci <i>E. coli</i>

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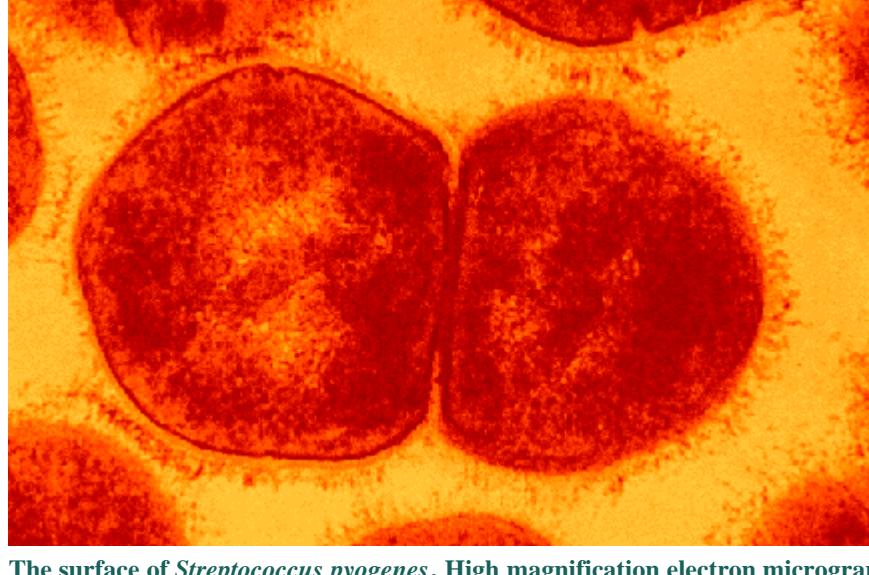
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BACTERIAL STRUCTURE IN RELATIONSHIP TO PATHOGENICITY

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The Importance of the Bacterial Surface

All of the various surface components of a bacterial cell are important in its ecology since they mediate the contact of the bacterium with its environment. The only "senses" that a bacterium has result from its immediate contact with its environment. It must use its surface components to assess the environment and respond in a way that supports its own existence and survival in that environment. The surface properties of a bacterium are determined by the exact molecular composition of its membrane and cell wall, including LPS, and the other surface structures such as flagella, fimbriae and capsules.

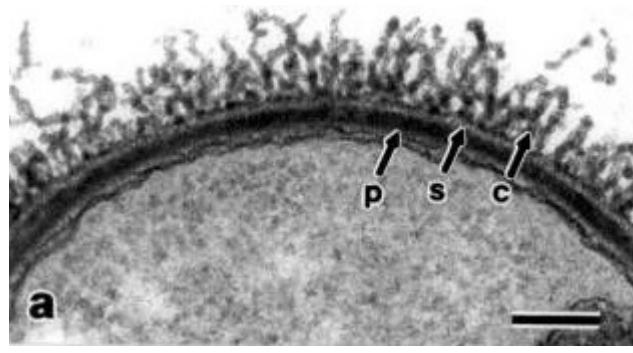


The surface of *Streptococcus pyogenes*. High magnification electron micrograph of an ultra-thin section by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology](#), Rockefeller University. At this magnification, especially in the cell on the left, the cell wall and cell surface fibrils, consisting mainly of M protein, are well defined. The interdigitation of these fibrils between neighboring cells of different chains can also be seen.

Bacterial **surface components** may have a primary biological function that has nothing to do with pathogenicity. Thus, the function of the LPS in the outer membrane of Gram-negative bacteria has to do with its permeability characteristics, rather than its toxicity for animals. However, there are endless examples wherein a bacterial surface component plays an indispensable role in the pathogenesis of infectious disease. Bacterial surface structures may act as (1) **permeability barriers** that allow selective passage of nutrients and exclusion of harmful substances (e.g. antimicrobial agents); (2) **adhesins** used to attach or adhere to specific surfaces or tissues; (3) **enzymes** to mediate specific reactions on the cell surface important in the survival of the organism; (4) **protective structures against phagocytic engulfment** or killing; (5) **antigenic disguises**; (6) "sensing proteins" that can respond to temperature, osmolarity, salinity, light, oxygen, nutrients, etc., resulting in a **molecular signal** to the genome of the cell that will cause expression of some determinant of virulence (e.g. an exotoxin).

In medical situations, the surface components of bacterial cells are major determinants of virulence for many

pathogens. Pathogens can colonize tissues, resist phagocytosis and the immune response, and induce inflammation, complement activation and immune responses in animals by means of various structural components.



The surface of *Bacillus anthracis*. From Mesnage, et al. *Journal of Bacteriology* (1998) 180, 52-58.

<http://www.pasteur.fr/recherche/unites/scme/Biblio/capsulea.htm>. The bacterial membrane is evident as the innermost layer surrounding the cytoplasm. **P** denotes the peptidoglycan cell wall. **S** refers to the S-layer which consists of two proteins including the major antigen. **C** denotes the poly-D-glutamic acid capsule that is exterior to and completely covers the S-layer proteins.

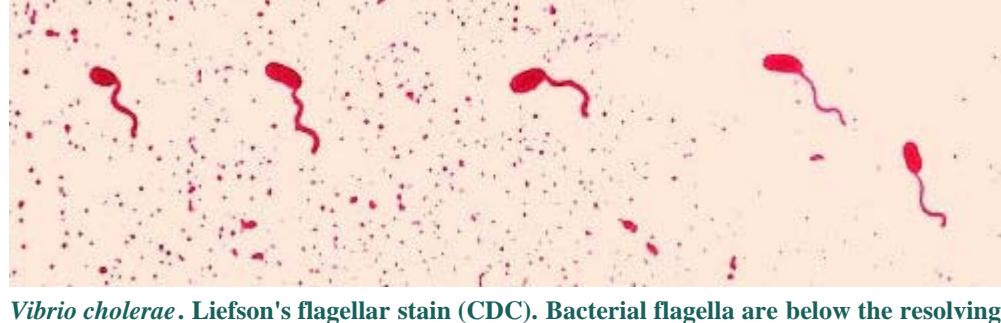
The Structure of the Bacterial Surface

Structurally, a bacterial cell has **three architectural regions**: **appendages** (proteins attached to the cell surface) in the form of flagella and 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. The surface components of a bacterium are the constituents of its cell envelope and appendages.

Flagella are filamentous protein structures attached to the cell surface that provide swimming movement for most motile bacterial cells. The diameter of a bacterial 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.

Bacteria 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). During **aerotaxis**, bacteria swim toward or away from O₂.

For a few pathogens motility is known to be a determinant of virulence. In the case of *Vibrio cholerae*, the vibrios apparently swim (laterally) into the intestinal mucosa to avoid being flushed out by the peristaltic action of the gut. Flagella are antigenic, and therefore, vulnerable to attack by host antibody molecules. Antibody molecules directed against flagellar antigens can agglutinate and/or immobilize bacterial cells, or possibly opsonize them from phagocytosis, which presumably would aid in host defense.



Vibrio cholerae. Loeffler's flagellar stain (CDC). Bacterial flagella are below the resolving power of the light microscope. In order to be visualized, the bacteria must be reacted with a stain that precipitates along the flagellar filaments, which increases their effective diameter to the point of resolution. *Vibrio cholerae* is motile by means of a single polar flagellum inserted into one pole of the cell.

Fimbriae and **Pili** are interchangeable terms used to designate short, hair-like structures on the surfaces of bacterial cells. Fimbriae are shorter and stiffer than flagella, and slightly smaller in diameter. Like flagella, they are composed of protein. A specialized type of pilus (always called a pilus), the F or **sex pilus**, mediates the transfer of DNA between mating bacteria, but the function of the smaller, more numerous common pili is quite different.

Inasmuch as many bacteria are able to exchange genes for virulence by means of conjugation, the sex pilus which confers the ability to conjugate, may well play a role in the their assembly of virulence determinants.

Common pili (almost always called **fimbriae**) are usually involved in adherence (attachment) of bacterial cells to surfaces in nature. In medical situations, they are major determinants of bacterial virulence because they allow pathogens to attach to (colonize) tissues and to resist attack by phagocytic white blood cells. As surface structures on the bacterial cell, the functions of fimbriae overlap with those of capsules discussed below. Fimbriae are also antigenic and secretory antibodies (IgA) will often block bacterial colonization, while circulating antibodies (IgG or IgM) will opsonize bacterial cells for phagocytosis.

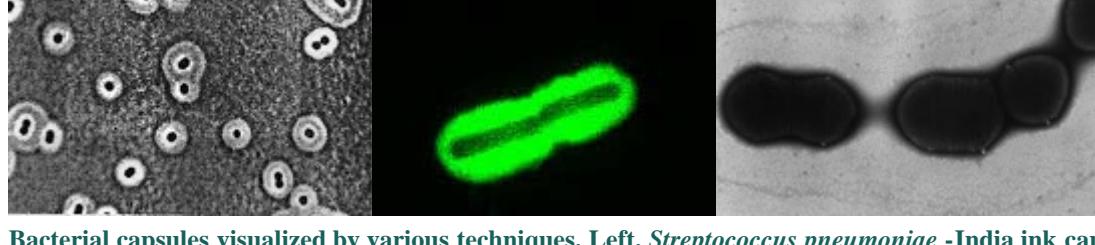


Neisseria gonorrhoeae. Electron micrograph by David M. Phillips, [Visuals Unlimited](#), with permission. This pathogen utilizes its fimbriae in order to initially colonize the urethral or cervical epithelium.

Most bacteria contain some sort of a polysaccharide layer outside of the cell wall or outer membrane. 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**. 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). Capsules, slime layers, and glycocalyx are known to mediate specific or non specific adherence of bacteria to particular surfaces. Capsules are known to protect bacteria from engulfment by predatory phagocytes and from attack by antimicrobial agents.

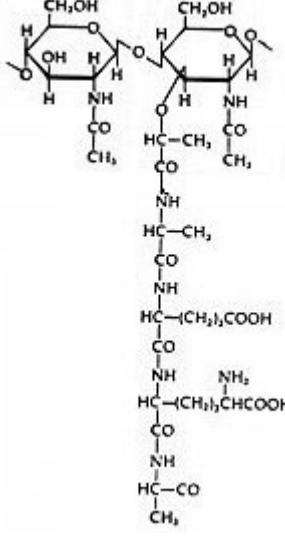
In nature, and in many medical situations, colonies of bacteria construct and live in a **biofilm**, made up principally of capsule material. A biofilm usually consists of a consortium (mixture) of bacteria living in a matrix of slime which is secreted by one of the bacterial members. Dental plaque is an example of a natural biofilm, as is a slimy mass of bacteria attached to a rock in a mountain stream. In medical situations, bacteria in a biofilm may have certain advantages over planktonic counterparts. For example, biofilm bacteria may be less susceptible to phagocytes, drugs, or neutralizing antibodies.

Many polysaccharide capsules possess an antigenic epitope so they will induce and react with host antibodies. Where the capsule is a main determinant of virulence of a pathogen (e.g. *Streptococcus pneumoniae*) antibodies against the bacterium neutralize its virulence.



Bacterial capsules visualized by various techniques. Left. *Streptococcus pneumoniae* -India ink capsule outline (K.Todar); Middle. *Bacillus anthracis* -fluorescent-tagged antibody (CDC); Right. *Streptococcus pyogenes* -transmission electron micrograph by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology](#), Rockefeller University. *S. pneumoniae* capsular material is composed of polysaccharide. The capsule is the pathogen's most important determinant of virulence because it allows the bacterial cells to escape phagocytosis in the lung. The *B. anthracis* capsule is composed of poly-D-glutamic acid. Its capsule is antiphagocytic, and it protects the bacteria from complement-mediated lysis in serum or blood. The capsule of *S. pyogenes* is composed of hyaluronic acid, the same polymer as found in human connective tissue. The capsule is an antigenic disguise that prevents recognition of the streptococci by phagocytes or the immune system.

The **cell wall** of a bacterium is an essential structure that protects the delicate cell protoplast from osmotic lysis. The cell wall of Bacteria consists of a polymer of disaccharides cross-linked by short chains of amino acids (peptides). This molecule is a type of **peptidoglycan** called **murein**. Murein is unique to Bacteria. In the **Gram-positive bacteria**, the cell wall is thick (15-80 nanometers), consisting of several layers of peptidoglycan complexed with molecules called **teichoic acids**. In the Gram-negative bacteria, 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**. Murein is a substance unique in nature to bacterial cell walls. Also, the outer membrane of Gram-negative bacteria invariably contains a unique component, **lipopolysaccharide (LPS or endotoxin)**, which is toxic to animals.



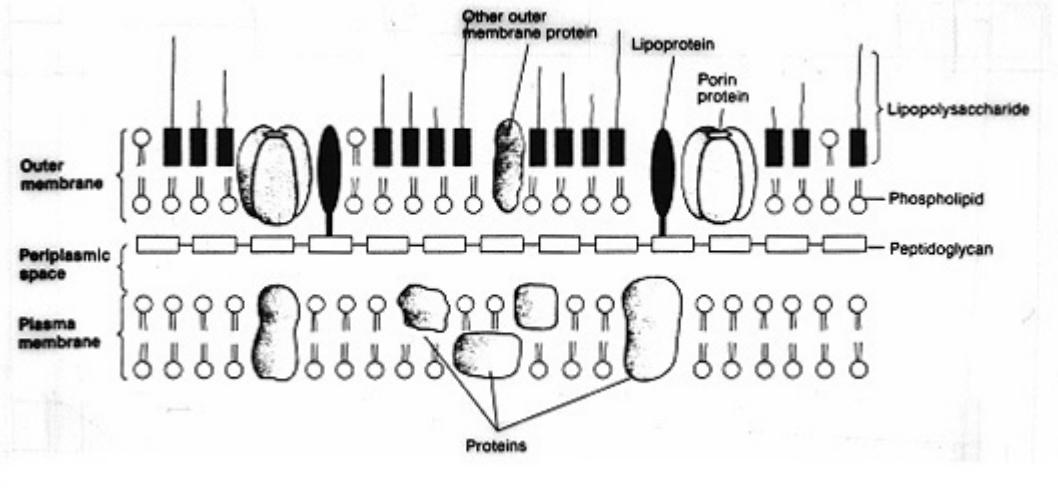
The structure of the muramic acid subunit in the peptidoglycan *Escherichia. coli*. The molecule consists of N-acetyl glucosamine (NAG) attached (via a beta 1,4 link) to N-acetyl-muramic acid (NAM). Attached to the NAM is a peptide chain, which (in the case of *E. coli*, as illustrated) consists of L-alanine, D-glutamate, diaminopimelic acid and D-alanine. Some antibiotics, including bacitracin, act by blocking the synthesis of the muramic acid subunit. Penicillin and related antibiotics (beta lactams), as well as vancomycin, block the assembly of the muropeptide subunits into the peptidoglycan polymer.

The cell wall is a complicated structure, fundamentally different in Gram-positive and Gram-negative bacteria. Cell wall components are major determinants of virulence in both groups of bacteria. **Endotoxin**, inherent to all Gram-negative bacteria, is toxic to animals in a variety of ways. Peptidoglycan and LPS, as well as some teichoic acids, induce the alternate complement pathway leading to inflammation. Teichoic acids and **O-specific polysaccharides** may be used as adhesins by Gram-positive and Gram-negative bacteria, respectively. Some cell wall components protect against phagocytic engulfment or digestion. Variations in the macromolecular structure of cell wall components may be at the basis of **antigenic variation** as well as specific host resistance to pathogens.



E. coli 0157. Transmission electron micrograph (CDC). O157 refers to the antigenic type of *E. coli* which, in this case, is based on the precise molecular structure of the O-specific polysaccharide in the cell wall LPS.

The essential outer membrane of Gram-negative bacteria is the target for attack by complement, hydrophobic agents and certain antibiotics. Murein (peptidoglycan) is dismantled by a host enzyme, lysozyme, found in most body fluids. Several antibiotics, mainly the beta lactams, exert their antimicrobial effect by blocking the synthesis and assembly of peptidoglycan.



Schematic drawing the outer membrane of a Gram-negative bacterium

The **membranes** of Bacteria are structurally similar to the cell membranes of eukaryotes, except that bacterial membranes consist of saturated or monounsaturated fatty acids (never polyunsaturated fatty acids) and do not normally contain sterols. The plasma membrane is an exceptionally dynamic structure in bacteria which mediates permeability, transport, secretion and energy generation. In terms of pathogenesis of a bacterium, it is absolutely dependent upon the integrity and function of its plasma membrane. The membrane might be responsible for secretion of toxins, resistance to antimicrobial agents, tactic responses or sensing other environmental signals to

turn on or off genes for virulence.

Endospores are bacterial structures (resting cells) 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.

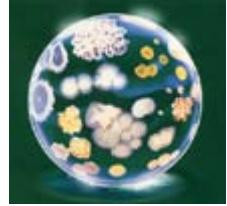
Endospores are formed by two genera of Gram-positive bacteria: *Bacillus*, the aerobic sporeformers, and *Clostridium*, the anaerobic sporeformers. Both genera contain pathogens, and the endospores produced by these bacteria invariably play some role in the toxicity, transmission or survival of the pathogen.



Spore stain of a *Bacillus* species. (CDC). Mature spores stain green whether free or still inside the vegetative sporangium. Vegetative cells and sporangia stain red. The Schaeffer-Fulton stain technique was applied. The primary stain, malachite green, is forced into the spores by heating the prepared slide to boiling for 4-5 minutes. After washing, the vegetative cells are counterstained with safranine.

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MECHANISMS OF BACTERIAL PATHOGENICITY: COLONIZATION AND INVASION

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Introduction

Microbial **pathogenicity** has been defined as the structural and biochemical mechanisms whereby microorganisms cause disease. Pathogenicity in bacteria may be associated with unique structural components of the cells (e.g. capsules, fimbriae, LPS or other cell wall components) or active secretion of substances that either damage host tissues or protect the bacteria against host defenses.

Infection may imply colonization, multiplication, invasion or persistence of a pathogen on or within a host, but disease (**infectious disease**) is used to describe an infection that causes significant overt damage to the host. There are two broad qualities of pathogenic bacteria underlie the means by which they cause disease: **invasiveness** and **toxigenesis**.

Invasiveness is the **ability to invade tissues**. This encompasses mechanisms for colonization (adherence and initial multiplication), ability to bypass or overcome host defense mechanisms, and the production of extracellular substances ("invasins") which facilitate the actual invasive process. This chapter deals with the first two aspects of invasiveness: colonization and invasion.

Toxigenesis is the **ability to produce toxins**. Toxic substances, both soluble and cell-associated, may be transported by blood and lymph and cause cytotoxic effects at tissue sites remote from the original point of invasion or growth.

COLONIZATION

The first stage of microbial infection is **colonization**: the establishment of the pathogen at the appropriate portal of entry. Pathogens usually colonize host tissues that are in contact with the external environment. Sites of entry in human hosts include the urogenital tract, the digestive tract, the respiratory tract and the conjunctiva. Organisms that infect these regions have usually developed tissue adherence mechanisms and some ability to overcome or withstand the constant pressure of the host defenses on the surface.

Bacterial Adherence to Mucosal Surfaces. In its simplest form, bacterial adherence or attachment to a eukaryotic cell or tissue surface requires the participation of two factors: a **receptor** and an **adhesin**. The receptors so far defined are usually specific carbohydrate or peptide residues on the eukaryotic cell surface. The bacterial adhesin is typically a macromolecular component of the bacterial cell surface which interacts with the host cell receptor. Adhesins and receptors usually interact in a complementary and specific fashion. Table 1 is a list of terms that are used in medical microbiology to refer to microbial adherence to surfaces or tissues.

TABLE 1. TERMS USED TO DESCRIBE ADHERENCE FACTORS IN HOST-PARASITE INTERACTIONS

ADHERENCE FACTOR	DESCRIPTION
Adhesin	A surface structure or macromolecule that binds a bacterium to a specific surface

Receptor	A complementary macromolecular binding site on a (eukaryotic) surface that binds specific adhesins or ligands
Lectin	Any protein that binds to a carbohydrate
Ligand	A surface molecule that exhibits specific binding to a receptor molecule on another surface
Mucous	The mucopolysaccharide layer of glucosaminoglycans covering animal cell mucosal surfaces
Fimbriae	Filamentous proteins on the surface of bacterial cells that may behave as adhesins for specific adherence
Common pili	Same as fimbriae
Sex pilus	A specialized pilus that binds prokaryotes together for the purpose of DNA transfer
Type 1 fimbriae	Fimbriae in <i>Enterobacteriaceae</i> which bind specifically to mannose terminated glycoproteins on eukaryotic cell surfaces
Glycocalyx	A layer of exopolysaccharide fibers on the surface of bacterial cells which may be involved in adherence to a surface
Capsule	A detectable layer of polysaccharide (rarely polypeptide) on the surface of a bacterial cell which may mediate specific or nonspecific attachment
Lipopolysaccharide (LPS)	A distinct cell wall component of the outer membrane of Gram-negative bacteria with the potential structural diversity to mediate specific adherence. Probably functions as an adhesin
Teichoic acids and lipoteichoic acids (LTA)	Cell wall components of Gram-positive bacteria that may be involved in nonspecific or specific adherence

Specific Adherence of Bacteria to Cell and Tissue Surfaces

Several types of observations provide indirect evidence for **specificity of adherence** of bacteria to host cells or tissues:

1. **Tissue tropism**: particular bacteria are known to have an apparent preference for certain tissues over others, e.g. *S. mutans* is abundant in dental plaque but does not occur on epithelial surfaces of the tongue; the reverse is true for *S. salivarius* which is attached in high numbers to epithelial cells of the tongue but is absent in dental plaque.
2. **Species specificity**: certain pathogenic bacteria infect only certain species of animals, e.g. *N. gonorrhoeae* infections are limited to humans; Enteropathogenic *E. coli* K-88 infections are limited to pigs; *E. coli* CFA I and CFA II infect humans; *E. coli* K-99 strain infects calves.; Group A streptococcal infections occur only in humans.
3. **Genetic specificity within a species**: certain strains or races within a species are genetically immune to a pathogen , e.g. Certain pigs are not susceptible to *E. coli* K-88 infections; Susceptibility to *Plasmodium vivax* infection (malaria) is dependent on the presence of the Duffy antigens on the host's redblood cells.

Although other explanations are possible, the above observations might be explained by the existence of specific interactions between microorganisms and eukaryotic tissue surfaces which allow microorganisms to become established on the surface.

Mechanisms of Adherence to Cell or Tissue Surfaces

The mechanisms for adherence may involve two steps:

1. **nonspecific adherence: reversible attachment** of the bacterium to the eukaryotic surface (sometimes called "docking")
2. **specific adherence: reversible permanent attachment** of the microorganism to the surface (sometimes called "anchoring").

The usual situation is that reversible attachment precedes irreversible attachment but in some cases, the opposite

situation occurs or specific adherence may never occur

Nonspecific adherence involves nonspecific attractive forces which allow approach of the bacterium to the eukaryotic cell surface. Possible interactions and forces involved are:

1. hydrophobic interactions
2. electrostatic attractions
3. atomic and molecular vibrations resulting from fluctuating dipoles of similar frequencies
4. Brownian movement
5. recruitment and trapping by biofilm polymers interacting with the bacterial glycocalyx (capsule)

Specific adherence involves permanent formation of many specific lock-and-key bonds between complementary molecules on each cell surface. Complementary receptor and adhesin molecules must be accessible and arranged in such a way that many bonds form over the area of contact between the two cells. Once the bonds are formed, attachment under physiological conditions becomes virtually irreversible.

Several types of experiments provide **direct evidence that receptor and/or adhesin molecules mediate specificity** of adherence of bacteria to host cells or tissues. These include:

1. The bacteria will bind isolated receptors or receptor analogs.
2. The isolated adhesins or adhesin analogs will bind to the eukaryotic cell surface.
3. Adhesion (of the bacterium to the eukaryotic cell surface) is inhibited by:
 - a. isolated adhesin or receptor molecules
 - b. adhesin or receptor analogs
 - c. enzymes and chemicals that specifically destroy adhesins or receptors
 - d. antibodies specific to surface components (i.e., adhesins or receptors)

Some Specific Bacterial Adhesins and their Receptors

The adhesins of *E. coli* are their common pili or fimbriae. A single strain of *E. coli* is known to be able to express several distinct types of fimbriae encoded by distinct regions of the chromosome or plasmids. This genetic diversity permits an organism to adapt to its changing environment and exploit new opportunities presented by different host surfaces. Many of the adhesive fimbriae of *E. coli* have probably evolved from fimbrial ancestors resembling Type-1 and type 4 fimbriae.

Type-1 fimbriae enable *E. coli* to bind to D-mannose residues on eukaryotic cell surfaces. Type-1 fimbriae are said to be "mannose-sensitive" since exogenous mannose blocks binding to receptors on red blood cells. Although the primary 17kDa fimbrial subunit is the major protein component of Type-1 fimbriae, the mannose-binding site is not located here, but resides in a minor protein (28-31kDa) located at the tips or inserted along the length of the fimbriae. By genetically varying the minor "tip protein" adhesin, the organisms can gain ability to adhere to different receptors. For example, tip proteins on pyelonephritis-associated (pap) pili recognize a galactose-galactose disaccharide, while tip proteins on S-fimbriae recognize sialic acid.

Pseudomonas, *Vibrio* and *Neisseria* possess a fimbrial protein subunit which contains methylated phenylalanine at its amino terminus. These "N-methylphenylalanine pili" have been established as virulence determinants in pathogenesis of *Pseudomonas aeruginosa* lung infection in cystic fibrosis patients. These type of fimbriae occur in *Neisseria gonorrhoeae* and their receptor is thought to be an oligosaccharide.

The adhesins of *Streptococcus pyogenes* are controversial. In 1972, Gibbons and his colleagues demonstrated that attachment of streptococci to the oral mucosa of mice is dependent on M protein. Olfek and Beachey argued that

lipoteichoic acid (LTA), rather than M protein, was responsible for streptococcal adherence to buccal epithelial cells. In 1996, Hasty and Courtney proposed a two-step model of attachment that involved both M protein and teichoic acids. They suggested that LTA loosely tethers streptococci to epithelial cells, and then M protein secures a firmer, irreversible association. In 1992, protein F was discovered and found to be a fibronectin binding protein. More recently, in 1998, M proteins M1 and M3 were also found to bind to fibronectin. Apparently, *S. pyogenes* produces multiple adhesins with varied specificities.

Staphylococcus aureus also binds to the amino terminus of fibronectin by means of a fibronectin-binding protein which occurs on the bacterial surface. Apparently *S. aureus* and Group A streptococci use different mechanisms but adhere to the same receptor on epithelial surfaces.

Treponema pallidum has three related surface adhesins (P1, P2 and P3) which bind to a four-amino acid sequence (Arg-Gly-Asp-Ser) of the cell-binding domain of fibronectin. It is not clear if *T. pallidum* uses fibronectin to attach to host surfaces or coats itself with fibronectin to avoid host defenses (phagocytes and immune responses).

TABLE 2. EXAMPLES OF SPECIFIC ATTACHMENTS OF BACTERIA TO HOST CELL OR TISSUE SURFACES

Bacterium	Adhesin	Receptor	Attachment site	Disease
<i>Streptococcus pyogenes</i>	Protein F	Amino terminus of fibronectin	Pharyngeal epithelium	Sore throat
<i>Streptococcus mutans</i>	Glycosyl transferase	Salivary glycoprotein	Pellicle of tooth	Dental caries
<i>Streptococcus salivarius</i>	Lipoteichoic acid	Unknown	Buccal epithelium of tongue	None
<i>Streptococcus pneumoniae</i>	Cell-bound protein	N-acetylhexosamine-galactose disaccharide	Mucosal epithelium	pneumonia
<i>Staphylococcus aureus</i>	Cell-bound protein	Amino terminus of fibronectin	Mucosal epithelium	Various
<i>Neisseria gonorrhoeae</i>	N-methylphenylalanine pili	Glucosamine-galactose carbohydrate	Urethral/cervical epithelium	Gonorrhea
<i>Enterotoxigenic E. coli</i>	Type-1 fimbriae	Species-specific carbohydrate(s)	Intestinal epithelium	Diarrhea
Uropathogenic <i>E. coli</i>	Type 1 fimbriae	Complex carbohydrate	Urethral epithelium	Urethritis
Uropathogenic <i>E. coli</i>	P-pili (pap)	Globobiose linked to ceramide lipid	Upper urinary tract	Pyelonephritis
<i>Bordetella pertussis</i>	Fimbriae ("filamentous hemagglutinin")	Galactose on sulfated glycolipids	Respiratory epithelium	Whooping cough
<i>Vibrio cholerae</i>	N-methylphenylalanine pili	Fucose and mannose carbohydrate	Intestinal epithelium	Cholera
<i>Treponema pallidum</i>	Peptide in outer membrane	Surface protein(fibronectin)	Mucosal epithelium	Syphilis
Mycoplasma	Membrane protein	Sialic acid	Respiratory epithelium	Pneumonia
Chlamydia	Unknown	Sialic acid	Conjunctival or urethral epithelium	Conjunctivitis or urethritis

INVASION

The invasion of a host by a pathogen may be aided by the production of bacterial extracellular substances which act against the host by breaking down primary or secondary defenses of the body. Medical microbiologists have long referred to these substances as **invasins**.^{*} Invasins are proteins (enzymes) that act locally to damage host cells and/or have the immediate effect of facilitating the growth and spread of the pathogen. The damage to the host as a result of this invasive activity may become part of the pathology of an infectious disease.

***Some modern texts and research articles have used the term invasin in a very specific manner to refer to certain bacterial proteins that aid the bacteria in their penetration of nonphagocytic host cells as an intracellular residence. These invasins are surface and/or diffusible proteins that promote actin rearrangement in the cytoskeleton of the host cells that stimulates engulfment of the bacteria. These types of invasins are utilized by intracellular pathogens such as *Listeria*, *Yersinia* and *Shigella*.**

The extracellular proteins produced by bacteria which promote their invasion are not clearly distinguished from some extracellular protein toxins ("exotoxins") which also damage the host. Invasins usually act at a short range (in the immediate vicinity of bacterial growth) and may not actually kill cells in their range of activity; exotoxins are often cytotoxic and may act at remote sites (removed from the site of bacterial growth). Also, exotoxins typically are more specific and more potent in their activity than invasins. Even so, some classic exotoxins (e.g. diphtheria toxin, anthrax toxin) may play some role in invasion in the early stages of an infection, and some invasins (e.g. staphylococcal leukocidin) have a relatively specific cytopathic effect.

A Survey of Bacterial Invasins

Spreading Factors

"Spreading Factors" is a descriptive term for a family of bacterial enzymes that affect the physical properties of tissue matrices and intercellular spaces, thereby promoting the spread of the pathogen.

Hyaluronidase is the original spreading factor. It is produced by streptococci, staphylococci, and clostridia. The enzyme attacks the interstitial cement ("ground substance") of connective tissue by depolymerizing hyaluronic acid.

Collagenase is produced by *Clostridium histolyticum* and *Clostridium perfringens*. It breaks down collagen, the framework of muscles, which facilitates gas gangrene due to these organisms.

Neuraminidase is produced by intestinal pathogens such as *Vibrio cholerae* and *Shigella dysenteriae*. It degrades neuraminic acid (also called sialic acid), an intercellular cement of the epithelial cells of the intestinal mucosa.

Streptokinase and **Staphylokinase** are produced by streptococci and staphylococci, respectively. Kinase enzymes convert inactive plasminogen to plasmin which digests fibrin and prevents clotting of the blood. The relative absence of fibrin in spreading bacterial lesions allows more rapid diffusion of the infectious bacteria.

Enzymes that Cause Hemolysis and/or Leucolysis

These enzymes usually act on the animal cell membrane by insertion into the membrane (forming a pore that results in cell lysis), or by enzymatic attack on phospholipids, which destabilizes the membrane. They may be referred to as **lecithinases** or **phospholipases**, and if they lyse red blood cells they are sometimes called **hemolysins**.

Leukocidins, produced by staphylococci and **streptolysin** produced by **streptococci** specifically lyse phagocytes and their granules. These latter two enzymes are also considered to be bacterial exotoxins.

Phospholipases, produced by *Clostridium perfringens* (i.e., alpha toxin), hydrolyze phospholipids in cell membranes by removal of polar head groups.

Lecithinases, also produced by *Clostridium perfringens*, destroy lecithin (phosphatidylcholine) in cell membranes.

Hemolysins, notably produced by staphylococci (i.e., alpha toxin), streptococci (i.e., streptolysin) and various clostridia, may be channel-forming proteins or phospholipases or lecithinases that destroy red blood cells and other cells (i.e., phagocytes) by lysis.

Staphylococcal coagulase

Coagulase, formed by *Staphylococcus aureus*, is a cell-associated and diffusible enzyme that converts fibrinogen to fibrin which causes clotting. Coagulase activity is almost always associated with pathogenic *S. aureus* and almost never associated with nonpathogenic *S. epidermidis*, which has led to much speculation as to its role as a determinant of virulence. Possibly, cell bound coagulase could provide an antigenic disguise if it clotted fibrin on the cell surface. Or a staphylococcal lesion encased in fibrin (e.g. a boil or pimple) could make the bacterial cells resistant to phagocytes or tissue bactericides or even drugs which might be unable to diffuse to their bacterial target.

Extracellular Digestive Enzymes

Heterotrophic bacteria, in general, produce a wide variety of extracellular enzymes including **proteases**, **lipases**, **glycohydrolases**, **nucleases**, etc., which are not clearly shown to have a direct role in invasion or pathogenesis. These enzymes presumably have other functions related to bacterial nutrition or metabolism, but may aid in invasion either directly or indirectly.

Toxins With Short-Range Effects Related to Invasion

Bacterial protein toxins which have adenylate cyclase activity, are thought to have immediate effects on host cells that promote bacterial invasion. One component of the anthrax toxin (**EF = Edema Factor**) is an **adenylate cyclase** that acts on nearby cells to cause increased levels of cyclic AMP and disruption of cell permeability. One of the toxins of *Bordetella pertussis*, the agent of whooping cough, has a similar effect. These toxins may contribute to invasion through their effects on macrophages or lymphocytes in the vicinity which are playing an essential role to contain the infection.

The following table summarizes the activities of many bacterial proteins that are noted for their contribution to bacterial invasion of tissues.

TABLE 3. SOME EXTRACELLULAR BACTERIAL PROTEINS THAT ARE CONSIDERED INVASINS

Invasin	Bacteria Involved	Activity
Hyaluronidase	Streptococci, staphylococci and clostridia	Degrades hyaluronic acid of connective tissue
Collagenase	<i>Clostridium</i> species	Dissolves collagen framework of muscles
Neuraminidase	<i>Vibrio cholerae</i> and <i>Shigella dysenteriae</i>	Degrades neuraminic acid of intestinal mucosa
Coagulase	<i>Staphylococcus aureus</i>	Converts fibrinogen to fibrin which causes clotting
Kinases	Staphylococci and streptococci	Converts plasminogen to plasmin which digests fibrin
Leukocidin	<i>Staphylococcus aureus</i>	Disrupts neutrophil membranes and causes discharge of lysosomal granules
Streptolysin	<i>Streptococcus pyogenes</i>	Repels phagocytes and disrupts phagocyte membrane and causes discharge of lysosomal granules
Hemolysins	Streptococci, staphylococci and clostridia	Phospholipases or lecithinases that destroy red blood cells (and other cells) by lysis
Lecithinases	<i>Clostridium perfringens</i>	Destroy lecithin in cell membranes
Phospholipases	<i>Clostridium perfringens</i>	Destroy phospholipids in cell membrane
Anthrax toxin	<i>Bacillus anthracis</i>	One component (EF) is an adenylate cyclase which causes increased levels of intracellular cyclic AMP
Pertussis toxin	<i>Bordetella pertussis</i>	One toxin component is an adenylate cyclase that acts locally

producing an increase in intracellular cyclic AMP

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MECHANISMS OF BACTERIAL PATHOGENICITY: Bacterial Defense Against Phagocytes

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Introduction

Some pathogenic bacteria are inherently able to resist the bactericidal components of host tissues, usually as a function of some structural property. For example, the poly-D-glutamate capsule of *Bacillus anthracis* protects the organisms against action of cationic proteins (defensins) in sera or in phagocytes. The outer membrane of Gram-negative bacteria is a permeability barrier that is not easily penetrated by hydrophobic compounds such as bile salts in the GI tract that are harmful to the bacteria. Pathogenic mycobacteria have a waxy cell wall that resists attack or digestion by most tissue bactericides. And intact lipopolysaccharides (LPS) of Gram-negative pathogens may protect the cells from complement-mediated lysis or the action of lysozyme.

Most successful pathogens, however, possess additional structural or biochemical features that allow them to resist the host cellular defense against them, i.e., the phagocytic and immune responses. If a pathogen breaches the host's surface defenses, it must then overcome the host's phagocytic response to succeed in an infection.

Ability of Pathogens to Avoid or Overcome Phagocytes

Microorganisms invading tissues are first and foremost exposed to phagocytes. Bacteria that readily attract phagocytes, and that are easily ingested and killed, are generally unsuccessful as pathogens. In contrast, most bacteria that are successful as pathogens interfere to some extent with the activities of phagocytes or in some way avoid their attention.

Bacterial pathogens have devised numerous and diverse strategies to avoid phagocytic engulfment and killing. Most are aimed at blocking one or more of the steps in phagocytosis, thereby halting the process. The process of phagocytosis is discussed in [Constitutive Host Defense against Bacterial Pathogens](#).

Avoiding Contact with Phagocytes

Bacteria can avoid the attention of phagocytes in a number of ways.

1. Pathogens may invade or **remain confined in regions inaccessible to phagocytes**. Certain internal tissues (e.g. the lumens of glands, the urinary bladder) and surface tissues (e.g. the skin) are not patrolled by phagocytes.
2. Some pathogens are able to **avoid provoking an overwhelming inflammatory response**. Without inflammation the host is unable to focus the phagocytic defenses.
3. Some bacteria or their products **inhibit phagocyte chemotaxis**. For example, Streptococcal streptolysin (which also kills phagocytes) suppresses neutrophil chemotaxis, even in very low concentrations. Fractions of *Mycobacterium tuberculosis* are known to inhibit leukocyte migration. The *Clostridium* ϕ toxin also inhibits neutrophil chemotaxis.
4. Some pathogens can cover the surface of the bacterial cell with a component which is seen as "self" by the host phagocytes and immune system. Such a strategy **hides the antigenic surface** of the bacterial cell. Phagocytes cannot recognize bacteria upon contact and the possibility of opsonization by antibodies to enhance phagocytosis is minimized. For example, pathogenic *Staphylococcus aureus* produces cell-bound coagulase which clots fibrin on

the bacterial surface. *Treponema pallidum*, the agent of syphilis, binds fibronectin to its surface. Group A streptococci are able to synthesize a capsule composed of hyaluronic acid. Hyaluronic acid is the ground substance (tissue cement) in connective tissue.

Inhibition of Phagocytic Engulfment

Some bacteria employ strategies to avoid engulfment (ingestion) if phagocytes do make contact with them. Many important pathogenic bacteria bear on their surfaces substances that inhibit phagocytic adsorption or engulfment. Clearly it is the bacterial surface that matters. Resistance to phagocytic ingestion is usually due to a component of the bacterial cell surface (cell wall, or fimbriae, or a capsule). Classical examples of antiphagocytic substances on the bacterial surface include:

1. **Polysaccharide capsules** of *S. pneumoniae*, *Haemophilus influenzae*, *Treponema pallidum* and *Klebsiella pneumoniae*.
2. **M protein** and **fimbriae** of Group A streptococci
3. **Surface slime** (polysaccharide) produced as a **biofilm** by *Pseudomonas aeruginosa*
4. **O polysaccharide** associated with LPS of *E. coli*
5. **K antigen** (acidic polysaccharides) of *E. coli* or the analogous **Vi antigen** of *Salmonella typhi*
6. Cell-bound or soluble **Protein A** produced by *Staphylococcus aureus*. Protein A attaches to the Fc region of IgG and blocks the cytophilic (cell-binding) domain of the Ab. Thus, the ability of IgG to act as an opsonic factor is inhibited, and opsonin-mediated ingestion of the bacteria is blocked.

Survival Inside of Phagocytes

Some bacteria survive inside of phagocytic cells, in either neutrophils or macrophages. Bacteria that can resist killing and survive or multiply inside of phagocytes are considered intracellular parasites. In this case, the environment of the phagocyte may be a protective one, protecting the bacteria during the early stages of infection or until they develop a full complement of virulence factors. The intracellular environment guards the bacteria against the activities of extracellular bactericides, antibodies, drugs, etc. Some bacteria that are intracellular parasites are listed in Table 1.

Table 1. BACTERIAL INTRACELLULAR PATHOGENS

Organism	Disease
<i>Mycobacterium tuberculosis</i>	Tuberculosis
<i>Mycobacterium leprae</i>	Leprosy
<i>Listeria monocytogenes</i>	Listeriosis
<i>Salmonella typhi</i>	Typhoid Fever
<i>Shigella dysenteriae</i>	Bacillary dysentery
<i>Yersinia pestis</i>	Plague
<i>Brucella</i> species	Brucellosis
<i>Legionella pneumophila</i>	Pneumonia
<i>Rickettsiae</i>	Typhus; Rocky Mountain Spotted Fever
<i>Chlamydia</i>	Chlamydia; Trachoma

Many intracellular parasites have special (genetically-encoded) mechanisms to get themselves into host cells that are nonphagocytic. Intracellular pathogens such as *Yersinia*, *Listeria*, *Salmonella*, *Shigella* and *Legionella* possess complex machinery for cellular invasion and intracellular survival. These systems involve various types of non-toxin virulence factors. Sometimes these factors are referred to as bacterial **invasins**. Still other bacteria such as *Bordetella pertussis* and *Streptococcus pyogenes*, have recently been discovered in the intracellular habitat of

epithelial cells.

Legionella pneumophila enters mononuclear phagocytes by depositing complement C3b on their surfaces and using that host protein to serve as the ligand for binding to macrophage cell surfaces. After ingestion, they remain in vacuoles that do not fuse with lysosomes apparently due to the influence of soluble substances produced by the bacteria.

Salmonella possesses an **invasin operon** (*inv A - H*) that encodes for factors that regulate their entry into host cells. Mutations in the operon yield organisms that can adhere to target cells without being internalized. This suggests that one or more of the *inv* proteins stimulates signal transduction in the host cell that results engulfment of the salmonellae. A similar invasin gene in *Yersinia* is known to encode a protein that both promotes adherence and activates the cytochalasin-dependent engulfment process. This invasin can confer invasive capacity on noninvasive *E. coli*, and even latex particles.

Intracellular parasites survive inside of phagocytes by virtue of mechanisms which interfere with the bactericidal activities of the host cell. Some of these bacterial mechanisms include:

1. Inhibition of fusion of the phagocytic lysosomes (granules) with the phagosome. The bacteria survive inside of phagosomes because they prevent the discharge of lysosomal contents into the phagosome environment. Specifically phagolysosome formation is inhibited in the phagocyte. This is the strategy employed by *Salmonella*, *M. tuberculosis*, *Legionella* and the chlamydiae.

-With *M. tuberculosis*, bacterial cell wall components (sulfatides) are thought to be released from the phagosome and modify lysosomal membranes to inhibit fusion.

-In *Chlamydia*, some element of the bacterial (elementary body) wall appears to modify the membrane of the phagosome in which it is contained.

-In *L. pneumophila*, as with the chlamydia, some structural feature of the bacterial cell surface, already present at the time of entry (ingestion), appears to modify the membranes of the phagosomes, thus preventing their merger with lysosomal granules. In *Legionella*, it is known that a single gene is responsible for the inhibition of phagolysosomal fusion.

-In *Salmonella typhimurium*, the pH that develops in the phagosome after engulfment actually induces bacterial gene products that are essential for their survival in macrophages.

2. Survival inside the phagolysosome. With some intracellular parasites, phagosome-lysosome fusion occurs, but the bacteria are resistant to inhibition and killing by the lysosomal constituents. Also, some extracellular pathogens can resist killing in phagocytes utilizing similar resistance mechanisms. Little is known of how bacteria can resist phagocytic killing within the phagocytic vacuole, but it may be due to the surface components of the bacteria or due to extracellular substances that they produce which interfere with the mechanisms of phagocytic killing. Some examples of how certain bacteria (both intracellular and extracellular pathogens) resist phagocytic killing are given below.

-*Mycobacterium leprae* grows inside phagocytic vacuoles even after extensive fusion with lysosomes.

-Mycobacteria (including *M. tuberculosis*) have waxy, hydrophobic cell wall and capsule components (mycolic acids), which are not easily attacked by lysosomal enzymes.

-Cell wall components (LPS?) of *Brucella abortus* apparently interfere with the intracellular bactericidal mechanisms of phagocytes.

-*B. abortus* and *Staphylococcus aureus* are vigorous catalase and superoxide dismutase producers, which might neutralize the toxic oxygen radicals that are generated by the NADPH oxidase and MPO systems in phagocytes. *S. aureus* also produces cell-bound pigments (carotenoids) that "quench" singlet oxygen produced in the phagocytic vacuole.

-The outer membrane and capsular components of Gram-negative bacteria (e.g. *Salmonella*, *Yersinia*, *Brucella*, *E. coli*) can protect the peptidoglycan layer from the lytic activity of lysozyme.

-Some pathogens (e.g. *Salmonella*, *E. coli*) are known to produce extracellular iron-binding compounds (**siderophores**) which can extract Fe+++ from lactoferrin (or transferrin) and supply iron to cells for growth.

-*Bacillus anthracis* resists killing and digestion by means of its capsule which is made up of polyD-glutamate. The "unnatural" configuration of this polypeptide affords resistance to attack or digestion by cationic proteins or by conventional proteases.

Escape from the phagosome. Early escape from the phagosome vacuole is essential for growth and virulence of some intracellular pathogens.

-This is a clever strategy employed by the Rickettsiae. *Rickettsia* enter host cells in membrane-bound vacuoles (phagosomes) but are free in the cytoplasm a short time later, perhaps in as little as 30 seconds. A bacterial enzyme, phospholipase A, may be responsible for dissolution of the phagosome membrane.

-*Listeria monocytogenes* relies on several molecules for early lysis of the phagosome to ensure their release into the cytoplasm. These include a pore-forming hemolysin (listeriolysin O) and two forms of phospholipase C. Once in the cytoplasm, *Listeria* induces its own movement through a remarkable process of host cell actin polymerization and formation of microfilaments within a comet-like tail.

-*Shigella* also lyses the phagosomal vacuole and induces cytoskeletal actin polymerization for the purpose of intracellular movement and cell-cell spread.

Products of Bacteria that Kill or Damage Phagocytes

One obvious strategy in defense against phagocytosis is direct attack by the bacteria upon the professional phagocytes. Any of the substances that pathogens produce that cause damage to phagocytes have been referred to as **aggressins**. Most of these are actually extracellular enzymes or toxins that kill phagocytes. Phagocytes may be killed by a pathogen before or after ingestion.

Killing Phagocytes Before Ingestion

Many Gram-positive pathogens, particularly the pyogenic cocci, secrete extracellular enzymes that kill phagocytes. Many of these enzymes are called **hemolysins** because their activity in the presence of red blood cells results in the lysis of the rbcs.

-Pathogenic streptococci produce **streptolysin**. Streptolysin O binds to cholesterol in membranes. The effect on neutrophils is to cause lysosomal granules to explode, releasing their contents into the cell cytoplasm.

-Pathogenic staphylococci produce **leukocidin**, which also acts on the neutrophil membrane and causes discharge of lysosomal granules.

-Extracellular proteins that inhibit phagocytosis include the **Exotoxin A** of *Pseudomonas aeruginosa* which kills macrophages, and the bacterial exotoxins that are adenylate cyclases (e.g. anthrax toxin EF and pertussis toxin AC) which decrease phagocytic activity.

Killing Phagocytes After Ingestion. Some bacteria exert their toxic action on the phagocyte after ingestion has taken place. They may grow in the phagosome and release substances which can pass through the phagosome membrane and cause discharge of lysosomal granules, or they may grow in the phagolysosome and release toxic substances which pass through the phagolysosome membrane to other target sites in the cell. Many bacteria that are the intracellular parasites of macrophages (e.g *Mycobacterium*, *Brucella*, *Listeria*) usually destroy macrophages in the end, but the mechanisms are not understood.

Other Antiphagocytic Strategies Used by Bacteria

The foregoing has been a discussion of the most commonly-employed strategies of bacterial defense against phagocytes. Although there are few clear examples, some other antiphagocytic strategies or mechanisms probably exist. For example, a pathogen may have a mechanism to inhibit the production of phagocytes or their release from the bone marrow.

A summary of bacterial mechanisms for interference with phagocytes is given in the table below.

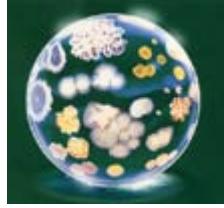
Table 2. BACTERIAL INTERFERENCE WITH PHAGOCYTES

BACTERIUM	TYPE OF INTERFERENCE	MECHANISM
<i>Streptococcus pyogenes</i>	Kill phagocyte	Streptolysin induces lysosomal discharge into cell cytoplasm
	Inhibit neutrophil chemotaxis	Streptolysin is chemotactic repellent
	Resist engulfment (unless Ab is present)	M Protein on fimbriae
	Avoid detection by phagocytes	Hyaluronic acid capsule
<i>Staphylococcus aureus</i>	Kill phagocyte	Leukocidin induces lysosomal discharge into cytoplasm
	Inhibit opsonized phagocytosis	Protein A blocks Fc portion of Ab; Polysaccharide capsule in some strains
	Resist killing	Carotenoids, catalase, superoxide dismutase detoxify toxic oxygen radicals
	Inhibit engulfment	Cell-bound coagulase hides ligands for phagocytic contact
<i>Bacillus anthracis</i>	Kill phagocyte	Anthrax toxin EF
	Resist killing	Capsular polyglutamate
<i>Streptococcus pneumoniae</i>	Resist engulfment (unless Ab is present)	Capsular polysaccharide
<i>Klebsiella pneumoniae</i>	Resist engulfment	Polysaccharide capsule
<i>Haemophilus influenzae</i>	Resist engulfment	Polysaccharide capsule
<i>Pseudomonas aeruginosa</i>	Kill phagocyte	Exotoxin A kills macrophages; Cell-bound leukocidin
	Resist engulfment	Alginate slime and biofilm polymers
<i>Salmonella typhi</i>	Resist engulfment and killing	Vi (K) antigen (microcapsule)
<i>Salmonella typhimurium</i>	Survival inside phagocytes	Bacteria develop resistance to low pH, reactive forms of oxygen, and host "defensins" (cationic proteins)
<i>Listeria monocytogenes</i>	Escape from phagosome	Listeriolysin, phospholipase C lyse phagosome membrane
<i>Clostridium perfringens</i>	Inhibit phagocyte chemotaxis	ø toxin
	Inhibit engulfment	Capsule
<i>Yersinia pestis</i>	Resist engulfment and/or killing	Protein capsule on cell surface
<i>Yersinia enterocolitica</i>	Kill phagocytes	Yop proteins injected directly into neutrophils
Mycobacteria	Resist killing and digestion	Cell wall components detoxify toxic oxygen radicals; prevent acidification of phagolysosome
<i>Mycobacterium tuberculosis</i>	Inhibit lysosomal fusion	Mycobacterial sulfatides modify lysosomes
<i>Legionella pneumophilia</i>	Inhibit phagosome-lysosomal fusion	Unknown
<i>Neisseria</i>	Inhibit phagolysosome formation;	

<i>gonorrhoeae</i>	possibly reduce respiratory burst	Involves outer membrane protein(porin) P.I
<i>Rickettsia</i>	Escape from phagosome	Phospholipase A
<i>Chlamydia</i>	Inhibit lysosomal fusion	Bacterial substance modifies phagosome
<i>Brucella abortus</i>	Resist killing	Cell wall substance (LPS?)
<i>Treponema pallidum</i>	Resist engulfment	Polysaccharide capsule material
<i>Escherichia coli</i>	Resist engulfment	O antigen (smooth strains); K antigen (acid polysaccharide)
	Resist engulfment and possibly killing	K antigen

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MECHANISMS OF BACTERIAL PATHOGENICITY: Bacterial Defense against the Host Immune Responses

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Bacterial Mechanisms to Overcome Host Immune Defenses

Antibody-mediated immunity (AMI) is the principal specific immune response effective against extracellular bacteria. The major protective immune response against intracellular bacteria is cell-mediated immunity (CMI).

On epithelial surfaces, the main antibacterial immune defense of the host is the protection afforded by secretory IgA. Once the epithelial surfaces have been penetrated, however, the major immune defenses of AMI and CMI are encountered.

If there is a way for an organism to successfully bypass or overcome the immune defenses, then some bacterial pathogen has probably "discovered" it. Bacteria evolve very rapidly in relation to their host, so that most of the feasible anti-host strategies are likely to have been tried out and exploited. Consequently, pathogenic bacteria have developed numerous ways to bypass or overcome the immune defenses of the host, which contribute to the virulence of the microbe and the pathology of the disease.

Immunological Tolerance to a Bacterial Antigen

Tolerance is a property of the host in which there is an immunologically-specific reduction in the immune response to a given Ag. Tolerance to a bacterial Ag does not involve a general failure in the immune response but a particular deficiency in relation to the specific antigen(s) of a given bacterium. If there is a depressed immune response to relevant antigens of a parasite, the process of infection is facilitated. Tolerance can involve either AMI or CMI or both arms of the immunological response.

Tolerance to an Ag can arise in a number of ways, but three are possibly relevant to bacterial infections.

1. **Fetal exposure to Ag.** If a fetus is infected at certain stages of immunological development, the microbial Ag may be seen as "self", thus inducing tolerance to the Ag which may persist even after birth.
2. **High persistent doses of circulating Ag.** Tolerance to a bacterium or one of its products might arise when large amounts of bacterial antigens are circulating in the blood.
3. **Molecular mimicry.** If a bacterial Ag is very similar to normal host "antigens", the immune responses to this Ag may be weak giving a degree of tolerance. Resemblance between bacterial Ag and host Ag is referred to as molecular mimicry. In this case the antigenic determinants of the bacterium are so closely related chemically to host "self" components that the immunological cells cannot distinguish between the two and an immune response cannot be raised. Some bacterial capsules are composed of polysaccharides (hyaluronic acid, sialic acid) so similar to host tissue polysaccharides that they are not immunogenic.

Antigenic Disguise

As already mentioned, some pathogens can hide their unique antigens from opsonizing antibodies or complement.

Bacteria may be able to coat themselves with host proteins such as fibrin, fibronectin, or even Ig molecules. In this way they are able to hide their own antigenic surface components from the immunological system.

S. aureus produces cell-bound **coagulase** and **clumping factor** that cause fibrin to clot and to deposit on the cell surface. It is possible that this disguises the bacteria immunologically so that they are not readily identified as antigens and targets for an immune response.

Protein A produced by *S. aureus*, and the analogous **Protein G** produced by *Streptococcus pyogenes*, bind the Fc portion of immunoglobulins, thus coating the bacteria with antibodies and canceling their opsonizing ability.

The **fibronectin** coat of *Treponema pallidum* provide an immunological disguise for the spirochetes.

In *E. coli* K1, that causes meningitis in newborns, a capsule composed predominantly of **sialic acid** provides an antigenic disguise, as does the hyaluronic acid capsule of *Streptococcus pyogenes*.

Immunosuppression

Some pathogens (mainly viruses and protozoa, rarely bacteria) cause immunosuppression in the infected host. This means that the host shows depressed immune responses to antigens in general, including those of the infecting pathogen. Suppressed immune responses are occasionally observed during chronic bacterial infections such as leprosy and tuberculosis.

In extreme forms of leprosy, caused by *Mycobacterium leprae*, there is poor response to leprosy antigens, as well as unrelated antigens. After patients have been successfully treated, immunological reactivity reappears, suggesting that general immunosuppression is in fact due to the disease.

In mild cases of leprosy there is frequently an associated immunological suppression that is specific for *M. leprae* antigens. This is separate from tolerance, since unique antigens (proteins) of *M. leprae* have been associated as the cause of this immunosuppression. The most likely explanations for this are due to (1) lack of costimulatory signals (interference with cytokine secretion); (2) activation of suppressor T cells; (3) disturbances in TH1/TH2 cell activities.

At present, little is known of the mechanisms by which pathogens inhibit immune responses. It seems probable that it is due to interference with the immune functions of B cells, T cells or macrophages. Since many intracellular bacteria infect macrophages, it might be expected that they compromise the role of these cells in an immunological response.

General immunosuppression induced in a host may be of immediate value to an invading pathogen, but it is of no particular significance (to the invader) if it merely promotes infection by unrelated microorganisms. Perhaps this is why it does not seem to be a commonly used strategy of the bacteria.

Persistence of a Pathogen at Bodily Sites Inaccessible to the Immune Response

Some pathogens can avoid exposing themselves to immune forces. Intracellular pathogens can evade host immune responses as long as they stay inside of infected cells and they do not allow microbial Ag to form on the cell surface. This is seen in macrophages infected with *Brucella*, *Listeria* or *M. leprae*. The macrophages support the growth of the bacteria and at the same time give them protection from immune responses. Some intracellular pathogens (*Yersinia*, *Shigella*) are residents of cells that are neither phagocytes nor APC's and their antigens are not displayed on the infected cell's surface.

Some pathogens persist on the luminal surfaces of the GI tract, oral cavity and the urinary tract, or the lumen of the salivary gland, mammary gland or the kidney tubule. If there is no host cell destruction, the pathogen may avoid inducing an inflammatory response, and there is no way in which sensitized lymphocytes or circulating antibodies can reach the site to eliminate the infection. Secretory IgA could react with surface antigens on bacterial cells, but the complement sequence would be unlikely to be activated and the cells would not be destroyed. Conceivably, IgA antibodies could immobilize bacteria by agglutination of cells or block adherence of bacteria to tissue or cell surfaces, but it is unlikely that IgA would kill bacteria directly or inhibit their growth.

Examples of some bacterial pathogens that grow at tissue sites generally inaccessible to the forces of AMI and

CMI are given below.

-*Streptococcus mutans*. The bacterium can initiate dental caries at any time after the eruption of the teeth, regardless of the immune status of the host. Either the host does not undergo an effective immune response or secretory IgA plays little role in preventing colonization and subsequent plaque development.

-*Vibrio cholerae* multiplies in the GI tract where the bacteria elaborate a toxin which causes loss of fluids and diarrhea in the host which is characteristic of the disease cholera. IgA antibodies against cellular antigens of the cholera vibrios are not completely effective in preventing infection by these bacteria as demonstrated by the relative ineffectiveness of the cholera vaccine prepared from phenol-killed vibrios.

-The carrier state of typhoid fever results from a persistent infection by the typhoid bacillus, *Salmonella typhi*. The organism is not eliminated during the initial infection and persists in the host for months, years or a life time. In the carrier state *S. typhi* is able to colonize the biliary tract (gall bladder) away from the immune forces, and be shed into urine and feces.

-Some bacteria cause persistent infections in the lumen of glands. *Brucella abortus* persistently infects mammary glands of cows and is shed in the milk. *Leptospira* multiplies persistently in the lumen of the kidney tubules of rats and is shed in the urine and remains infectious.

Induction of Ineffective Antibody

Many types of antibody are formed against a given Ag, and some bacterial components may display various antigenic determinants. Antibodies tend to range in their capacity to react with Ag (the ability of specific Ab to bind to an Ag is called **avidity**). If Abs formed against a bacterial Ag are of low avidity, or if they are directed against unimportant antigenic determinants, they may have only weak antibacterial action. Such "**ineffective**" (non-neutralizing) Abs might even aid a pathogen by combining with a surface Ag and blocking the attachment of any functional Abs that might be present.

In the case of *Neisseria gonorrhoeae* the presence of antibody to an outer membrane protein called rmp interferes with the serum bactericidal reaction and in some way compromises the surface defenses of the female urogenital tract. Increased susceptibility to reinfection is highly correlated with the presence of circulating rmp antibodies.

Antibodies Absorbed by Soluble Bacterial Antigens

Some bacteria can liberate antigenic surface components in a soluble form into the tissue fluids. These soluble antigens are able to combine with and "neutralize" antibodies before they reach the bacterial cells. For example, small amounts of endotoxin (LPS) may be released into surrounding fluids by Gram-negative bacteria.

Autolysis of Gram-negative or Gram-positive bacteria may release antigenic surface components in a soluble form. *Streptococcus pneumoniae* and *Neisseria meningitidis* are known to release capsular polysaccharides during growth in tissues. They are found in the serum of patients with pneumococcal pneumonia and in the cerebrospinal fluid of patients with meningitis. Theoretically, these released surface antigens could "mop up" antibody before it reached the bacterial surface which should be an advantage to the pathogen. These soluble bacterial cell wall components are powerful antigens and complement activators so they contribute in a major way to the pathology observed in meningitis and pneumonia.

Protein A, produced by *S. aureus* may remain bound to the staphylococcal cell surface or it may be released in a soluble form. Protein A will bind to the Fc region of IgG. On the cell surface, protein A binds IgG in the wrong orientation to exert its antibacterial activity, and soluble protein A agglutinates and partially inactivates IgG.

Local Interference with Antibody Activity

There are probably several ways that pathogens interfere with the antibacterial action of antibody molecules. Some pathogens produce enzymes that destroy antibodies.

Neisseria gonorrhoeae, *N. meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Streptococcus mutans*, which can grow on the surfaces of the body, produce **IgA proteases** that inactivate secretory IgA by cleaving the molecule at the hinge region, detaching the Fc region of the immunoglobulin. Soluble forms of

Protein A produced *S. aureus* agglutinate immunoglobulin molecules and partially inactivate IgG.

Antigenic Variation

One way bacteria can avoid forces of the immune response is to periodically changing antigens, i.e., to undergo antigenic variation. Some bacteria avoid the host antibody response by changing from one type of fimbriae to another, or by switching fimbrial tips. This makes the original AMI response obsolete by using new fimbriae that do not bind the previous antibodies. Pathogenic bacteria can vary (change) other surface proteins, especially outer membrane proteins, that are the targets of antibodies.

Antigens may vary or change within the host during the course of an infection, or alternatively antigens may vary among multiple strains (antigenic types) of a parasite in the population. Antigenic variation is an important mechanism used by pathogenic microorganisms for escaping the neutralizing activities of antibodies. Antigenic variation usually results from site-specific inversions or gene conversions or gene rearrangements in the DNA of the microorganisms.

Borrelia recurrentis is a spirochete that causes the human disease relapsing fever. The disease is characterized by episodes of fever which relapse (come and go) for a period of weeks or months. After infection, the bacteria multiply in tissues and cause a febrile illness until the onset of an immune response a week or so later. Bacteria then disappear from the blood because of antibody mediated phagocytosis, lysis, agglutination, etc., and the fever falls. Then an antigenically distinct mutant arises in the infected individual, multiplies, and in 4-10 days reappears in the blood and there is another febrile attack. The immune system is stimulated and responds by conquering the new antigenic variant, but the cycle continues such that there may be up to 10 febrile episodes before final recovery. With each attack a new antigenic variant of the bacterium appears and a new set of antibodies is formed in the host. Thus, this bacterium can change its antigens during the course of an infection in a single host, and this variation in bacterial antigens contributes significantly to the course of the infection.

Neisseria gonorrhoeae can change fimbrial antigens during the course of an infection. During initial stages of an infection, adherence to epithelial cells of the cervix or urethra is mediated by pili (fimbriae). Equally efficient attachment to phagocytes would be undesirable. Rapid switching on and off of the genes controlling pili are therefore necessary at different stages of the infection, and *N. gonorrhoeae* is capable of undergoing this type of "pili switching" or **phase variation**. Genetically controlled changes in outer membrane proteins also occur in the course of an infection. This finely controlled expression of the genes for pili and surface proteins changes the adherence pattern to different host cells, increases resistance to cervical proteases, increases resistance to phagocytosis and immune lysis, and is presumably necessary for successful infection.

Many pathogenic bacteria exist in nature as **multiple antigenic types** or serotypes, meaning that they are variant strains of the same pathogenic species. For example, there are multiple serotypes of *Salmonella typhimurium* based on differences in cell wall (O) antigens or flagellar (H) antigens. There are 80 different antigenic types of *Streptococcus pyogenes* based on M-proteins on the cell surface. There are over one hundred strains of *Streptococcus pneumoniae* depending on their capsular polysaccharide antigens. Based on minor differences in surface structure chemistry there are multiple serotypes of *Vibrio cholerae*, *Staphylococcus aureus*, *Escherichia coli*, *Neisseria gonorrhoeae* and an assortment of other bacterial pathogens. Antigenic variation is prevalent among pathogenic viruses as well.

If the immune response is the main defense against a pathogen, then being able to shed old antigens and present new ones to the immune system might allow infection or continued invasion by the pathogen to occur. Furthermore, the infected host would seem to be the ideal selective environment for the emergence of new antigenic variants of bacteria. Perhaps this explains why many bacteria exist in a great variety of antigenic types.

Evading Complement

Antibodies that are bound to bacterial surfaces will activate complement by the classical pathway and bacterial polysaccharides activate complement by the alternative pathway. Bacteria in serum and other tissues, especially Gram-negative bacteria, need protection from the antimicrobial effects of complement.

One role of **capsules** in bacterial virulence is to protect the bacteria from complement activation and the ensuing inflammatory response. Polysaccharide capsules can hide bacterial components such as LPS or peptidoglycan

which can induce the alternate complement pathway. Some bacterial capsules are able to inhibit formation of the C3b complex on their surfaces, thus avoiding C3b opsonization and subsequent formation of C5b and the membrane attack complex (MAC) on the bacterial cell surface. Capsules that contain sialic acid (a common component of host cell glycoproteins), such as found in *Neisseria meningitidis*, have this effect.

One of the principal targets of complement on Gram-negative bacteria is LPS. It serves as the attachment site for C3b and triggers the alternative pathway of activation. It also binds C5b.

LPS can be modified by pathogens in two ways that affects its interaction with complement.

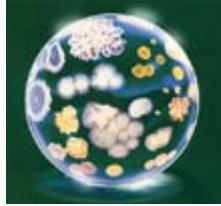
First, by attachment of sialic acid residues to the LPS O antigen, a bacterium can prevent the formation of C3 convertase just as capsules that contain sialic acid can do so. Both *Neisseria meningitidis* and *Haemophilus influenzae*, which cause bacterial meningitis, are able to covalently attach sialic acid residues to their O antigens resulting in resistance to MAC. Second, LPS with long, intact O antigen side-chains can prevent effective MAC killing. Apparently the MAC complex is held too far from the vulnerable outer membrane to be effective.

Bacteria that are not killed and lysed in serum by the complement MAC are said to be **serum resistant**. As might be expected many of the Gram-negative bacteria that cause systemic infections, (bacteremia or septicemia) are serum resistant. Gram-positive bacteria are naturally serum-resistant since their cells are not enclosed in an outer membrane.

Other ways that pathogens are able to inhibit the activity of complement is to destroy one or more of the components of complement. *Pseudomonas aeruginosa* produces an extracellular **elastase** enzyme that inactivates components of complement.

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MECHANISMS OF BACTERIAL PATHOGENICITY: PROTEIN TOXINS

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Bacterial Toxigenesis

Toxigenesis, or the ability to produce toxins, is an underlying mechanism by which many bacterial pathogens produce disease. At a chemical level, there are two types of bacterial toxins, **lipopolysaccharides**, which are associated with the cell walls of Gram-negative bacteria, and **proteins**, which are released from bacterial cells and may act at tissue sites removed from the site of bacterial growth. The **cell-associated** lipopolysaccharide (**LPS**) toxins are referred to as **endotoxins** and the **extracellular** diffusible toxins are referred to as **exotoxins**.

Endotoxins are cell-associated substances that are structural components of the outer membrane of Gram-negative bacteria. However, endotoxins may be released from growing bacterial cells or from cells which are lysed as a result of effective host defense (e.g. lysozyme) or the activities of certain antibiotics (e.g. penicillins and cephalosporins). **Exotoxins** are usually secreted by bacteria but in some cases they are released by lysis of the bacterial cell. Hence, either type of bacterial toxin may ultimately act in close association with the cells that produce the toxin, or at tissue sites remote from the original point of bacterial invasion or growth. Some bacterial toxins may also act at the site of colonization and play a role in invasion.

BACTERIAL PROTEIN TOXINS

Exotoxins are typically soluble proteins secreted by living bacteria during exponential growth. The production of the toxin is generally specific to a particular bacterial species that produces the disease associated with the toxin (e.g. only *Clostridium tetani* produces tetanus toxin; only *Corynebacterium diphtheriae* produces the diphtheria toxin). Usually, virulent strains of the bacterium produce the toxin while nonvirulent strains do not, and the toxin is the major determinant of virulence (e.g. tetanus and diphtheria). At one time it was thought that exotoxin production was limited mainly to Gram-positive bacteria, but both Gram-positive and Gram-negative bacteria produce soluble protein toxins.

Bacterial protein toxins are the most powerful human poisons known and retain high activity at very high dilutions. The lethality of the most potent bacterial exotoxins is compared to the lethality of strychnine, snake venom, and endotoxin in Table 1 below.

TABLE 1. LETHALITY OF BACTERIAL PROTEIN TOXINS

Toxin	Toxic Dose (mg)	Host	Lethal toxicity compared with:		
			Strychnine	Endotoxin	Snake Venom
Botulism Type D	0.8x10 ⁻⁸	Mouse	3x10 ⁶	3x10 ⁷	3x10 ⁵
Tetanus	4x10 ⁻⁸	Mouse	1x10 ⁶	1x10 ⁷	1x10 ⁵
<i>Shigella</i> Neurotoxin	2.3x10 ⁻⁶	Rabbit	1x10 ⁶	1x10 ⁷	1x10 ⁵
Diphtheria	6x10 ⁻⁵	Guinea Pig	2x10 ³	2x10 ⁴	2x10 ²

The **protein toxins resemble enzymes** in a number of ways. Like enzymes, bacterial exotoxins are **denatured by**

heat, acid and proteolytic enzymes; they have a high biological activity (most **act catalytically**); and they exhibit **specificity of action**.

As enzymes attack specific substrates, so bacterial protein toxins are highly specific in the substrate utilized and in their mode of action. The **substrate** (in the host) may be a component of tissue cells, organs, or body fluid. Usually the site of damage caused by the toxin indicates the location of the substrate for that toxin. Terms such as **enterotoxin**, **neurotoxin**, **leukocidin** or **hemolysin** are sometimes used to indicate the target site of some well-defined protein toxins.

Certain protein toxins have very **specific cytotoxic activity** (i.e., they attack specific types of cells). For example, tetanus or botulinum toxins attack only neurons. But some toxins (as produced by staphylococci, streptococci, clostridia, etc.) have fairly **broad cytotoxic activity** and cause nonspecific death of all sorts of cells and tissues, eventually resulting in **necrosis**. Toxins that are phospholipases act in this way. They cleave phospholipids which are regular components of host cell membranes, resulting in the death of the cell by leakage of cellular contents. This is also true of pore-forming **hemolysins** and **leukocidins**.

A few bacterial toxins that obviously bring about the death of an animal are known simply as **lethal toxins**, and even though the tissues affected and the target sites may be known, the precise mechanism by which death occurs is not understood (e.g. anthrax toxin LF).

Bacterial protein toxins are strongly **antigenic**. In vivo, specific antibody (**antitoxin**) neutralizes the toxicity of these bacterial proteins. However, in vitro, specific antitoxin may not fully inhibit their enzymatic activity. This suggests that the antigenic determinant of the toxin may be distinct from the active (enzymatic) portion of the protein molecule. The degree of neutralization of the enzymatic site may depend on the distance from the antigenic site on the molecule. However, since the toxin is fully neutralized in vivo, this suggests that other host factors must play a role in nature.

Protein toxins are inherently **unstable**: in time they lose their toxic properties but retain their antigenic ones. This was first discovered by Ehrlich and he coined the term **toxoid** for this product. **Toxoids** are detoxified toxins which retain their antigenicity and their immunizing capacity. The formation of toxoids can be accelerated by treating toxins with a variety of reagents including formalin, iodine, pepsin, ascorbic acid, ketones, etc. The mixture is maintained at 37 degrees at pH range 6 to 9 for several weeks. The resulting toxoids can be used for **artificial immunization** against diseases caused by pathogens where the primary determinant of bacterial virulence is toxin production. Toxoids are the immunizing agents against diphtheria and tetanus that are part of the DPT vaccine.

A plus B subunit Arrangement of Protein Toxins

Many protein toxins, notably those that act intracellularly (with regard to host cells), consist of two components: one component (**subunit A**) is responsible for the **enzymatic activity** of the toxin; the other component (**subunit B**) is concerned with **binding** to a specific receptor on the host cell membrane and transferring the enzyme across the membrane. The enzymatic component is not active until it is released from the native (A+B) toxin. Isolated A subunits are enzymatically active but lack binding and cell entry capability. Isolated B subunits may bind to target cells (and even block the binding of the native toxin), but they are nontoxic.

There are a variety of ways that toxin subunits may be synthesized and arranged: **A + B** indicates that the toxin is synthesized and secreted as two separate protein subunits that interact at the target cell surface; **A-B** or **A-5B** indicates that the A and B subunits are synthesized separately, but associated by noncovalent bonds during secretion and binding to their target; **5B** indicates that the binding domain of the protein is composed of 5 identical subunits. **A/B** denotes a toxin synthesized as a single polypeptide, divided into A and B domains, that may be separated by proteolytic cleavage.

Attachment and Entry of Toxins

There are at least **two mechanisms of toxin entry into target cells**.

In one mechanism called **direct entry**, the B subunit of the native (A+B) toxin binds to a specific receptor on the target cell and induces the formation of a pore in the membrane through which the A subunit is transferred into the cell cytoplasm.

In an alternative mechanism, the native toxin binds to the target cell and the A+B structure is taken into the cell by

the process of **receptor-mediated endocytosis (RME)**. The toxin is internalized in the cell in a membrane-enclosed vesicle called an **endosome**. H⁺ ions enter the endosome lowering the internal pH which causes the A+B subunits to separate. Somehow, the B subunit affects the release of the A subunit from the endosome so that it will reach its target in the cell cytoplasm. The B subunit remains in the endosome and is recycled to the cell surface. In both cases (above) a large protein molecule must insert into and cross a membrane lipid bilayer (either the cell membrane or the endosome membrane). This activity is reflected in the ability of most A+B or A/B toxins, or their B components, to insert into artificial lipid bilayers, creating ion permeable pathways.

A few bacterial toxins (e.g. diphtheria) are known to utilize both direct entry and RME to enter into host cells, which is not surprising since both mechanisms are variations on a theme. Bacterial toxins with similar enzymatic mechanisms may enter their target cells by different mechanisms. Thus, the diphtheria toxin and *Pseudomonas* exotoxin A, which have identical mechanisms of enzymatic activity, enter their host cells in slightly different ways. The adenylate cyclase toxin of *Bordetella pertussis* and the anthrax toxin (Edema Factor) of *Bacillus anthracis* act similarly to catalyze the production of cAMP from host cell intracellular ATP reserves. However, the anthrax toxin enters cells by receptor mediated endocytosis, whereas the pertussis adenylate cyclase traverses the cell membrane directly.

The **specific receptors** for the B subunit of the toxin on target cells or tissues are usually sialogangliosides (glycoproteins) called **G-proteins** on the cell membrane. For example, the cholera toxin utilizes the ganglioside GM1, and tetanus toxin utilizes ganglioside GT1 and/or GD1b as receptors on host cells.

Control of Synthesis and the Release of Protein Toxins

The regulation of synthesis and secretion of many bacterial toxins is tightly controlled by regulatory elements that are sensitive to environmental signals. For example, the production of diphtheria toxin is totally repressed by the availability of adequate amounts of iron in the medium for bacterial growth. Only under conditions of limiting amounts of iron in the growth medium does toxin production become derepressed. The expression of cholera toxin and related virulence factors (adhesins) is controlled by environmental osmolarity and temperature. In *B. pertussis*, induction of different virulence components is staggered, such that attachment factors are produced initially to establish the infection, and toxins are synthesized and released later to counter the host defenses and promote bacterial survival.

The processes by which protein toxins are assembled and secreted by bacterial cells are also variable. Many of the classic exotoxins are synthesized with an NH terminal leader (signal) sequence consisting of a few (1-3) charged amino acids and a stretch (14-20) of hydrophobic amino acids. The signal sequence may bind and insert into the cytoplasmic membrane during translation such that the polypeptide is secreted while being synthesized. The signal peptide is cleaved as the toxin (protein) is released into the periplasm. Alternatively, the toxin may be synthesized intracytoplasmically, then bound to a leader sequence for passage across the membrane. Frequently, chaperone proteins are required to guide this process. Some multicomponent toxins, such as the cholera toxin, have their subunits synthesized and secreted separately into the periplasm where they are assembled. In Gram-negative bacteria, the outer membrane poses an additional permeability barrier that a protein toxin usually has to mediate if it is to be released in a soluble form. It has been proposed that some Gram-negative exotoxins (e.g. *E. coli* ST enterotoxin) might be released in membrane vesicles composed of outer membrane components. Since these vesicles presumably would possess the outer membrane associated attachment factors, they could act as smart bombs capable of specifically interacting with and possibly entering target cells to release their contents of toxin.

Diphtheria toxin

The best known and studied bacterial toxin is the diphtheria toxin, produced by *Corynebacterium diphtheriae*. Diphtheria toxin is a bacterial exotoxin of the A/B prototype. It is produced as single polypeptide chain with a molecular weight of 60,000 daltons. The function of the protein is distinguishable into two parts: subunit A, with a m.w. of 21,000 daltons, contains the enzymatic activity for inhibition of elongation factor-2 involved in host protein synthesis; subunit B, with a m.w. of 39,000 daltons, is responsible for binding to the membrane of a susceptible host cell.

In vitro, the native toxin is produced in an inactive form which can be activated by the proteolytic enzyme trypsin in the presence of thiol (reducing agent). The enzymatic activity of Fragment A is masked in the intact toxin. Fragment B is required to enable Fragment A to reach the cytoplasm of susceptible cells. The C terminal

end of Fragment B is hydrophilic and contains determinants that interact with specific membrane receptors on sensitive cell membranes and the N-terminal end of Fragment B is strongly hydrophobic. The specific membrane receptor for the B fragment has recently been shown to be a transmembranous heparin-binding protein on the susceptible cell's surface.

The diphtheria toxin enters its target cells by either direct entry or receptor mediated endocytosis. The first step is the irreversible binding of the C-terminal hydrophilic portion of Fragment B (AA 432-535) to the receptor. During RME the whole toxin is then taken up in an endocytic vesicle. In the endocytic vesicle, the pH drops to about 5 which allows unfolding of the A and B chains. This exposes hydrophobic regions of both the A and B chains that can insert into the vesicle membrane. The result is exposure of the A chain to the cytoplasmic side of the membrane. There, reduction and proteolytic cleavage releases the A chain in the cytoplasm. A is released as an extended chain but regains its active (enzymatic) globular conformation in the cytoplasm. The A chain catalyzes the ADP ribosylation of elongation factor-2 (EF-2).

Diphtheria toxin is very potent in its action; a single molecule of subunit A within a cell is lethal and a single diphtheria bacillus is thought to be able to produce about 5,000 molecules per hour. The toxin (subunit A) utilizes NAD as a substrate: it catalyzes the attachment of the ADP- ribose portion of NAD to the elongation factor which inactivates its function in protein synthesis.

Other considerations

In keeping with the observation that genetic information for functions not involved in viability of bacteria is frequently located extrachromosomally, the genes encoding toxin production are generally located on **plasmids** or in **lysogenic bacteriophages**. Thus, the processes of genetic exchange in bacteria, notably **conjugation** and **transduction**, can mobilize these genetic elements between strains of bacteria, and therefore may play a role in determining the pathogenic potential of a bacterium. **Horizontal transfer** of genetic elements that encode virulence also occurs between species of bacteria, which could explain how *E. coli* and *Vibrio cholerae* produce a nearly-identical diarrhea-inducing toxin

Why certain bacteria produce such potent toxins is mysterious and is analogous to asking why an organism should produce an antibiotic. The production of a toxin may play a role in adapting a bacterium to a particular niche, but it is not essential to the viability of the organism. Most toxigenic bacteria are free-living in Nature and in associations with humans in a form which is phenotypically identical to the toxigenic strain but lacking the ability to produce the toxin.

There is conclusive evidence for the pathogenic role of diphtheria, tetanus and botulinum toxins, various enterotoxins, staphylococcal toxic shock syndrome toxin, and streptococcal erythrogenic toxin. And there is good evidence for the pathological involvement of pertussis toxin, anthrax toxin, shiga toxin and the necrotizing toxins of clostridia, in bacterial disease.

A summary of bacterial protein toxins and their activities is given in Tables 2 and 3.

Details of the mechanisms of action of these toxins and their involvement in the pathogenesis of disease is discussed in chapters with the specific bacterial pathogens.

For more information on bacterial toxins go to this website: [Bacterial Toxins: Friends or Foes?](#)

TABLE 2. ACTIVITIES OF EXTRACELLULAR BACTERIAL PROTEIN TOXINS

NAME OF TOXIN	BACTERIA INVOLVED	ACTIVITY
Anthrax toxin (EF)	<i>Bacillus anthracis</i>	Edema Factor (EF) is an adenylate cyclase that causes increased levels in intracellular cyclic AMP in phagocytes and formation of ion-permeable pores in membranes (hemolysis)
Adenylate cyclase toxin	<i>Bordetella pertussis</i>	Acts locally to increase levels of cyclic AMP in phagocytes and formation of ion-permeable pores in membranes (hemolysis)
Cholera enterotoxin (ctx)	<i>Vibrio cholerae</i>	ADP ribosylation of G proteins stimulates adenylate cyclase and increases cAMP in cells of the GI tract, causing secretion of water and electrolytes

E. coli LT toxin	<i>Escherichia coli</i>	Similar to cholera toxin
E. coli ST toxin	<i>Escherichia coli</i>	Stimulates guanylate cyclase and promotes secretion of water and electrolytes from intestinal epithelium
Shiga toxin	<i>Shigella dysenteriae</i>	Enzymatically cleaves rRNA resulting in inhibition of protein synthesis in susceptible cells
Perfringens enterotoxin	<i>Clostridium perfringens</i>	Stimulates adenylate cyclase leading to increased cAMP in epithelial cells
Botulinum toxin	<i>Clostridium botulinum</i>	Zn ⁺⁺ dependent protease that inhibits neurotransmission at neuromuscular synapses resulting in flaccid paralysis
Tetanus toxin	<i>Clostridium tetani</i>	Zn ⁺⁺ dependent protease that Inhibits neurotransmission at inhibitory synapses resulting in spastic paralysis
Diphtheria toxin (dtx)	<i>Corynebacterium diphtheriae</i>	ADP ribosylation of elongation factor 2 leads to inhibition of protein synthesis in target cells
Exotoxin A	<i>Pseudomonas aeruginosa</i>	Inhibits protein synthesis; similar to diphtheria toxin
Anthrax toxin (LF)	<i>Bacillus anthracis</i>	Lethal Factor (LF) is a Zn ⁺⁺ dependent protease that induces cytokine release and is cytotoxic to cells by an unknown mechanism
Pertussis toxin (ptx)	<i>Bordetella pertussis</i>	ADP ribosylation of G proteins blocks inhibition of adenylate cyclase in susceptible cells
Staphylococcus enterotoxins*	<i>Staphylococcus aureus</i>	Massive activation of the immune system, including lymphocytes and macrophages, leads to emesis
Toxic shock syndrome toxin (TSST-1)*	<i>Staphylococcus aureus</i>	Acts on the vascular system causing inflammation, fever and shock
Exfoliatin toxin*	<i>Staphylococcus aureus</i>	Cleavage within epidermal cells (intraepidermal separation)
Erythrogenic toxin (streptococcal pyrogenic exotoxin SPE)*	<i>Streptococcus pyogenes</i>	Same as TSST - inflammation, fever and shock; can cause localized erythematous reactions

* The pyrogenic exotoxins produced by *Staphylococcus aureus* and *Streptococcus pyogenes* have been designated as superantigens. They represent a family of molecules with the ability to elicit massive activation of the immune system. These proteins share the ability to stimulate T cell proliferation by interaction with Class II MHC molecules on APCs and specific V beta chains of the T cell receptor. The important feature of this interaction is the resultant production of IL-1, TNF, and other lymphokines which appear to be the principal mediators of disease processes associated with these toxins.

TABLE 3. ENZYMATIC ACTIVITY AND BIOLOGICAL EFFECTS OF SOME BACTERIAL EXOTOXINS

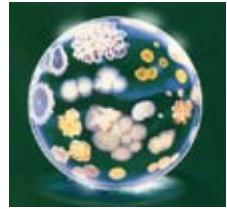
TOXIN (subunit arr)*	ENZYMATIC ACTIVITY	BIOLOGICAL EFFECTS
Cholera toxin(A-5B)	ADP ribosylates adenylate cyclase Gs regulatory protein	Activates adenylate cyclase; increased levels of intracellular cAMP promote secretion of fluid and electrolytes in intestinal epithelium leading to diarrhea
Diphtheria toxin (A/B)	ADP ribosylates elongation factor 2	Inhibits protein synthesis in animal cells resulting in death of the cells
Pertussis toxin (A-5B)	ADP ribosylates adenylate cyclase Gi regulatory protein	Blocks inhibition of adenylate cyclase; increased levels of cAMP effect hormone activity and reduce phagocytic activity
<i>E. coli</i> heat-labile toxin LT (A-5B)	ADP ribosylates adenylate cyclase Gs regulatory protein	Similar or identical to cholera toxin
<i>E. coli</i> heat stable toxin ST	ST toxins, of which there several types, are small polypeptides ranging in size from 18 to	Stimulates guanylate cyclase in epithelial cells of the GI tract resulting in intra-cellular accumulation of cyclic GMP which has a net

	72 AA, and probably lack enzymatic activity	
Shiga toxin (A/5B)	Glycosidase cleavage of ribosomal RNA (cleaves a single Adenine base from the 28S rRNA)	secretory effect on cells and leads to diarrhea
<i>Pseudomonas</i> Exotoxin A (A/B)	ADP ribosylates elongation factor 2 analogous to diphtheria toxin	Inactivates the mammalian 60S ribosomal subunit and leads to inhibition of protein synthesis and death of the susceptible cell
Botulinum toxin (A/B)	Zn ⁺⁺ dependent protease acts on synaptobrevin at motor neuron ganglioside	Inhibits protein synthesis in susceptible cells, resulting in death of the cells
Tetanus toxin(A/B)	Zn ⁺⁺ dependent protease acts on synaptobrevin in central nervous system	Inhibits presynaptic acetylcholine release from peripheral cholinergic neurons resulting in flaccid paralysis
Anthrax toxin LF (A2+B)	A1 (Lethal Factor=LF) is a Zn ⁺⁺ dependent protease with an unknown substrate; A2 (Edema Factor=EF) is an adenylate cyclase	Inhibits neurotransmitter release from inhibitory neurons in the CNS resulting in spastic paralysis
<i>Bordetella</i> <i>pertussis</i> AC toxin (A/B) and Bacillus anthracis EF (A1+B)	Calmodulin-regulated adenylate cyclases that catalyze the formation of cyclic AMP from ATP in susceptible cells, and the formation of ion-permeable pores in cell membranes	B subunit, called the Protective Antigen (PA), plus LF induces cytokine release and death of target cells or experimental animals
<i>Staphylococcus</i> <i>aureus</i> Exfoliatin B (?)	Cleaves desmoglein 1, a cadherin found in desmosomes in the epidermis	Increases cAMP in phagocytes leading to inhibition of phagocytosis by neutrophils and macrophages; hemolysis or leukolysis

* **toxin subunit arrangements:** **A-B or A-5B** indicates subunits synthesized separately and associated by noncovalent bonds; **A/B** denotes subunit domains of a single protein that may be separated by proteolytic cleavage; **A+B** indicates subunits synthesized and secreted as separate protein subunits that interact at the target cell surface; **5B** indicates that the binding domain is composed of 5 identical subunits.

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MECHANISMS OF BACTERIAL PATHOGENICITY: ENDOTOXINS

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BACTERIAL ENDOTOXINS

Endotoxins are part of the outer membrane of the cell wall of Gram-negative bacteria. Endotoxins are invariably associated with Gram-negative bacteria whether the organisms are pathogens or not. Although the term "endotoxin" is occasionally used to refer to any cell-associated bacterial toxin, it is properly reserved to refer to the **lipopolysaccharide** complex associated with the outer membrane of Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, and other leading pathogens.

The biological activity of endotoxin is associated with the lipopolysaccharide (LPS). **Toxicity** is associated with the lipid component (**Lipid A**) and **immunogenicity** is associated with the **polysaccharide** components. The cell wall antigens (**O antigens**) of Gram-negative bacteria are components of LPS. LPS elicits a variety of inflammatory responses in an animal. Because it activates complement by the alternative (properdin) pathway, it is often part of the pathology of Gram-negative bacterial infections.

The relationship of endotoxins to the bacterial cell surface is illustrated in Figure 1 below.

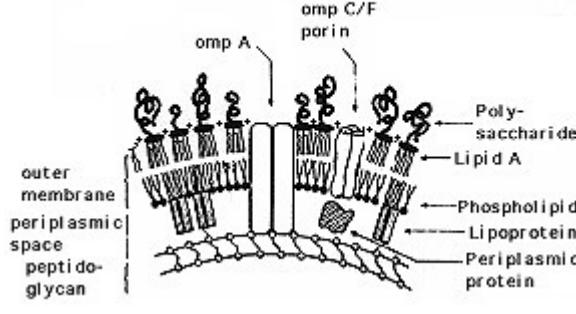


Figure 1. Structure of the cell surface of a Gram-negative bacterium

In vivo, Gram-negative bacteria probably release minute amounts of endotoxin while growing. It is known, that small amounts of endotoxin may be released in a soluble form, especially by young cultures. However, for the most part, endotoxins remain associated with the cell wall until disintegration of the bacteria. In vivo , this results from autolysis of the bacteria, external lysis mediated by complement and lysozyme, and phagocytic digestion of bacterial cells.

Compared to the classic exotoxins of bacteria, endotoxins are less potent and less specific in their action, since they do not act enzymatically. Endotoxins are heat stable (boiling for 30 minutes does not destabilize endotoxin), but

certain powerful oxidizing agents such as superoxide, peroxide and hypochlorite, degrade them. Endotoxins, although antigenic, cannot be converted to toxoids. A comparison of the properties of bacterial endotoxins and classic exotoxins is shown in Table 1.

Table 1. Characteristics of bacterial endotoxins and classic exotoxins

PROPERTY	ENDOTOXIN	EXOTOXIN
CHEMICAL NATURE	Lipopolysaccharide(mw = 10kDa)	Protein (mw = 50-1000kDa)
RELATIONSHIP TO CELL	Part of outer membrane	Extracellular, diffusible
DENATURED BY BOILING	No	Usually
ANTIGENIC	Yes	Yes
FORM TOXOID	No	Yes
POTENCY	Relatively low (>100ug)	Relatively high (1 ug)
SPECIFICITY	Low degree	High degree
ENZYMATIC ACTIVITY	No	Usually
PYROGENICITY	Yes	Occasionally

The Role of LPS in the Outer Membrane of Gram-negative Bacteria

The function of the outer membrane of Gram-negative bacteria is to act as permeability barrier. The outer membrane is impermeable to large molecules and hydrophobic compounds from the environment. Endotoxin (LPS) is located on the outer face of the membrane, where it mediates contact with the environment. LPS is essential to the function of the outer membrane, and as a structural component of the cell, it may play several roles in the pathogenesis of Gram-negative bacterial infections. First, it is a permeability barrier that is permeable only to low molecular weight, hydrophilic molecules. In the *E. coli* the *ompF* and *ompC* porins exclude passage of all hydrophobic molecules and any hydrophilic molecules greater than a molecular weight of about 700 daltons. This prevents penetration of the bacteria by bile salts and other toxic molecules from the GI tract. It also a barrier to lysozyme and many antimicrobial agents. Second, it impedes destruction of the bacterial cells by serum components and phagocytic cells. Third, LPS plays an important role as a surface structure in the interaction of the pathogen with its host. For example, LPS may be involved in adherence (colonization), or resistance to phagocytosis, or antigenic shifts that determine the course and outcome of an infection.

Chemical Nature of Endotoxin

Most of the work on the chemical structure of endotoxin has been done with species of *Salmonella* and *E. coli*. LPS can be extracted from whole cells by treatment with 45% phenol at 90°. Mild hydrolysis of LPS yields Lipid A plus polysaccharide.

Lipopolysaccharides are complex amphiphilic molecules with a mw of about 10kDa, that vary widely in chemical composition both between and among bacterial species. The general architecture of LPS is shown in Figure 2. The general structure of *Salmonella* LPS is shown in Figure 3 and the complete structure of *Salmonella* lipid A is illustrated in Figure 4.

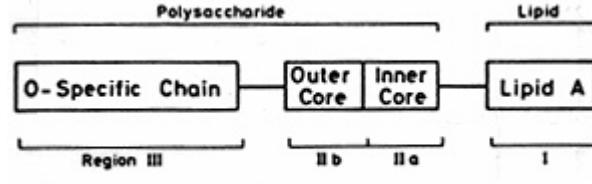


Figure 2. General architecture of Lipopolysaccharide

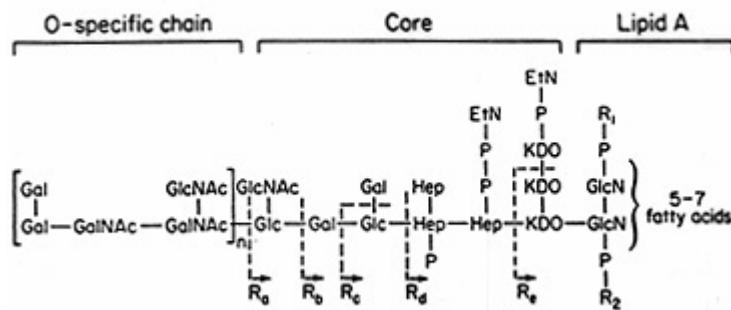


Figure 3. General Structure of *Salmonella* LPS

Glc = glucose; GlcNac = N-acetyl- glucosamine; Gal = galactose; Hep = heptose; P = phosphate; Etn = ethanolamine; R1 and R2 = phoshethanolamine or aminoarabinose. Ra to Re indicate incomplete forms of LPS. The Rd2 phenotype (not shown) would have only a single heptose unit. The Rc, Rd2, and Rd1 mutants lack the phosphate group attached to Hep.

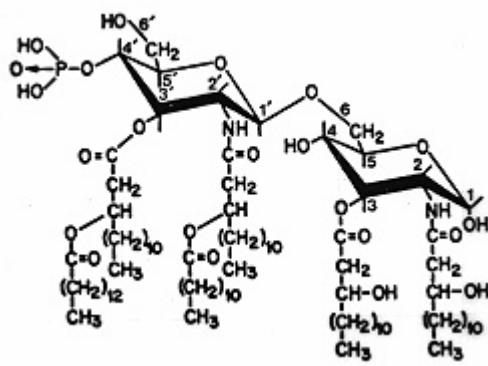


Figure 4. Complete structure of the Lipid A Moiety of LPS of *S. typhimurium*, *S. minnesota*, and *E. coli*

LPS consists of three components or regions, **Lipid A**, an **R polysaccharide** and an **O polysaccharide**.

Region I. Lipid A is the lipid component of LPS. It contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. Usually 6 FA are found. All FA in Lipid A are saturated. Some FA are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The structure of Lipid A is highly conserved among Gram-negative bacteria. Among *Enterobacteriaceae* Lipid A is virtually constant.

Region II. Core (R) antigen or R polysaccharide is attached to the 6 position of one NAG. The R antigen consists of a short chain of sugars. For example: KDO - Hep - Hep - Glu - Gal - Glu - GluNAc -

Two unusual sugars are usually present, heptose and 2-keto-3-deoxyoctanoic acid (KDO), in the core polysaccharide. KDO is unique and invariably present in LPS and so has been an indicator in assays for LPS (endotoxin).

With minor variations, the core polysaccharide is common to all members of a bacterial genus (e.g. *Salmonella*), but it is structurally distinct in other genera of Gram-negative bacteria. *Salmonella*, *Shigella* and *Escherichia* have similar but not identical cores.

Region III. Somatic (O) antigen or O polysaccharide is attached to the core polysaccharide. It consists of repeating oligosaccharide subunits made up of 3 - 5 sugars. The individual chains vary in length ranging up to 40 repeat units. The O polysaccharide is much longer than the core polysaccharide, and it maintains the hydrophilic domain of the LPS molecule. A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in the O polysaccharide.

Great variation occurs in the composition of the sugars in the O side chain between species and even strains of Gram-negative bacteria. At least 20 different sugars are known to occur and many of these sugars are characteristically unique dideoxyhexoses, which occur in nature only in Gram-negative cell walls. Variations in sugar content of the O polysaccharide contribute to the wide variety of antigenic types of *Salmonella* and *E. coli* and presumably other strains of Gram-negative species. Particular sugars in the structure, especially the terminal ones, confer immunological specificity of the O antigen, in addition to "smoothness" (colony morphology) of the strain. Loss of the O specific region by mutation results in the strain becoming a "rough" (colony morphology) or R strain.

The elucidation of the structure of LPS (Figure 3) relied heavily on the availability of mutants each blocked at a particular step in LPS synthesis. The biosynthesis of LPS is strictly sequential. The core sugars are added sequentially to Lipid A by successive additions, and the O side chain is added last, one preassembled subunit at a time. The properties of mutants producing incomplete LPS molecules suggests the nature and biological functions performed by various parts of the LPS molecule.

Loss of the O antigen results in loss of virulence suggesting that this portion is important during a host-parasite interaction. It is known that such "rough" mutants are more susceptible to phagocytosis and serum bactericidal reactions.

Loss of the more proximal parts of the core, as in "deep rough" mutants (i.e. in Rd1, Rd2, and Re mutants in Figure 3) makes the strains sensitive to a range of hydrophobic compounds, including antibiotics, detergents, bile salts and mutagens. This area contains a large number of charged groups and is thought to be important in maintaining the permeability properties of the outer membrane.

Mutants in the assembly of Lipid A cannot be isolated except as conditional lethal mutants and this region must therefore be essential for cell viability. The innermost region of LPS, consisting of Lipid A and three residues of KDO, appears to be essential for viability, presumably for assembling the outer membrane.

LPS and virulence of Gram-negative bacteria

Both Lipid A (the toxic component of LPS) and the polysaccharide side chains (the nontoxic but immunogenic portion of LPS) act as determinants of virulence in Gram-negative bacteria.

The O polysaccharide and virulence

Virulence and the property of "smoothness", associated with an intact **O polysaccharide**, are regularly associated in many bacterial infections. The involvement of the polysaccharide chain in virulence is shown by the fact that small changes in the sugar sequences in the side chains of LPS result in major changes in virulence. How are the polysaccharide side chains involved in the expression of virulence? There are a number of possibilities:

1. Smooth antigens could allow organisms to **adhere** specifically to certain tissues, especially epithelial tissues.
2. Smooth antigens probably allow **resistance to phagocytes**, since rough mutants are more readily engulfed and destroyed by phagocytes.
3. The hydrophilic O polysaccharides could act as water-solubilizing **carriers for toxic Lipid A**. It is known that the exact structure of the polysaccharide can greatly influence water binding capacity at the cell surface.
4. The O antigens could provide **protection from damaging reactions with antibody and complement**. Rough strains of Gram-negative bacteria derived from virulent strains are generally non virulent. Smooth strains have polysaccharide "whiskers" which bear O antigens projecting from the cell surface. The O antigens are the key targets for the action of host antibody and complement, but when the reaction takes place at the tips of the

polysaccharide chains, a significant distance external to the general bacterial cell surface, complement fails to have its normal lytic effect. Such bacteria are virulent because of this resistance to immune forces of the host. If the projecting polysaccharide chains are shortened or removed, antibody reacts with antigens on the general bacterial surface, or very close to it, and complement can lyse the bacteria. This contributes to the loss of virulence in "rough" colonial strains.

5. The O-polysaccharide or **O antigen** is the basis of **antigenic variation** among many important Gram-negative pathogens including *E. coli*, *Salmonella* and *Vibrio cholerae*. Antigenic variation guarantees the existence of multiple serotypes of the bacterium, so that it is afforded multiple opportunities to infect its host if it can bypass the immune response against a different serotype. Furthermore, even though the O polysaccharides are strong antigens, they seldom elicit immune responses which give full protection to the host against secondary challenge with specific endotoxin.

Lipid A and virulence

Endotoxins are toxic to most mammals, and regardless of the bacterial source, all endotoxins produce the same range of biological effects in the animal host. Most of our knowledge of the biological activities of endotoxins derives not from the study of natural disease but by challenge of experimental animals.

The injection of living or killed Gram-negative cells, or purified LPS, into experimental animals causes a wide spectrum of nonspecific pathophysiological reactions such as: **fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death**. Injection of fairly small doses of endotoxin results in death in most mammals. The sequence of events follows a regular pattern: (1) latent period; (2) physiological distress (diarrhea, prostration, shock); (3) death. How soon death occurs varies on the dose of the endotoxin, route of administration, and species of animal. Animals vary in their susceptibility to endotoxin.

The physiological effects of endotoxin are thought to be mediated by **Lipid A**. Since Lipid A is embedded in the outer membrane of bacterial cells, it probably only exerts its toxic effects when released from multiplying cells in a soluble form, or when the bacteria are lysed as a result of autolysis, complement and the membrane attack complex (MAC), ingestion and killing by phagocytes, or killing with certain types of antibiotics.

It is thought that LPS released into the bloodstream by lysing Gram-negative bacteria is first bound by certain plasma proteins identified as **LPS-binding proteins**. The LPS-binding protein complex interacts with CD14 receptors on monocytes and macrophages and other types of receptors on endothelial cells. In **monocytes and macrophages** three types of events are triggered during their interaction with LPS:

1. **Production of cytokines**, including IL-1, IL-6, IL-8, tumor necrosis factor (TNF) and platelet-activating factor. These in turn stimulate production of prostaglandins and leukotrienes. These are powerful mediators of inflammation and septic shock that accompanies endotoxin toxemia. LPS activates macrophages to enhanced phagocytosis and cytotoxicity. Macrophages are stimulated to produce and release lysosomal enzymes, IL-1 ("endogenous pyrogen"), and tumor necrosis factor (TNFalpha), as well as other cytokines and mediators.

2. **Activation of the complement cascade**. C3a and C5a cause histamine release (leading to vasodilation) and effect neutrophil chemotaxis and accumulation. The result is inflammation.

3. **Activation of the coagulation cascade**. Initial activation of Hageman factor (blood-clotting Factor XII) can activate several humoral systems resulting in

- a. coagulation: a blood clotting cascade that leads to coagulation, thrombosis, acute disseminated intravascular coagulation, which depletes platelets and various clotting factors resulting in internal bleeding.
- b. activation of the complement alternative pathway (as above, which leads to inflammation)
- c. plasmin activation which leads to fibrinolysis and hemorrhaging.
- d. kinin activation releases bradykinins and other vasoactive peptides which causes hypotension.

The net effect is to induce inflammation, intravascular coagulation, hemorrhage and shock.

LPS also acts as a **B cell mitogen** stimulating the polyclonal differentiation and multiplication of B-cells and the secretion of immunoglobulins, especially IgG and IgM.

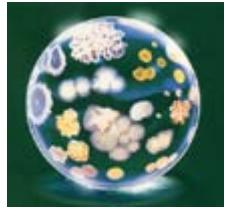
The physiological activities of LPS are mediated mainly by the Lipid A component of LPS. Lipid A is a powerful biological response modifier that can stimulate the mammalian immune system. During infectious disease caused

by Gram-negative bacteria, endotoxins released from, or part of, multiplying cells have similar effects on animals and significantly contribute to the symptoms and pathology of the disease encountered.

The primary structure of Lipid A has been elucidated and Lipid A has been chemically synthesized. Its biological activity appears to depend on a peculiar conformation that is determined by the glucosamine disaccharide, the PO₄ groups, the acyl chains, and also the KDO-containing inner core.

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Antimicrobial Agents Used in Treatment of Infectious Disease

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Introduction

Most microbiologists distinguish two groups of antimicrobial agents used in the treatment of infectious disease: **antibiotics**, which are natural substances produced by certain groups of microorganisms, and **chemotherapeutic agents**, which are chemically synthesized. A hybrid substance is a **semisynthetic antibiotic**, wherein a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties. Furthermore, some antimicrobial compounds, originally discovered as products of microorganisms, can be synthesized entirely by chemical means. They might be referred to as **synthetic antibiotics** to distinguish them from the chemotherapeutic agents.

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 an antimicrobial agent, from a host 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 host. 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.

Characteristics of Antibiotics

Antibiotics are low-molecular weight substances that are produced as secondary metabolites by certain groups of microorganisms, especially *Streptomyces*, *Bacillus*, and a few molds (*Penicillium* and *Cephalosporium*) that are inhabitants of soils (Table 1). 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 is affected by a certain antibiotic is expressed as its **spectrum of action**. Antibiotics effective against prokaryotes 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**.

A clinically-useful antibiotic should have as many of these characteristics as possible.

- It should have a wide spectrum of activity with the ability to destroy or inhibit many different species of pathogenic organisms.
- It should be nontoxic to the host and without undesirable side effects.
- It should be nonallergenic to the host.
- It should not eliminate the normal flora of the host.
- It should be able to reach the part of the human body where the infection is occurring.
- It should be inexpensive and easy to produce.

- It should be chemically-stable (have a long shelf-life).
- Microbial resistance is uncommon and unlikely to develop.

Kinds of Antimicrobial Agents and their Primary Modes of Action

The table below is a summary of the classes of antibiotics and their properties including their biological source and mode of action.

Table 1. Classes of antibiotics and their properties

Chemical class	Examples	Biological source	Spectrum (effective against)	Mode of action
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
Carboxyphenems	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. <i>Staphylococcus aureus</i>	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
Lincomycins	Clindamycin	Streptomyces lincolnensis	Gram-positive and Gram-negative bacteria esp. anaerobic <i>Bacteroides</i>	Inhibits translation (protein synthesis)
Macrolides	Erythromycin	Streptomyces erythreus	Gram-positive bacteria, Gram-negative bacteria not enterics, <i>Neisseria</i> , <i>Legionella</i> , <i>Mycoplasma</i>	Inhibits translation (protein synthesis)
Polypeptides	Polymyxin	<i>Bacillus polymyxa</i>	Gram-negative bacteria	Damages cytoplasmic membranes
	Bacitracin	<i>Bacillus subtilis</i>	Gram-positive bacteria	Inhibits steps in murein (peptidoglycan) biosynthesis and assembly
		Streptomyces		Inactivate membranes

Polyenes	Amphotericin	nodosus	Fungi	containing sterols
	Nystatin	Streptomyces noursei	Fungi (Candida)	Inactivate membranes containing sterols
Rifamycins	Rifampicin	Streptomyces mediterranei	Gram-positive and Gram-negative bacteria, <i>Mycobacterium tuberculosis</i>	Inhibits transcription (bacterial RNA polymerase)
Tetracyclines	Tetracycline	Streptomyces species	Gram-positive and Gram-negative bacteria, <i>Rickettsias</i>	Inhibit translation (protein synthesis)
Semisynthetic tetracycline	Doxycycline		Gram-positive and Gram-negative bacteria, <i>Rickettsias</i> , <i>Ehrlichia</i> , <i>Borrelia</i>	Inhibit translation (protein synthesis)
Chloramphenicol	Chloramphenicol	Streptomyces venezuelae	Gram-positive and Gram-negative bacteria	Inhibits translation (protein synthesis)

Antimicrobial Agents Used in the Treatment of Infectious Disease

The following discussion of antibiotics and chemotherapeutic organizes the antimicrobial agents based on their mode of action in bacterial cells.

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 are stereochemically related to D-alanyl-D-alanine which is a substrate for the last step in peptidoglycan synthesis, the final cross-linking between peptide side chains. Penicillins bind to and inhibit the carboxypeptidase and transpeptidase enzymes that are required for this step in peptidoglycan biosynthesis. Beta lactam antibiotics are normally bactericidal and require that cells be actively growing in order to exert their toxicity.

Different beta lactams differ in their spectrum of activity and their effect on Gram-negative rods, as well as their toxicity, stability in the human body, rate of clearance from blood, whether they can be taken orally, ability to cross the blood-brain barrier, and susceptibility to bacterial beta-lactamases.

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 of the molecule (6-aminopenicillanic acid) which can be modified chemically by the addition of side chains. 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. **Amoxicillin** 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, amoxicillin 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.

Cephalosporins are beta lactam antibiotics with a similar mode of action to penicillins that are produced by species of *Cephalosporium*. They 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*.

Two other classes of beta lactams are the **carbapenems** and **monobactams**. The latter are particularly useful for the treatment of allergic individuals. A person who becomes allergic to penicillin usually becomes allergic to the cephalosporins and the carbapenems as well. Such individuals can still be treated with the monobactams, which are structurally different so as not to induce allergy.

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.

Cycloserine inhibits the early stages of murein synthesis where D-alanyl-D-alanine is added to the growing peptide side chain. The antibiotic resembles D-alanine in spatial structure, and it competitively inhibits the racemase reaction that converts L-alanine to D-alanine and the synthetase reaction that joins two D-alanine molecules. The affinity of cycloserine for these enzymes is about a hundred times greater than that of D-alanine. Cycloserine enters bacterial cells by means of an active transport system for glycine and can reach a relatively high intracellular concentration. This concentrating effect, along with its high affinity for susceptible enzymes, enables cycloserine to function as a very effective antimicrobial agent. However, it is fairly toxic and has limited use as a secondary drug for tuberculosis.

Glycopeptides, such as the antibiotic **vancomycin**, appear to inhibit both transglycosylation and transpeptidation reactions during peptidoglycan assembly. They bind to the muropeptide subunit as it is transferred out of the cell cytoplasm and inhibit subsequent polymerization reactions. Vancomycin is not effective against Gram-negative bacteria because it cannot penetrate their outer membrane. However, it has become important in clinical usage for treatment of infections by strains of *Staphylococcus aureus* that are resistant to virtually all other antibiotics.

Cell membrane inhibitors

These antibiotics 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 polymyxia*. Polymyxin is effective mainly against Gram-negative bacteria and is usually limited to topical usage. Polymyxin binds to membrane phospholipids and thereby interferes with membrane function. Polymyxin is occasionally given for urinary tract infections caused by *Pseudomonas* strains 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.

Protein synthesis inhibitors

Many therapeutically useful antibiotics owe their action to inhibition of some step in the complex process of protein synthesis. Their attack is always at one of the events occurring on the ribosome and never at 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.

Streptomycin binds to 30S subunit of the bacterial ribosome, specifically to the S12 protein which is involved in the initiation of protein synthesis. Experimentally, streptomycin has been shown to prevent the initiation of protein synthesis by blocking the binding of initiator N-formylmethionine tRNA to the ribosome. It also prevents the normal dissociation of ribosomes into their subunits, leaving them mainly in their 70S form and preventing the formation of polysomes. The overall effect of streptomycin seems to be one of distorting the ribosome so that it no longer can carry out its normal functions. This evidently accounts for its antibacterial activity but does not explain its bactericidal effects, which distinguishes streptomycin and other aminoglycosides from most other protein synthesis inhibitors.

Kanamycin and **tobramycin** have been reported to bind to the ribosomal 30S subunit and to prevent it from joining to the 50S subunit during protein synthesis. They may have a bactericidal effect because this leads to cytoplasmic accumulation of dissociated 30S subunits, which is apparently lethal to the cells.

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** (a mixture of 3 components) 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 or synthetically. **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. *Pseudomonas aeruginosa* is less sensitive but is generally susceptible to tetracycline concentrations that are obtainable in the bladder. 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 the use of **doxycycline** in the treatment of Lyme disease.

Some newly discovered members of the tetracycline family (e.g. chelocardin) have been shown to act by inserting into the bacterial membrane, not by inhibiting protein synthesis.

Chloramphenicol is a protein synthesis inhibitor 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 prokaryotic cells and have 70S ribosomes, they are subject to inhibition by some of the protein synthesis inhibitors including chloramphenicol. 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 **macrolide** family of antibiotics is characterized by structures that 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.

Lincomycin and **clindamycin** are a miscellaneous group of protein synthesis inhibitors with activity similar to the macrolides. **Lincomycin** has activity against Gram-positive bacteria and some Gram-negative bacteria (*Neisseria*, *H. influenzae*). **Clindamycin** is a derivative of lincomycin, with the same range of antimicrobial activity, but it is considered more effective. It is frequently used as a penicillin substitute and is effective against Gram-negative anaerobes (e.g. *Bacteroides*).

Effects on Nucleic Acids

Some antibiotics and 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 prokaryotes and some medical utility are the **quinolones** 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 *Proteus* species which are common causes of UTI. It is not usually effective against *Pseudomonas aeruginosa*, and Gram-positive bacteria are resistant.

Some quinolones have a broadened spectrum against Gram-positive bacteria. The **fluoroquinolone**, Cipro (ciprofloxacin) was recently touted as the drug of choice for treatment and prophylaxis of anthrax, which is caused by a Gram-positive bacillus.

The **rifamycins** are a comparatively new group of antibiotics, 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 the bacterial RNA polymerase and is inactive towards DNA polymerase or RNA polymerase from animal cells. 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 the cerebrospinal fluid so it is useful for treatment of bacterial meningitis.

Competitive Inhibitors

Many of the synthetic chemotherapeutic agents are **competitive inhibitors** of essential metabolites or growth factors that are needed in bacterial metabolism. Hence, these types of antimicrobial agents are sometimes referred to as **anti-metabolites** or **growth factor analogs**, since they are designed to specifically inhibit an essential metabolic pathway in the bacterial pathogen. At a chemical level, competitive inhibitors are structurally similar to a bacterial growth factor or metabolite, but they do not fulfill their metabolic function in the cell. Some are bacteriostatic and some are bactericidal. Their selective toxicity is based on the premise that the bacterial pathway does not occur in the host.

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 tetrahydrofolic 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: (**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, similar in activity to the sulfonamides. PAS was once a primary anti-tuberculosis drug, but now it is a secondary agent, having been largely replaced by ethambutol.

Microorganisms that Produce Antibiotics

The molds, ***Penicillium*** and ***Cephalosporium***, produce Beta-lactam antibiotics, i.e., penicillin, cephalosporin, and their relatives.

Actinomycetes, mainly ***Streptomyces*** species: produce tetracyclines, aminoglycosides (streptomycin and its relatives), macrolides (erythromycin and its relatives), chloramphenicol, ivermectin, rifamycins, and most other clinically-useful antibiotics that are not beta-lactams.

Bacillus species, such as *B. polymyxa* and *Bacillus subtilis* produce polypeptide antibiotics (e.g. polymyxin and bacitracin).

These organisms all have in common that they live in a soil habitat and they form some sort of a spore or resting structure. It is not known why these microorganisms produce antibiotics. It may rest in the obvious, i.e., the antibiotics afford the microbes some nutritional advantage in their habitat by antagonism against the competition. However, it may rest on the subtle: i.e., the antibiotics act as some sort of hormone or signal molecule associated with sporulation or dormancy or germination.

Antibiotics are secondary metabolites of microorganisms and they are produced at the same time that the cells begin sporulation processes. Antibiotics tend to be rather large, complicated, organic molecules and may require as many as 30 separate enzymatic steps to synthesize. The maintenance of a substantial component of the bacterial genome devoted solely to the synthesis of an antibiotic leads one to the conclusion that the process (or molecule) is important, if not essential, to the survival of these organisms in their natural habitat.

Most of the microorganisms that produce antibiotics are resistant to the action of their own antibiotic, although the organisms are affected by other antibiotics, and their antibiotic may be effective against closely-related strains. Generally speaking, how or why bacteria are resistant to their own antibiotics is also unknown, but the mechanisms may be similar to resistance that develops in medically-important bacteria.

Microbial Resistance to Antibiotics

The first antibiotic, penicillin, was discovered in 1929 by Sir Alexander Fleming who observed inhibition of staphylococci on an agar plate contaminated by a *Penicillium* mold. World War II (and the inevitable bacterial infections that occurred in war-related wounds) was an important impetus to study the chemotherapeutic value of penicillin. 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. It is important to note that a significant fraction of all human infections are caused by these two bacteria (i.e., strep throat, pneumonia, septicemia, skin infections, wound infections, scarlet fever, toxic shock syndrome). Penicillin had unbelievable ability to kill these bacterial pathogens without harming the host that harbored them.

Resistance to penicillin in some strains of staphylococci was recognized almost immediately after introduction of the drug. Resistance to penicillin today occurs in as many as 80% of all strains of *Staphylococcus aureus* and some strains of *S. aureus* have been isolated that are resistant to virtually all clinically-available antibiotics. Surprisingly, *Streptococcus pyogenes* (Group A strep) has never fully developed resistance to penicillin, and it remains a reasonable choice antibiotic for many types of streptococcal infections. Interestingly, penicillin has 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 to penicillin 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. Today, drug-resistant strains of *M. tuberculosis* are threatening to break through in one of the world's most prevalent infectious diseases.

The Genetic 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 for most bacterial genes is approximately 10^{-8} . This means that if a bacterial population doubles from 10^8 cells to 2×10^8 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, a bacterium like *E. coli* develops genetic resistance through the process of mutation and selection and then donates these genes to some other bacterium through one of several processes for genetic exchange that exist in bacteria (below).

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 Mechanisms of Bacterial Resistance to Antibiotics

section in progress

The Medical Problem of Bacterial Resistance

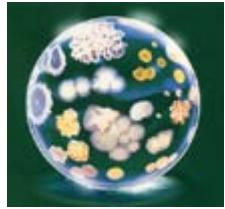
Obviously, if a bacterial pathogen is able to develop or acquire resistance to an antibiotic, then that substance becomes useless in the treatment of infectious disease caused by that pathogen (unless the resistance can somehow be overcome with secondary measures). So as pathogens develop resistance, humanity 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). Broad patterns of resistance exist in these pathogens, and it seems likely that new antibiotics will soon be needed 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.

It is said that the discovery and use of antibiotics and immunization procedures against infectious disease are two developments in the field of microbiology that have contributed about twenty years to the average life span of humans in developed countries where these practices are employed. While the greater part of this span in time is probably due to vaccination, most of us are either still alive or have family members who are still alive because an antibiotic conquered an infectious disease that otherwise would have killed the individual. If we want to retain this medical luxury in our society, we must be vigilant and proactive: we must fully understand how and why antimicrobial agents work, and why they don't work, and realize that we must maintain a stride ahead of microbial pathogens that can only be contained by antibiotic chemotherapy.

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Bacterial resistance to Antibiotics

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Definition of an antibiotic

Antibiotics are substances produced by microorganisms that kill or inhibit other microorganisms. Antibiotics are products of the earth, more specifically of soil; they are byproducts of cellular metabolism; antibiotics are "all natural".

History of antibiotics and antibiotic chemotherapy

The first antibiotic, penicillin, was discovered in 1929 by Sir Alexander Fleming who observed inhibition of staphylococci on an agar plate contaminated by a *Penicillium* mold. World War II (and the inevitable bacterial infections that occurred in war-related wounds) was an important impetus to study the chemotherapeutic value of penicillin. 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. It is important to note that a significant fraction of all human infections are caused by these two bacteria (i.e., strep throat, pneumonia, septicemia, skin infections, wound infections, scarlet fever, toxic shock syndrome). Penicillin had unbelievable ability to kill these bacterial pathogens without harming the host that harbored them. This brings to light the fundamental principle of antimicrobial chemotherapy that may be relevant in this symposium, i.e., **selective toxicity**: Antibiotics used in the treatment of disease must be effective against the pathogenic microorganism and not the host; the host (patient taking the antibiotic) must essentially be **resistant** to the action of the drug.

Resistance to penicillin in some strains of staphylococci was recognized almost immediately after introduction of the drug. (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. Interestingly, penicillin has 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 to penicillin because their vulnerable cell wall is protected by an outer membrane that prevents permeation of the penicillin molecule.

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These organisms all have in common that they live in a soil habitat and they form some sort of a spore or resting structure. It is not known why these microorganisms produce antibiotics but it may rest in the obvious : affording them some nutritional advantage in their habitat by antagonizing the competition; or the subtle: acting as some sort of hormone or signal molecule associated with sporulation or dormancy or germination. Antibiotics are secondary metabolites of microorganisms and they are produced at the same time that the cells begin sporulation processes. Antibiotics tend to be rather large, complicated, organic molecules and may require as many as 30 separate enzymatic steps to synthesize. The maintenance of a substantial component of the bacterial genome devoted solely to the synthesis of an antibiotic leads one to the conclusion that the process (or molecule) is important, if not essential, to the survival of these organisms in their natural habitat.

Most of the microorganisms that produce antibiotics are resistant to the action of their own antibiotic, although the organisms are affected by other antibiotics, and their antibiotic may be effective against closely-related strains. Generally speaking, how or why bacteria are resistant to their own antibiotics is also unknown, but it may be worth pondering or studying if we are to understand the cellular and molecular basis of resistance.

The basis of microbial 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.

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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".

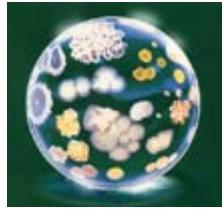
Not only is there a problem in finding new antibiotics to fight old diseases (because resistant strains of bacteria have emerged), there is a parallel problem to find new antibiotics to fight new diseases. In the past two decades, many "new" bacterial diseases have been discovered (Legionnaire's disease,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

Conclusions

It is said that the discovery and use of antibiotics and immunization procedures against infectious disease are two developments in the field of microbiology that have contributed about twenty years to the average life span of humans in developed countries where these practices are employed. While the greater part of this span in time is probably due to vaccination, most of us are either still alive or have family members who are still alive because an antibiotic conquered an infectious disease that otherwise would have killed the individual. If we want to retain this medical luxury in our society we must be vigilant and proactive: we must fully understand how and why antimicrobial agents work, and why they don't work, and realize that we must maintain a stride ahead of microbial pathogens that can only be contained by antibiotic chemotherapy.

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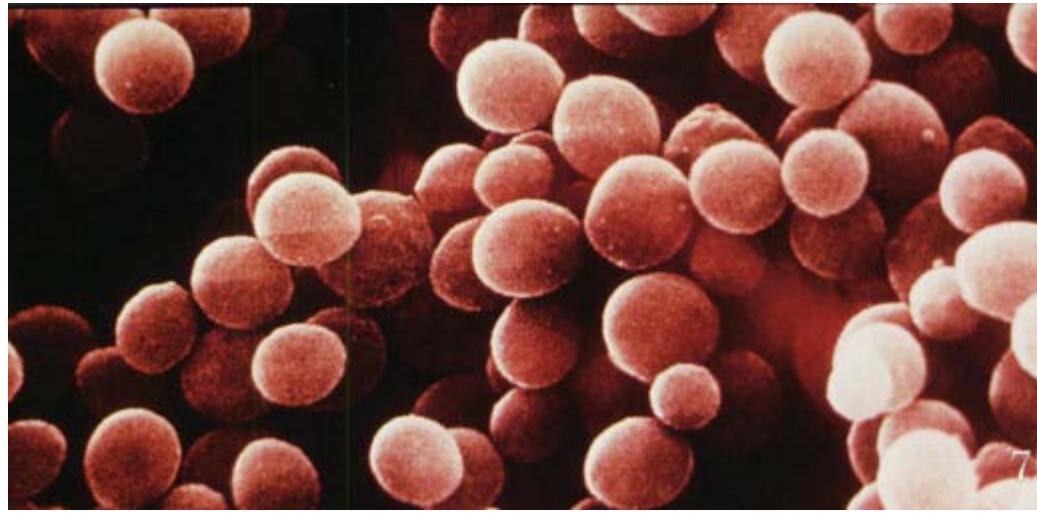
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Staphylococcus

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Staphylococcus aureus. Electron micrograph from [Visuals Unlimited](#), with permission.

The Staphylococci

Staphylococci are Gram-positive spherical bacteria that occur in microscopic clusters resembling grapes. Bacteriological culture of the nose and skin of normal humans invariably yields staphylococci. In 1884, Rosenbach described the two pigmented colony types of staphylococci and proposed the appropriate nomenclature: *Staphylococcus aureus* (yellow) and *Staphylococcus albus* (white). The latter species is now named *Staphylococcus epidermidis*. Although more than 20 species of *Staphylococcus* are described in Bergey's Manual (2001), only *Staphylococcus aureus* and *Staphylococcus epidermidis* are significant in their interactions with humans. *S. aureus* colonizes mainly the nasal passages, but it may be found regularly in most other anatomical locales. *S. epidermidis* is an inhabitant of the skin.

Taxonomically, the genus *Staphylococcus* is in the Bacterial family *Staphylococcaceae*, which includes three lesser known genera, *Gamella*, *Macrococcus* and *Salinicoccus*. The best-known of its nearby phylogenetic relatives are the members of the genus *Bacillus* in the family *Bacillaceae*, which is on the same level as the family *Staphylococcaceae*. The *Listeriaceae* are also a nearby family.

Staphylococcus aureus forms a fairly large yellow colony on rich medium, *S. epidermidis* has a relatively small white colony. *S. aureus* is often hemolytic on blood agar; *S. epidermidis* is non hemolytic. Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation that yields principally lactic acid. The bacteria are catalase-positive and oxidase-negative. *S. aureus* can grow at a temperature range of 15 to 45 degrees and at NaCl concentrations as high as 15 percent. Nearly all strains of *S. aureus* produce the enzyme coagulase: nearly all strains of *S. epidermidis* lack this enzyme. *S. aureus* should always be considered a potential pathogen; most strains of *S. epidermidis* are nonpathogenic and may even play a protective role in their host as normal flora. *Staphylococcus epidermidis* may be a pathogen in the hospital environment.

Staphylococci are perfectly spherical cells about 1 micrometer in diameter. They grow in clusters because

staphylococci divide in two planes. The configuration of the cocci helps to distinguish staphylococci from streptococci, which are slightly oblong cells that usually grow in chains (because they divide in one plane only). The catalase test is important in distinguishing streptococci (catalase-negative) from staphylococci, which are vigorous catalase-producers. The test is performed by adding 3% hydrogen peroxide to a colony on an agar plate or slant. Catalase-positive cultures produce O₂ and bubble at once. The test should not be done on blood agar because blood itself contains catalase.

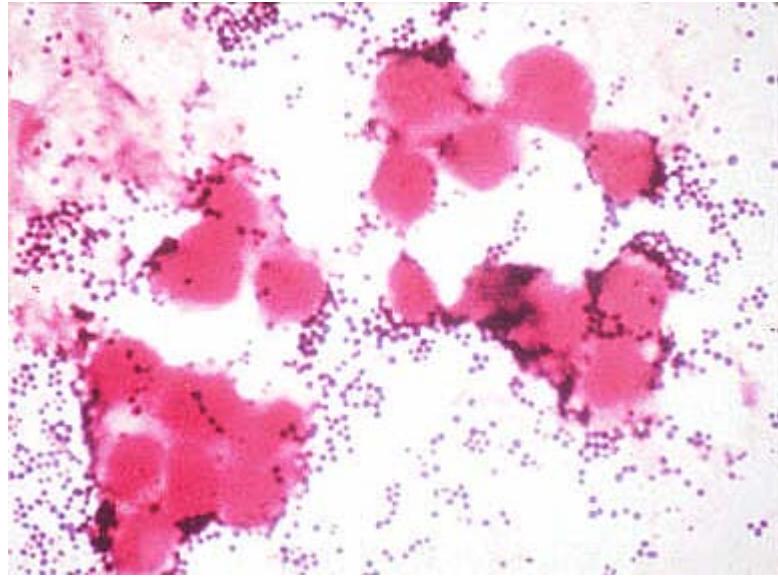


FIGURE 1. Gram stain of *Staphylococcus aureus* in pustular exudate

Table 1. Important phenotypic characteristics of *Staphylococcus aureus*

Gram-positive, cluster-forming coccus
nonmotile, nonsporeforming facultative anaerobe
fermentation of glucose produces mainly lactic acid
ferments mannitol (distinguishes from <i>S. epidermidis</i>)
catalase positive
coagulase positive
golden yellow colony on agar
normal flora of humans found on nasal passages, skin and mucous membranes
pathogen of humans, causes a wide range of suppurative infections, as well as food poisoning and toxic shock syndrome

Pathogenesis of *S. aureus* infections

Staphylococcus aureus causes a variety of suppurative (pus-forming) infections and toxinoses in humans. It causes superficial skin lesions such as **boils**, **stytes** and **furunculosis**; more serious infections such as **pneumonia**, **mastitis**, **phlebitis**, **meningitis**, and **urinary tract infections**; and deep-seated infections, such as **osteomyelitis** and **endocarditis**. *S. aureus* is a major cause of **hospital acquired (nosocomial) infection** of surgical wounds and infections associated with indwelling medical devices. *S. aureus* causes **food poisoning** by releasing enterotoxins into food, and **toxic shock syndrome** by release of superantigens into the blood stream.

S. aureus expresses many potential **virulence factors**: (1) **surface proteins** that promote colonization of host tissues; (2) **invasins** that promote bacterial spread in tissues (**leukocidin**, **kinases**, **hyaluronidase**); (3) **surface factors** that inhibit phagocytic engulfment (**capsule**, **Protein A**); (4) **biochemical properties** that enhance their survival in phagocytes (**carotenoids**, **catalase** production); (5) **immunological disguises** (**Protein A**, **coagulase**, **clotting factor**); and (6) **membrane-damaging toxins** that lyse eukaryotic cell membranes (**hemolysins**, **leukotoxin**, **leukocidin**); (7) **exotoxins** that damage host tissues or otherwise provoke symptoms of disease (**SEA-**

G, TSST, ET (8) inherent and acquired resistance to antimicrobial agents.

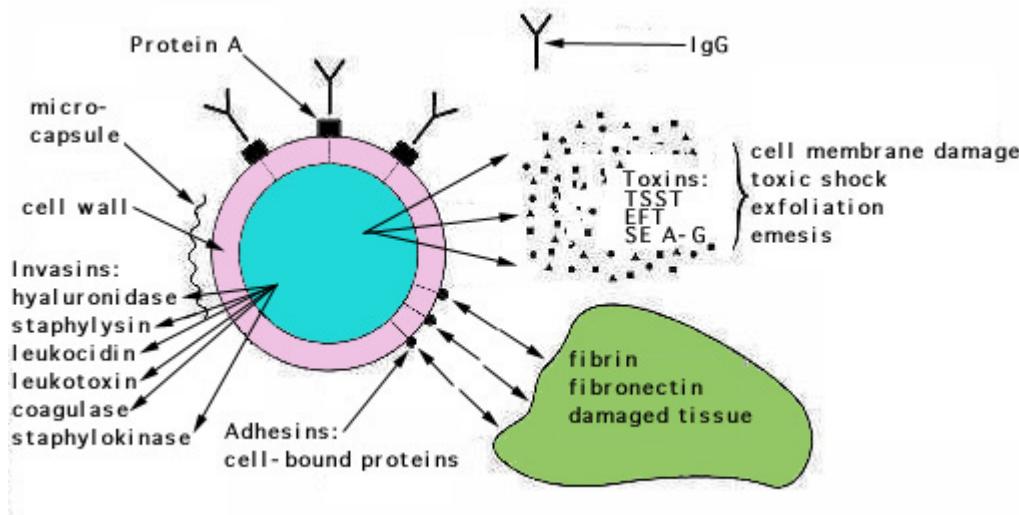


FIGURE 2. Virulence determinants of *Staphylococcus aureus*

For the majority of diseases caused by *S. aureus*, pathogenesis is multifactorial, so it is difficult to determine precisely the role of any given factor. However, there are correlations between strains isolated from particular diseases and expression of particular virulence determinants, which suggests their role in a particular disease. The application of molecular biology has led to advances in unraveling the pathogenesis of staphylococcal diseases. Genes encoding potential virulence factors have been cloned and sequenced, and many protein toxins have been purified. With some staphylococcal toxins, symptoms of a human disease can be reproduced in animals with the purified protein toxins, lending an understanding of their mechanism of action.

Human staphylococcal infections are frequent, but usually remain localized at the portal of entry by the normal host defenses. The portal may be a hair follicle, but usually it is a break in the skin which may be a minute needle-stick or a surgical wound. Foreign bodies, including sutures, are readily colonized by staphylococci, which may make infections difficult to control. Another portal of entry is the respiratory tract. Staphylococcal pneumonia is a frequent complication of influenza. The localized host response to staphylococcal infection is inflammation, characterized by an elevated temperature at the site, swelling, the accumulation of pus, and necrosis of tissue. Around the inflamed area, a fibrin clot may form, walling off the bacteria and leukocytes as a characteristic pus-filled boil or abscess. More serious infections of the skin may occur, such as furuncles or impetigo. Localized infection of the bone is called osteomyelitis. Serious consequences of staphylococcal infections occur when the bacteria invade the blood stream. A resulting septicemia may be rapidly fatal; a bacteremia may result in seeding other internal abscesses, other skin lesions, or infections in the lung, kidney, heart, skeletal muscle or meninges.

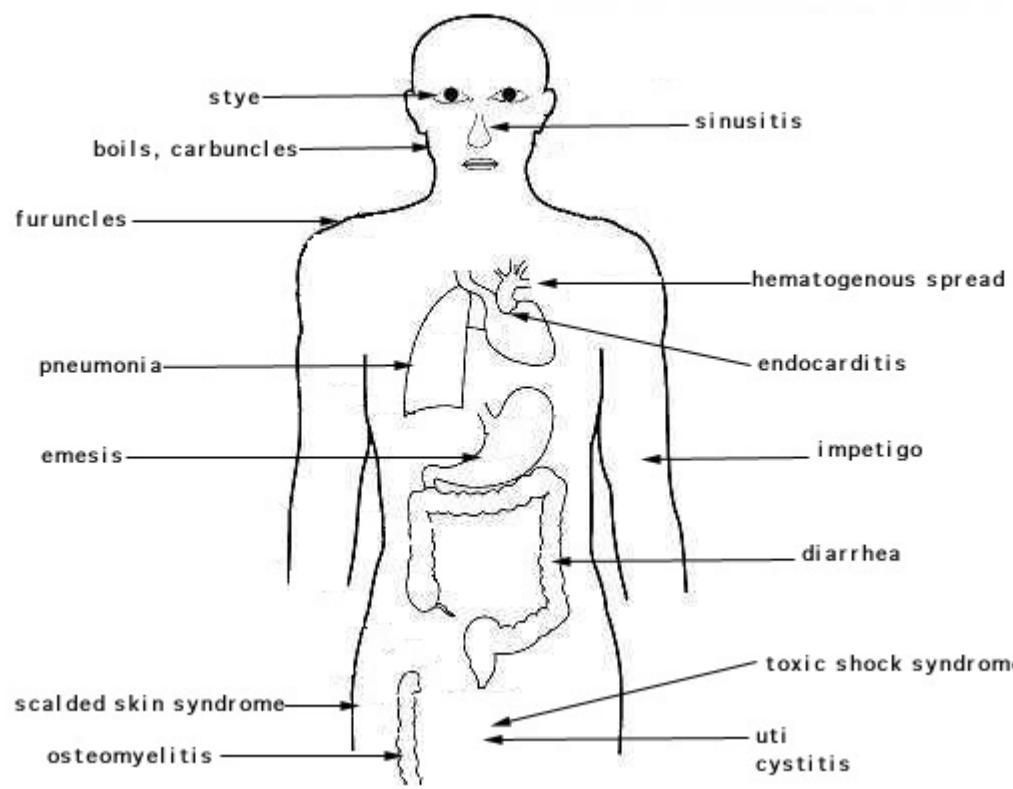


FIGURE 3. Sites of infection and diseases caused by *Staphylococcus aureus*

Adherence to Host Cell Proteins

S. aureus cells express on their **surface proteins** that promote attachment to host proteins such as laminin and fibronectin that form the extracellular matrix of epithelial and endothelial surfaces. In addition, most strains express a fibrin/fibrinogen binding protein (clumping factor) which promotes attachment to blood clots and traumatized tissue. Most strains of *S. aureus* express both fibronectin and fibrinogen-binding proteins. In addition, an adhesin that promotes attachment to collagen has been found in strains that cause osteomyelitis and septic arthritis. Interaction with collagen may also be important in promoting bacterial attachment to damaged tissue where the underlying layers have been exposed.

Evidence that staphylococcal matrix-binding proteins are virulence factors has come from studying defective mutants in adherence assays. Mutants defective in binding to fibronectin and to fibrinogen have reduced virulence in a rat model for endocarditis, and mutants lacking the collagen-binding protein have reduced virulence in a mouse model for septic arthritis, suggesting that bacterial colonization is ineffective. Furthermore, the isolated ligand-binding domain of the fibrinogen, fibronectin and collagen receptors strongly blocks attachment of bacterial cells to the corresponding host proteins.

Invasion

The invasion of host tissues by staphylococci apparently involves the production of a huge array of extracellular proteins, some of which may occur also as cell-associated proteins. These proteins are described below with some possible explanations for their role in invasive process.

Membrane-damaging toxins

a-toxin (a-hemolysin) The best characterized and most potent membrane-damaging toxin of *S. aureus* is a-toxin. It is expressed as a monomer that binds to the membrane of susceptible cells. Subunits then oligomerize to form heptameric rings with a central pore through which cellular contents leak.

In humans, platelets and monocytes are particularly sensitive to a-toxin. Susceptible cells have a specific receptor for a-toxin which allows the toxin to bind causing small pores through which monovalent cations can pass. The

mode of action of alpha hemolysin is likely by osmotic lysis.

β-toxin is a sphingomyelinase which damages membranes rich in this lipid. The classical test for β-toxin is lysis of sheep erythrocytes. The majority of human isolates of *S. aureus* do not express β-toxin. A lysogenic bacteriophage is known to encode the toxin.

d-toxin is a very small peptide toxin produced by most strains of *S. aureus*. It is also produced by *S. epidermidis*. The role of d-toxin in disease is unknown.

Leukocidin is a multicomponent protein toxin produced as separate components which act together to damage membranes. Leukocidin forms a hetero-oligomeric transmembrane pore composed of four LukF and four LukS subunits, thereby forming an octameric pore in the affected membrane. Leukocidin is hemolytic, but less so than alpha hemolysin.

Only 2% of all of *S. aureus* isolates express leukocidin, but nearly 90% of the strains isolated from severe dermonecrotic lesions express this toxin, which suggests that it is an important factor in necrotizing skin infections.

Coagulase and clumping factor

Coagulase is an extracellular protein which binds to prothrombin in the host to form a complex called staphylothrombin. The protease activity characteristic of thrombin is activated in the complex, resulting in the conversion of fibrinogen to fibrin. Coagulase is a traditional marker for identifying *S. aureus* in the clinical microbiology laboratory. However, there is no overwhelming evidence that it is a virulence factor, although it is reasonable to speculate that the bacteria could protect themselves from phagocytic and immune defenses by causing localized clotting.

There is some confusion in the literature concerning coagulase and clumping factor, the fibrinogen-binding determinant on the *S. aureus* cell surface. Partly the confusion results from the fact that a small amount of coagulase is tightly bound on the bacterial cell surface where it can react with prothrombin leading to fibrin clotting. However, genetic studies have shown unequivocally that coagulase and clumping factor are distinct entities. Specific mutants lacking coagulase retain clumping factor activity, while clumping factor mutants express coagulase normally.

Staphylokinase

Many strains of *S. aureus* express a plasminogen activator called staphylokinase. This factor lyses fibrin. The genetic determinant is associated with lysogenic bacteriophages. A complex formed between staphylokinase and plasminogen activates plasmin-like proteolytic activity which causes dissolution of fibrin clots. The mechanism is identical to streptokinase, which is used in medicine to treat patients suffering from coronary thrombosis. As with coagulase, there is no strong evidence that staphylokinase is a virulence factor, although it seems reasonable to imagine that localized fibrinolysis might aid in bacterial spreading.

Other extracellular enzymes

S. aureus can express proteases, a lipase, a deoxyribonuclease (DNase) and a fatty acid modifying enzyme (FAME). The first three probably provide nutrients for the bacteria, and it is unlikely that they have anything but a minor role in pathogenesis. However, the FAME enzyme may be important in abscesses, where it could modify anti-bacterial lipids and prolong bacterial survival.

Avoidance of Host Defenses

S. aureus expresses a number of factors that have the potential to interfere with host defense mechanisms. This includes both structural and soluble elements of the bacterium.

Capsular Polysaccharide

The majority of clinical isolates of *S. aureus* express a surface polysaccharide of either serotype 5 or 8. This has

been called a microcapsule because it can be visualized only by electron microscopy unlike the true capsules of some bacteria which are readily visualized by light microscopy. *S. aureus* strains isolated from infections express high levels of the polysaccharide but rapidly lose the ability when cultured in the laboratory. The function of the capsule in virulence is not entirely clear. Although it does impede phagocytosis in the absence of complement, it also impedes colonization of damaged heart valves, perhaps by masking adhesins.

Protein A

Protein A is a surface protein of *S. aureus* which binds IgG molecules by their Fc region. In serum, the bacteria will bind IgG molecules in the wrong orientation on their surface which disrupts opsonization and phagocytosis. Mutants of *S. aureus* lacking protein A are more efficiently phagocytosed in vitro, and mutants in infection models have diminished virulence.

Leukocidin

S. aureus can express a toxin that specifically acts on polymorphonuclear leukocytes. Phagocytosis is an important defense against staphylococcal infection so leukocidin should be a virulence factor.

Exotoxins

S. aureus can express several different types of protein toxins which are probably responsible for symptoms during infections. Those which damage the membranes of cells were discussed above under **Invasion**. Some will lyse erythrocytes, causing hemolysis, but it is unlikely that hemolysis is a relevant determinant of virulence in vivo. Leukocidin causes membrane damage to leukocytes, but is not hemolytic.

Systemic release of a-toxin causes septic shock, while enterotoxins and TSST-1 are superantigens that may cause toxic shock. Staphylococcal enterotoxins cause emesis (vomiting) when ingested and the bacterium is a leading cause of food poisoning.

The exfoliatin toxin causes the scalded skin syndrome in neonates, which results in widespread blistering and loss of the epidermis. There are two antigenically distinct forms of the toxin, ETA and ETB. The toxins have esterase and protease activity and apparently target a protein which is involved in maintaining the integrity of the epidermis.

Superantigens: enterotoxins and toxic shock syndrome toxin

S. aureus secretes two types of toxin with superantigen activity, **enterotoxins**, of which there are six antigenic types (named SE-A, B, C, D, E and G), and **toxic shock syndrome toxin (TSST-1)**. Enterotoxins cause diarrhea and vomiting when ingested and are responsible for staphylococcal food poisoning. TSST-1 is expressed systemically and is the cause of toxic shock syndrome (TSS). When expressed systemically, enterotoxins can also cause toxic shock syndrome. In fact, enterotoxins B and C cause 50% of non-menstrual cases of TSS. TSST-1 is weakly related to enterotoxins, but it does not have emetic activity. TSST-1 is responsible for 75% of TSS, including all menstrual cases. TSS can occur as a sequel to any staphylococcal infection if an enterotoxin or TSST-1 is released systemically and the host lacks appropriate neutralizing antibodies.

Superantigens stimulate T cells non-specifically without normal antigenic recognition (Figure 4). Up to one in five T cells may be activated, whereas only 1 in 10,000 are stimulated during a usual antigen presentation. Cytokines are released in large amounts, causing the symptoms of TSS. Superantigens bind directly to class II major histocompatibility complexes of antigen-presenting cells outside the conventional antigen-binding groove. This complex recognizes only the V_b element of the T cell receptor. Thus any T cell with the appropriate V_b element can be stimulated, whereas normally, antigen specificity is also required in binding.

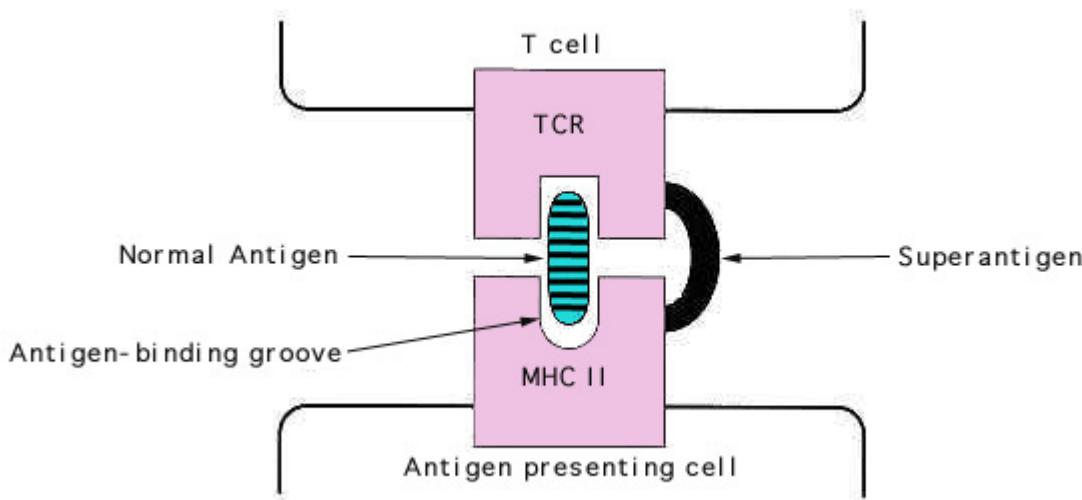


FIGURE 4. Superantigens and the non-specific stimulation of T cells. Superantigens bind directly to class II major histocompatibility complexes (MHC II) of antigen-presenting cells outside the normal antigen-binding groove. Up to one in five T cells may be activated. Cytokines are released in large amounts, causing the symptoms of toxic shock.

Exfoliatin toxin (ET)

The exfoliatin toxin, associated with scalded skin syndrome, causes separation within the epidermis, between the living layers and the superficial dead layers. The separation is through the stratum granulosum of the epidermis. This is probably why healing occurs with little scarring although the risks of fluid loss and secondary infections are increased. Staphylococcal exfoliative toxin B has been shown to specifically cleave desmoglein 1, a cadherin that is found in desmosomes in the epidermis.

Pathogenic *Staphylococcus epidermidis*

In contrast to *S. aureus*, little is known about mechanisms of pathogenesis of *S. epidermidis* infections. Adherence is obviously a crucial step in the initiation of foreign body infections. Bacteria-plastic interactions are probably important in colonization of catheters, and a polysaccharide adhesion (PS/A) has been identified. In addition, when host proteins deposit on the implanted device *S. epidermidis* will bind to fibronectin.

A characteristic of many pathogenic strains of *S. epidermidis* is the production of a slime resulting in biofilm formation. The slime is predominantly a secreted teichoic acid, normally found in the cell wall of the staphylococci. This ability to form a biofilm on the surface of a prosthetic device is probably a significant determinant of virulence for these bacteria.

Resistance of Staphylococci to Antimicrobial Drugs

Hospital strains of *S. aureus* are usually resistant to a variety of different antibiotics. A few strains are resistant to all clinically useful antibiotics except vancomycin, and vancomycin-resistant strains are increasingly-reported. The term **MRSA** refers to **Methicillin resistant *Staphylococcus aureus***. Methicillin resistance is widespread and most methicillin-resistant strains are also multiply resistant. A plasmid associated with vancomycin resistance has been detected in *Enterococcus faecalis* which can be transferred to *S. aureus* in the laboratory, and it is speculated that this transfer may occur naturally (e.g. in the gastrointestinal tract). In addition, *S. aureus* exhibits resistance to antiseptics and disinfectants, such as quaternary ammonium compounds, which may aid its survival in the hospital environment.

Staphylococcal disease has been a perennial problem in the hospital environment since the beginning of the antibiotic era. During the 1950's and early 1960's, staphylococcal infection was synonymous with nosocomial infection. Gram-negative bacilli (e.g. *E. coli* and *Pseudomonas aeruginosa*) have replaced the staphylococci as the most frequent causes of nosocomial infections, although the staphylococci have remained a problem, especially in

surgical wounds.. *S aureus* responded to the introduction of antibiotics by the usual bacterial means to develop drug resistance: (1) mutation in chromosomal genes followed by selection of resistant strains and (2) acquisition of resistance genes as extrachromosomal plasmids, transducing particles, transposons, or other types of DNA inserts. *S. aureus* expresses its resistance to drugs and antibiotics through a variety of mechanisms.

Beginning with the use of the penicillin in the 1940's, drug resistance has developed in the staphylococci within a very short time after introduction of an antibiotic into clinical use. Some strains are now resistant to most conventional antibiotics, and there is concern that new antibiotics have not been forthcoming. New strategies in the pharmaceutical industry to find antimicrobial drugs involve identifying potential molecular targets in cells (such the active sites of enzymes involved in cell division), then developing inhibitors of the specific target molecule. Hopefully, this approach will turn up new antimicrobial agents for the battle against staphylococcal infections. In fact, in the past two years alternatives to vancomycin have been approved with the increase in VRSA (vancomycin resistant *S. aureus*) isolates.

Host Defense against Staphylococcal Infections

Phagocytosis is the major mechanism for combatting staphylococcal infection. Antibodies are produced which neutralize toxins and promote opsonization. However, the bacterial capsule and protein A may interfere with phagocytosis. Biofilm growth on implants is also impervious to phagocytosis. Staphylococci may be difficult to kill after phagocytic engulfment because they produce carotenoids and catalase which neutralize singlet oxygen and superoxide which are primary phagocytic killing mechanisms within the phagolysosome.

Treatment

Hospital acquired infection is often caused by antibiotic resistant strains (MRSA) and can only be treated with vancomycin or an alternative. Until recently, infections acquired outside hospitals have been treated with penicillinase-resistant β -lactams. However, many of the community acquired (CA) Staphylococcal infections are now methicillin resistant. Particularly in Georgia, Texas, and California, the prevalence of CA-MRSA is widespread. Over 60% of abscess isolates from the emergency department of an Austin, Texas hospital yielded MRSA. These organisms are uniformly resistant to penicillins and cephalosporins. The infections have been treated with combination therapy using sulfa drugs and minocycline or rifampin.

Vaccines

No vaccine is yet available that stimulates active immunity against staphylococcal infections in humans. A vaccine based on fibronectin binding protein induces protective immunity against mastitis in cattle and might also be used as a vaccine in humans.

Hyperimmune serum or monoclonal antibodies directed towards surface components (e.g., capsular polysaccharide or surface protein adhesions) could theoretically prevent bacterial adherence and promote phagocytosis by opsonization of bacterial cells. Also, human hyperimmune serum could be given to hospital patients before surgery as a form of passive immunization.

When the precise molecular basis of the interactions between staphylococcal adhesins and host tissue receptors is known it might be possible to design compounds that block the interactions and thus prevent bacterial colonization. These could be administered systemically or topically.

In February, 2002, an experimental bivalent vaccine against *Staphylococcus aureus* was reported to be safe and immunogenic for approximately 40 weeks in patients with end-stage renal disease undergoing hemodialysis. The vaccine called **StaphVAX** is composed of *S. aureus* type 5 and 8 capsular polysaccharides conjugated to nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A. In randomized trials, one injection of the vaccine was administered to 892 hemodialysis patients. Between weeks 3 and 40, 11 cases of *S. aureus* bacteremia were diagnosed in the vaccinated group compared with 26 cases in a control group. Nearly 90% of patients receiving the vaccine generated antibodies to the two capsular polysaccharides. A decrease in vaccine efficacy after week 40 correlated with a decrease in *S. aureus* antibodies. The investigators did not believe that use of StaphVAX would be limited to hemodialysis patients. For example, the vaccine might be used in cases where healthy individuals come into the hospital for elective surgery, such as a joint replacement. Such patients do not require protection for

the rest of their lives; what they need is protection for a short period while they are in the hospital. The vaccine manufacturer will experiment with booster shots to maintain immunity for longer periods of time, and with passive immunization for such at-risk populations as premature infants. They hope to gain FDA approval for the vaccine in 2006.

Table 2. Possible virulence determinants expressed in the pathogenesis of *Staphylococcus aureus* infections

boils and pimples (folliculitis)

Colonization: cell-bound (protein) adhesins

Invasion: **Invasins:** staphylokinase

Other extracellular enzymes (proteases, lipases, nucleases, collagenase, elastase. etc.)

Resistance to phagocytosis: coagulase, leukocidin

Resistance to immune responses: coagulase

Toxigenesis: cytotoxic toxins (hemolysins and leukocidin)

pneumonia

Colonization: cell-bound (protein) adhesins

Invasion:

Invasins: staphylokinase, hyaluronidase

Other extracellular enzymes (proteases, lipases, nucleases, collagenase, elastase. etc.)

Resistance to phagocytosis: coagulase, leukocidin, hemolysins, carotenoids, superoxide dismutase, catalase, growth at low pH

Resistance to immune responses: coagulase, antigenic variation

Toxigenesis: Cytotoxic toxins (hemolysins and leukocidin)

food poisoning (emesis or vomiting)

Toxigenesis: Enterotoxins A-G

septicemia (invasion of the bloodstream)

Invasion:

Invasins: staphylokinase, hyaluronidase

Other extracellular enzymes (proteases, lipases, nucleases, collagenase, elastase. etc.)

Resistance to phagocytosis: coagulase, protein A, leukocidin, hemolysins, carotenoids, superoxide dismutase, catalase, growth at low pH

Resistance to immune responses: coagulase, protein A, antigenic variation

Toxigenesis: cytotoxic toxins (hemolysins and leukocidin)

osteomyelitis (invasion of bone)

Colonization: cell-bound (protein) adhesins

Invasion:

Invasins: staphylokinase, hyaluronidase

Other extracellular enzymes (proteases, lipases, nucleases, collagenase, elastase. etc.)

Resistance to phagocytosis: coagulase, protein A, leukocidin, hemolysins, carotenoids, superoxide dismutase, catalase, growth at low pH

Resistance to immune responses: coagulase, protein A, antigenic variation

Toxigenesis: cytotoxic toxins (hemolysins and leukocidin)

toxic shock syndrome

Colonization: cell-bound (protein) adhesins

Resistance to immune responses: coagulase, antigenic variation

Toxigenesis: TSST toxin, Enterotoxins A-G

surgical wound infections

Colonization: cell-bound (protein) adhesins

Invasion:

Invasins: staphylokinase, hyaluronidase

Other extracellular enzymes (proteases, lipases, nucleases, collagenase, elastase. etc.)

Resistance to phagocytosis: coagulase, protein A, leukocidin, hemolysins, carotenoids, superoxide dismutase,

catalase, growth at low pH

Resistance to immune responses: coagulase, protein A, antigenic variation

Toxigenesis: cytotoxic toxins (hemolysins and leukocidin)

scalded skin syndrome (analogous to scarlet fever)

Colonization: cell-bound (protein) adhesins

Invasion:

Invasins: staphylokinase, hyaluronidase

Other extracellular enzymes (proteases, lipases, nucleases, collagenase, elastase. etc.)

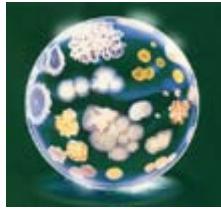
Resistance to phagocytosis: coagulase, leukocidin, hemolysins

Resistance to immune responses: coagulase, antigenic variation

Toxigenesis: Exfoliatin toxin

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Streptococcus pyogenes

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Introduction

Streptococcus pyogenes (**Group A streptococcus**) is a Gram-positive, nonmotile, nonsporeforming coccus that occurs in chains or in pairs of cells. Individual cells are round-to-ovoid cocci, 0.6-1.0 micrometer in diameter (Figure 1). Streptococci divide in one plane and thus occur in pairs or (especially in liquid media or clinical material) in chains of varying lengths. The metabolism of *S. pyogenes* is fermentative; the organism is a catalase-negative aerotolerant anaerobe (facultative anaerobe), and requires enriched medium containing blood in order to grow. Group A streptococci typically have a capsule composed of hyaluronic acid and exhibit beta (clear) hemolysis on blood agar.

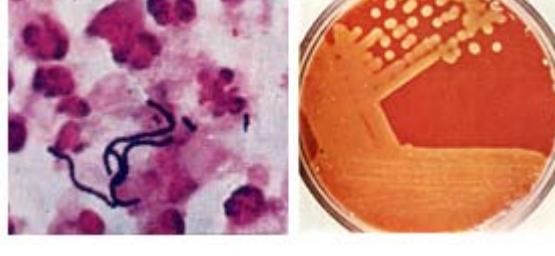


Figure 1. *Streptococcus pyogenes*. Left. Gram stain of *Streptococcus pyogenes* in a clinical specimen. Right. Colonies of *Streptococcus pyogenes* on blood agar exhibiting beta (clear) hemolysis.

Streptococcus pyogenes is one of the most frequent pathogens of humans. It is estimated that between 5-15% of normal individuals harbor the bacterium, usually in the respiratory tract, without signs of disease. As normal flora, *S. pyogenes* can infect when defenses are compromised or when the organisms are able to penetrate the constitutive defenses. When the bacteria are introduced or transmitted to vulnerable tissues, a variety of types of **suppurative infections** can occur.

In the last century, infections by *S. pyogenes* claimed many lives especially since the organism was the most important cause of **puerperal fever** (sepsis after childbirth). **Scarlet fever** was formerly a severe complication of streptococcal infection, but now, because of antibiotic therapy, it is little more than streptococcal **pharyngitis** accompanied by rash. Similarly, **erysipelas** (a form of cellulitis accompanied by fever and systemic toxicity) is less common today. However, there has been a recent increase in variety, severity and **sequelae** of *Streptococcus pyogenes* infections, and a resurgence of **severe invasive infections**, prompting descriptions of "flesh eating bacteria" in the news media. A complete explanation for the decline and resurgence is not known. Today, the pathogen is of major concern because of the occasional cases of rapidly progressive disease and because of the small risk of serious sequelae in untreated infections. These diseases remain a major worldwide health concern, and effort is being directed toward clarifying the risk and mechanisms of these sequelae and identifying rheumatogenic and nephritogenic strains of streptococci.

Acute *Streptococcus pyogenes* infections may present as **pharyngitis** (**strep throat**), **scarlet fever** (rash), **impetigo** (infection of the superficial layers of the skin) or **cellulitis** (infection of the deep layers of the skin). Invasive, toxicogenic infections can result in **necrotizing fasciitis**, **myositis** and **streptococcal toxic shock syndrome**. Patients may also develop immune-mediated **post-streptococcal sequelae**, such as acute **rheumatic fever** and acute

glomerulonephritis, following acute infections caused by *Streptococcus pyogenes*.

Streptococcus pyogenes produces a wide array of **virulence factors** and a very large number of diseases. Virulence factors of Group A streptococci include: (1) **M protein**, fibronectin-binding protein (**Protein F**) and **lipoteichoic acid** for adherence; (2) **hyaluronic acid capsule** as an immunological disguise and to inhibit phagocytosis; **M-protein** to inhibit phagocytosis (3) **invasins** such as **streptokinase**, **streptodornase** (DNase B), **hyaluronidase**, and **streptolysins**; (4) exotoxins, such as **pyrogenic (erythrogenic) toxin** which causes the rash of **scarlet fever** and systemic **toxic shock syndrome**.

Classification of Streptococci

Hemolysis on blood agar

The type of hemolytic reaction displayed on blood agar has long been used to classify the streptococci. **Beta-hemolysis** is associated with complete lysis of red cells surrounding the colony, whereas **alpha-hemolysis** is a partial or "green" hemolysis associated with reduction of red cell hemoglobin. Nonhemolytic colonies have been termed gamma-hemolytic. Hemolysis is affected by the species and age of red cells, as well as by other properties of the base medium. **Group A streptococci are nearly always beta-hemolytic**; related Group B can manifest alpha, beta or gamma hemolysis. Most strains of *S. pneumoniae* are alpha-hemolytic but can cause β-hemolysis during anaerobic incubation. Most of the oral streptococci and enterococci are non hemolytic. The property of hemolysis is not very reliable for the absolute identification of streptococci, but it is widely used in rapid screens for identification of *S. pyogenes* and *S. pneumoniae*.

Antigenic types

The cell surface structure of Group A streptococci is among the most studied of any bacteria (Figure 2). The cell wall is composed of repeating units of N-acetylglucosamine and N-acetylmuramic acid, the standard peptidoglycan. Historically, the definitive identification of streptococci has rested on the serologic reactivity of "cell wall" polysaccharide antigens as originally described by Rebecca Lancefield. **Eighteen group-specific antigens (Lancefield groups) were established**. The Group A polysaccharide is a polymer of N-acetylglucosamine and rhamnose. Some group antigens are shared by more than one species. This polysaccharide is also called the **C substance** or **group carbohydrate antigen**.

Pathogenesis

Streptococcus pyogenes owes its major success as a pathogen to its ability to colonize and rapidly multiply and spread in its host while evading phagocytosis and confusing the immune system.

Acute diseases associated with *Streptococcus pyogenes* occur chiefly in the **respiratory tract, bloodstream**, or the **skin**. Streptococcal disease is most often a respiratory infection (pharyngitis or tonsillitis) or a skin infection (pyoderma). Some strains of streptococci show a predilection for the respiratory tract; others, for the skin. Generally, streptococcal isolates from the pharynx and respiratory tract do not cause skin infections. Figure 3 describes the pathogenesis of *S. pyogenes* infections.

S. pyogenes is the leading cause of uncomplicated bacterial **pharyngitis** and **tonsillitis** commonly referred to a **strep throat**. Other respiratory infections include **sinusitis**, **otitis**, and **pneumonia**.

Infections of the skin can be superficial (**impetigo**) or deep (**cellulitis**). Invasive streptococci cause **joint or bone infections**, destructive **wound infections** (**necrotizing fasciitis**) and **myositis**, **meningitis** and **endocarditis**. Two **post streptococcal sequelae**, **rheumatic fever** and **glomerulonephritis**, may follow streptococcal disease, and occur in 1-3% of untreated infections. These conditions and their pathology are not attributable to dissemination of bacteria, but to aberrant immunological reactions to Group A streptococcal antigens. **Scarlet fever** and **streptococcal toxic shock syndrome** are systemic responses to circulating bacterial toxins.

The **cell surface** of *Streptococcus pyogenes* accounts for many of the bacterium's determinants of virulence, especially those concerned with colonization and evasion of phagocytosis and the host immune responses. The surface of *Streptococcus pyogenes* is incredibly complex and chemically-diverse. Antigenic components include

capsular polysaccharide (C-substance), cell wall **peptidoglycan** and **lipoteichoic acid (LTA)**, and a variety of surface proteins, including **M protein**, **fimbrial proteins**, **fibronectin-binding proteins**, (e.g. **Protein F**) and cell-bound **streptokinase**.

The cytoplasmic membrane of *S. pyogenes* contains some antigens similar to those of human cardiac, skeletal, and smooth muscle, heart valve fibroblasts, and neuronal tissues, resulting in **molecular mimicry** and a tolerant or suppressed immune response by the host.

The cell envelope of a Group A streptococcus is illustrated in Figure 2. The complexity of the surface can be seen in several of the electron micrographs of the bacterium that accompany this article.

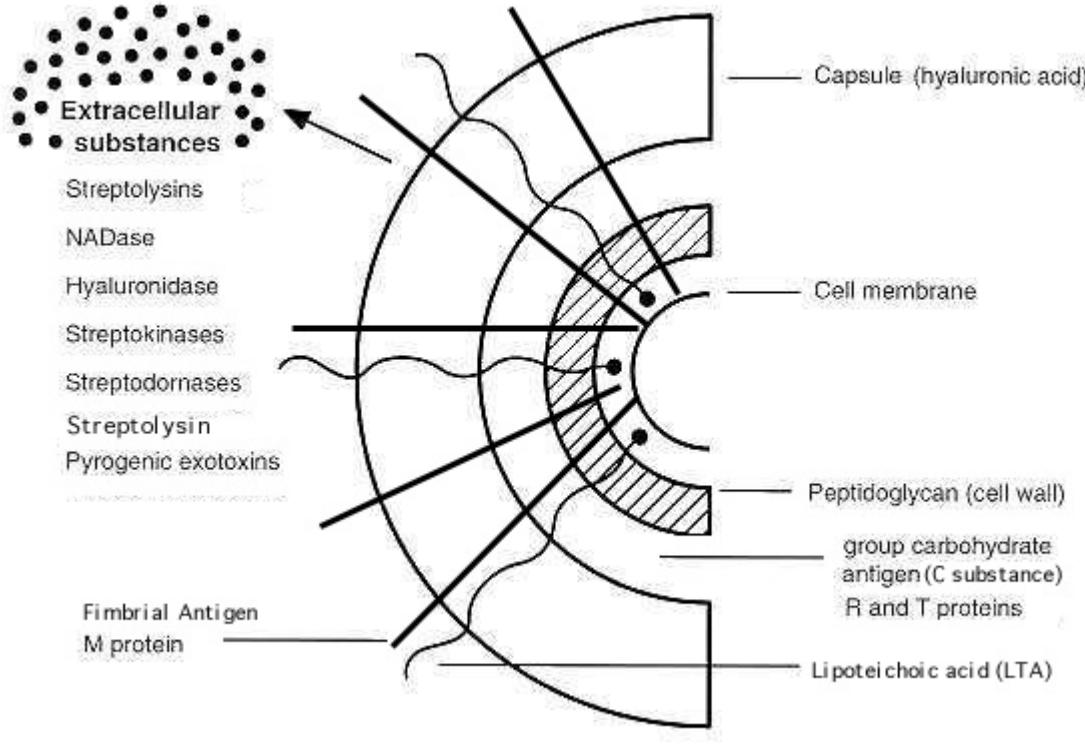


Figure 2. Cell surface structure of *Streptococcus pyogenes* and secreted products involved in virulence.

In Group A streptococci, the **R** and **T** proteins are used as epidemiologic markers and have no known role in virulence. The group carbohydrate antigen (composed of N-acetylglucosamine and rhamnose) has been thought to have no role in virulence, but emerging strains with increased invasive capacity produce a very mucoid colony, suggesting a role of the capsule in virulence.

The **M proteins** are clearly virulence factors associated with both colonization and resistance to phagocytosis. More than 50 types of *S. pyogenes* M proteins have been identified on the basis of antigenic specificity, and it is the M protein that is the major cause of antigenic shift and antigenic drift in the Group A streptococci. The M protein (found in fimbriae) also binds fibrinogen from serum and blocks the binding of complement to the underlying peptidoglycan. This allows survival of the organism by inhibiting phagocytosis.

The streptococcal M protein, as well as peptidoglycan, N-acetylglucosamine, and group-specific carbohydrate, contain antigenic epitopes that mimic those of mammalian muscle and connective tissue. As mentioned above, the cell surface of recently emerging strains of streptococci is distinctly mucoid (indicating that they are highly encapsulated). These strains are also rich in surface M protein. The M proteins of certain M-types are considered **rheumatogenic** since they contain antigenic epitopes related to heart muscle, and they therefore may lead to autoimmune rheumatic carditis (rheumatic fever) following an acute infection.

The Hyaluronic Acid Capsule

The **capsule** of *S. pyogenes* is non antigenic since it is composed of **hyaluronic acid**, which is chemically similar

to that of host connective tissue. This allows the bacterium to hide its own antigens and to go unrecognized as antigenic by its host. The Hyaluronic acid capsule also prevents opsonized phagocytosis by neutrophils or macrophages.

Adhesins

Colonization of tissues by *S. pyogenes* is thought to result from a failure in the constitutive defenses (normal flora and other nonspecific defense mechanisms) which allows establishment of the bacterium at a portal of entry (often the upper respiratory tract or the skin) where the organism multiplies and causes an inflammatory purulent lesion.

It is now realized that *S. pyogenes* (like many other bacterial pathogens) produces multiple adhesins with varied specificities. There is evidence that *Streptococcus pyogenes* utilizes **lipoteichoic acids (LTA)**, **M protein**, and multiple **fibronectin-binding proteins** in its repertoire of adhesins. LTA is anchored to proteins on the bacterial surface, including the M protein. Both the M proteins and lipoteichoic acid are supported externally to the cell wall on fimbriae and appear to mediate bacterial adherence to host epithelial cells. The fibronectin-binding protein, **Protein F**, has also been shown to mediate streptococcal adherence to the amino terminus of fibronectin on mucosal surfaces.

Identification of *Streptococcus pyogenes* adhesins has long been a subject of conflict and debate. Most of the debate was between proponents of the LTA model and those of the M protein model. In 1972, Gibbons and his colleagues proposed that attachment of streptococci to the oral mucosa of mice is dependent on M protein. However, Olfek and Beachey argued that lipoteichoic acid (LTA), rather than M protein, was responsible for streptococcal adherence to buccal epithelial cells. In 1996, Hasty and Courtney proposed a two-step model of attachment that involved both M protein and teichoic acids. They suggested that LTA loosely tethers streptococci to epithelial cells, and then M protein and/or other fibronectin (Fn)-binding proteins secure a firmer, irreversible association. The first streptococcal fibronectin-binding protein (Sfb) was demonstrated in 1992. Shortly thereafter, protein F was discovered. Most recently (1998), the M1 and M3 proteins were shown to bind fibronectin.

Extracellular products: invasins and exotoxins

Colonization of the upper respiratory tract and acute pharyngitis may spread to other portions of the upper or lower respiratory tracts resulting in infections of the middle ear (otitis media), sinuses (sinusitis), or lungs (pneumonia). In addition, meningitis can occur by direct extension of infection from the middle ear or sinuses to the meninges or by way of bloodstream invasion from the pulmonary focus. Bacteremia can also result in infection of bones (osteomyelitis) or joints (arthritis). During these aspects of acute disease the streptococci bring into play a variety of secretory proteins that mediate their invasion.

For the most part, streptococcal invasins and protein toxins interact with mammalian blood and tissue components in ways that kill host cells and provoke a damaging inflammatory response. The soluble extracellular growth products and toxins of *Streptococcus pyogenes* (see Figure 2, above), have been studied intensely. **Streptolysin S** is an oxygen-stable leukocidin; **Streptolysin O** is an oxygen-labile leukocidin. NADase is also leukotoxic. **Hyaluronidase** (the original "spreading factor") can digest host connective tissue hyaluronic acid, as well as the organism's own capsule. **Streptokinases** participate in fibrin lysis. **Streptodornases A-D** possess deoxyribonuclease activity; Streptodornases B and D possess ribonuclease activity as well. **Protease** activity similar to that in *Staphylococcus aureus* has been shown in strains causing soft tissue necrosis or toxic shock syndrome. This large repertoire of products is important in the pathogenesis of *S. pyogenes* infections. Even so, antibodies to these products are relatively insignificant in protection of the host.

The streptococcal invasins act in a variety of ways summarized in Table 1 at the end of this article. Streptococcal invasins lyse eukaryotic cells, including red blood cells and phagocytes; they lyse other host macromolecules, including enzymes and informational molecules; they allow the bacteria to spread among tissues by dissolving host fibrin and intercellular ground substances.

Pyrogenic Exotoxins

Three **streptococcal pyrogenic exotoxins (SPE)**, formerly known as **Erythrogenic toxin**, are recognized: types A, B, C. These toxins act as **superantigens** by a mechanism similar to those described for staphylococci. As antigens, they do not require processing by antigen presenting cells. Rather, they stimulate T cells by binding class II MHC

molecules directly and nonspecifically. With superantigens about 20% of T cells may be stimulated (vs 1/10,000 T cells stimulated by conventional antigens) resulting in massive detrimental cytokine release. SPE A and SPE C are encoded by lysogenic phages; the gene for SPE B is located on the bacterial chromosome.

The erythrogenic toxin is so-named for its association with scarlet fever which occurs when the toxin is disseminated in the blood. Re-emergence in the late 1980's of exotoxin-producing strains of *S. pyogenes* has been associated with a **toxic shock-like syndrome** similar in pathogenesis and manifestation to staphylococcal toxic shock syndrome, and with other forms of invasive disease associated with severe tissue destruction. The latter condition is termed **necrotizing fasciitis**. Outbreaks of sepsis, toxic shock and necrotizing fasciitis have been reported at increasing frequency. The destructive nature of wound infections prompted the popular press to refer to *S. pyogenes* as "flesh-eating bacteria" and "ski-eating streptococci". The increase in invasive streptococcal disease was associated with emergence of a highly virulent serotype M1 which is disseminated world-wide. The M1 strain produces the erythrogenic toxin (Spe A), thought to be responsible for toxic shock, and the enzyme cysteine protease which is involved in tissue destruction. Because clusters of toxic shock were also associated with other serotypes, particularly M3 strains, it is believed that unidentified host factors may also have played an important role in the resurgence of these dangerous infections.

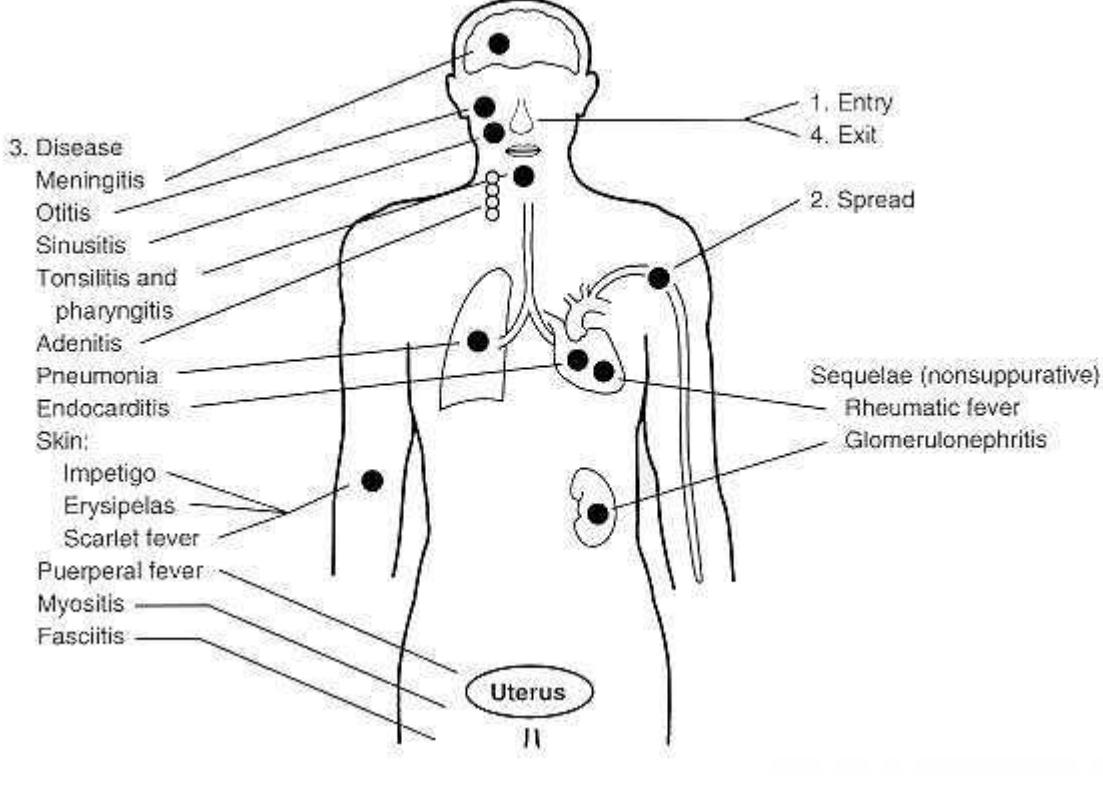


FIGURE 3. Pathogenesis of *Streptococcus pyogenes* infections. Adapted from Baron's Medical Microbiology Chapter 13, *Streptococcus* by Maria Jevitz Patterson.

Post streptococcal sequelae

Infection with *Streptococcus pyogenes* can give rise to serious **nonsuppurative sequelae**: acute **rheumatic fever** and acute **glomerulonephritis**. These pathological events begin 1-3 weeks after an acute streptococcal illness, a latent period consistent with an immune-mediated etiology. Whether all *S. pyogenes* strains are rheumatogenic is controversial; however, clearly not all strains are nephritogenic.

Acute **rheumatic fever** is a sequel only of pharyngeal infections, but acute **glomerulonephritis** can follow infections of the pharynx or the skin. Although there is no adequate explanation for the precise pathogenesis of acute rheumatic fever, an abnormal or enhanced immune response seems essential. Also, persistence of the organism on pharyngeal tissues (i.e., the tonsils) is associated with an increased likelihood of rheumatic fever. Acute rheumatic fever can result in permanent damage to the heart valves. Less than 1% of sporadic streptococcal pharyngitis infections result in acute rheumatic fever; however, recurrences are common, and life-long antibiotic prophylaxis is recommended following a single case.

The occurrence of cross-reactive antigens in *S. pyogenes* and heart tissues possibly explains the autoimmune responses that develop following some infections. The antibody mediated immune (AMI) response (i.e., level of serum antibody) is higher in patients with rheumatic fever than in patients with uncomplicated pharyngitis. In addition, cell-mediated immunity (CMI) seems to play a role in the pathology of acute rheumatic fever.

Acute glomerulonephritis results from deposition of antigen-antibody-complement complexes on the basement membrane of kidney glomeruli. The antigen may be streptococcal in origin or it may be a host tissue species with antigenic determinants similar to those of streptococcal antigen (cross-reactive epitopes for endocardium, sarcolemma, vascular smooth muscle). The incidence of acute glomerulonephritis in the United States is variable, perhaps due to cycling of nephritogenic strains, but it appears to be decreasing. Recurrences are uncommon, and prophylaxis following an initial attack is unnecessary.

Host defenses

S. pyogenes is usually an **exogenous secondary invader**, following viral disease or disturbances in the normal bacterial flora. In the normal human the skin is an effective barrier against invasive streptococci, and nonspecific defense mechanisms prevent the bacteria from penetrating beyond the superficial epithelium of the upper respiratory tract. These mechanisms include mucociliary movement, coughing, sneezing and epiglottal reflexes.

The **host phagocytic system** is a second line of defense against streptococcal invasion. Organisms can be opsonized by activation of the classical or alternate complement pathway and by anti-streptococcal antibodies in the serum. *S. pyogenes* is rapidly killed following phagocytosis enhanced by specific antibody. The bacteria do not produce catalase or significant amounts of superoxide dismutase to inactivate the oxygen metabolites (hydrogen peroxide, superoxide) produced by the oxygen-dependent mechanisms of the phagocyte. Therefore, they are quickly killed after engulfment by phagocytes. The streptococcal defense must be one to stay out of phagocytes.

In immune individuals, IgG antibodies reactive with M protein promote phagocytosis which results in killing of the organism. This is the major mechanism by which AMI is able to terminate Group A streptococcal infections. **M protein vaccines** are a major candidate for use against rheumatic fever, but certain M protein types cross-react antigenically with the heart and themselves may be responsible for rheumatic carditis. This risk of autoimmunity has prevented the use of Group A streptococcal vaccines. However, since the cross-reactive epitopes of the M-protein are now known, it appears that limited anti-streptococcal vaccines are on the horizon.

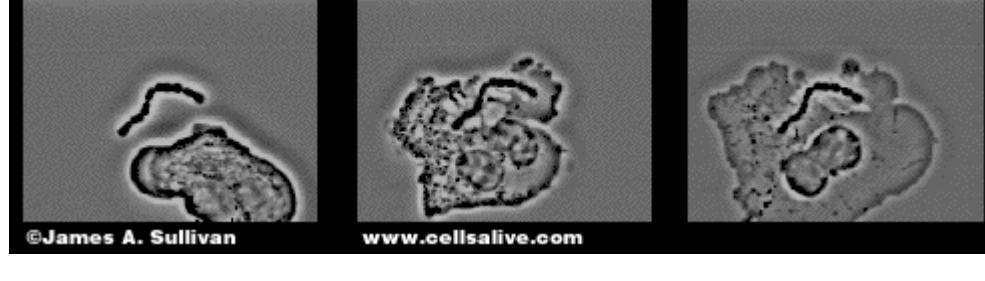


FIGURE 4. Phagocytosis of *Streptococcus pyogenes* by a macrophage. CELLS alive!

The hyaluronic acid capsule allows the organism to evade opsonization. The capsule is also an antigenic disguise that hides bacterial antigens and is non antigenic to the host. Actually, the hyaluronic acid outer surface of *S. pyogenes* is weakly antigenic, but it does not result in stimulation of protective immunity. The only protective immunity that results from infection by Group A streptococcus comes from the development of type-specific antibody to the M protein of the fimbriae, which protrude from the cell wall through the capsular structure. This antibody, which follows respiratory and skin infections, is persistent. Presumably, protective levels of specific IgA is produced in the respiratory secretions while protective levels of IgG are formed in the serum. Sometimes, intervention of an infection with effective antibiotic treatment precludes the development of this persistent antibody. This accounts, in part, for recurring infections in an individual by the same streptococcal strain. Antibody to the erythrogenic toxin involved in scarlet fever is also long lasting.

Treatment and prevention

Penicillin is still uniformly effective in treatment of Group A streptococcal disease. It is important to identify and treat Group A streptococcal infections in order to prevent sequelae. No effective vaccine has been produced, but specific M-protein vaccines are being tested.

Table 1. Summary of virulence determinants of *Streptococcus pyogenes*

Adherence (colonization) surface macromolecules

M protein

Lipoteichoic acid (LTA)

Protein F and Sfb (fibronectin-binding proteins)

Enhancement of spread in tissues

Hyaluronidase hydrolyses hyaluronic acid, part of the ground substance in host tissues.

Proteases

Streptokinase lyses fibrin

Evasion of phagocytosis

Capsule: hyaluronic acid is produced.

C5a peptidase: C5a enhances chemotaxis of phagocytes .

M protein is a fibrillar surface protein. Its distal end bears a negative charge that interferes with phagocytosis. It also blocks complement deposition on the cell surface. Mutations during the course of infection alter the structure of M proteins, rendering some antibodies ineffective. Strains that persist in carriers frequently exhibit altered M proteins.

Leukocidins, including streptolysin S and streptolysin O, are proteins secreted by the streptococci to kill phagocytes (and probably to release nutrients for their growth)

Defense against host immune responses

Antigenic disguise and tolerance provided by hyaluronic acid capsule

Antigenic variation. Antibody against M protein (antigen) is the only effective protective antibody, but there are more than 50 different M types, and subsequent infections may occur with a different M serotype.

Production of toxins and other systemic effects

Toxic shock: Exotoxin is superantigen that binds directly to MHC II (without being processed) and binds abnormally to the T cell receptor of many (up to 20% of) T cells. Exaggerated production of cytokines causes the signs of shock: fever, rash, low blood pressure. aberrant interaction between toxin, macrophage, and T cells.

Induction of circulating, cross-reactive antibodies

Some of the antibodies produced during infection by certain strains of streptococci cross-react with certain host tissues. These antibodies can indirectly damage host tissues, even after the organisms have been cleared, and cause autoimmune complications.

Table 2. Summary of diseases caused by *Streptococcus pyogenes*

Suppurative conditions (active infections associated with pus) occur in the throat, skin, and systemically.

Throat

Streptococcal pharyngitis is acquired by inhaling aerosols emitted by infected individuals. The symptoms reflect the

inflammatory events at the site of infection. A few (1-3%) people develop rheumatic fever weeks after the infection has cleared.

Skin

Impetigo involves the infection of epidermal layers of skin. Pre-pubertal children are the most susceptible. Cellulitis occurs when the infection spreads subcutaneous tissues. Erysipelas is the infection of the dermis. About 5% of patients will develop more disseminated disease. Necrotizing fasciitis involves infection of the fascia and may proceed rapidly to underlying muscle.

Systemic

Scarlet fever is caused by production of erythrogenic toxin by a few strains of the organism.

Toxic shock is caused by a few strains that produce a toxic shock-like toxin.

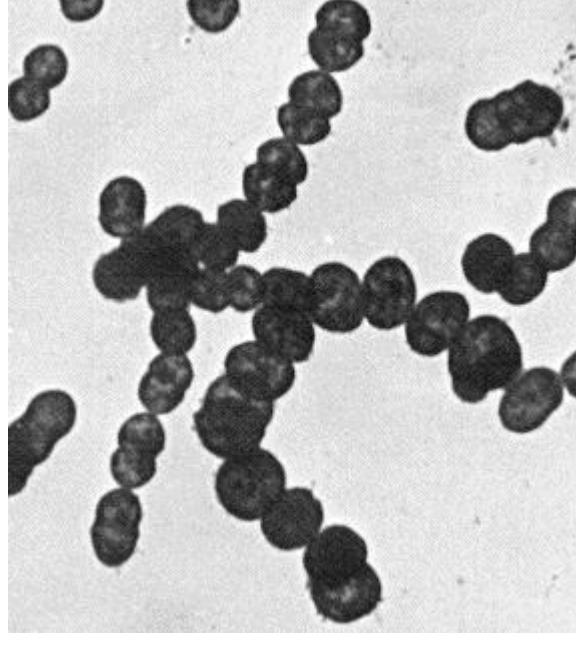
Non-suppurative Sequelae

Some of the antibodies produced during the above infections cross-react with certain host tissues. These can indirectly damage host tissues, even after the organisms have been cleared, and cause non suppurative complications.

Rheumatic fever. M protein cross reacts with sarcolemma. Antibodies cross-react with heart tissue, fix complement, and cause damage.

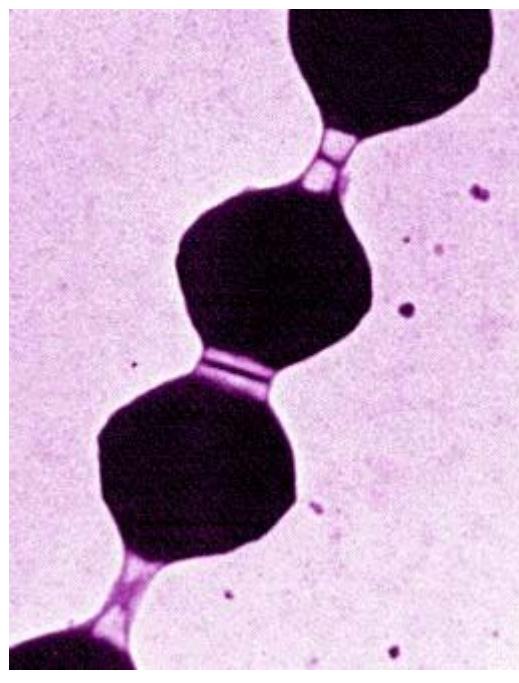
Glomerulonephritis. Antigen-antibody complexes may be deposited in kidney, fix complement, and damage glomeruli. Only a few M-types are nephritogenic.

Gallery of electron micrographs of *Streptococcus pyogenes* from [The Laboratory of Pathogenesis and Immunology](#) at Rockefeller University, the home of research on *Streptococcus pyogenes*



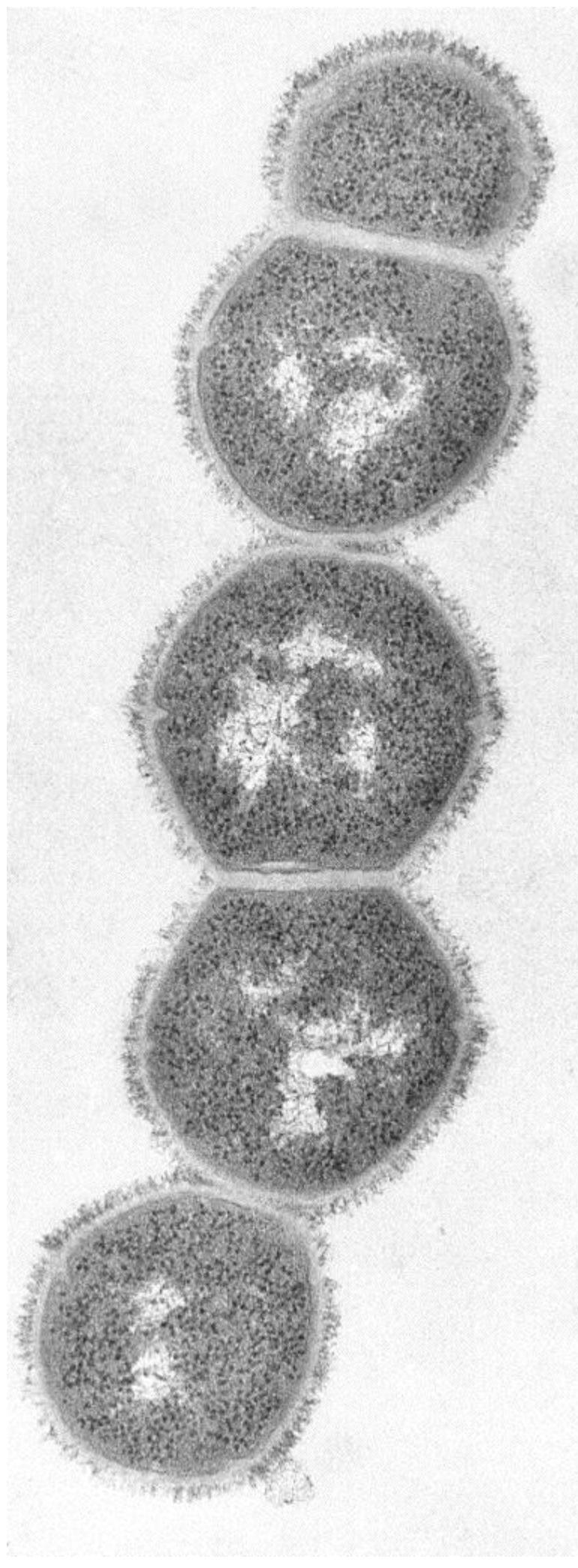
Critical point dried whole group A streptococci (*Streptococcus pyogenes*) viewed directly by transmission electron microscopy (TEM 6,500X). Chains of streptococci are clearly evident. To remove cell surface proteins, cells were treated with trypsin prior to preparation and mounting. Strain: D471; M-type 6. Electron micrograph of *Streptococcus pyogenes* by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology](#), Rockefeller University.

Streptococcus

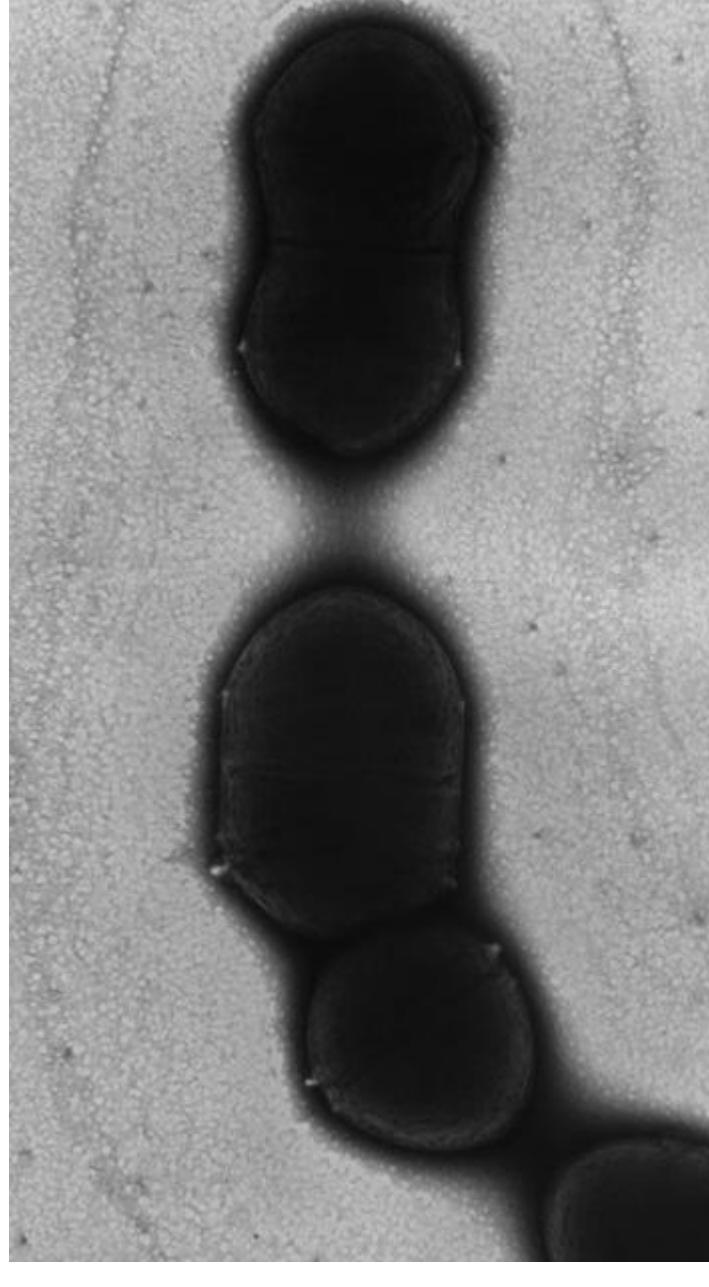


Dividing streptococci (12,000X). Electron micrograph of *Streptococcus pyogenes* by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology](#), Rockefeller University.

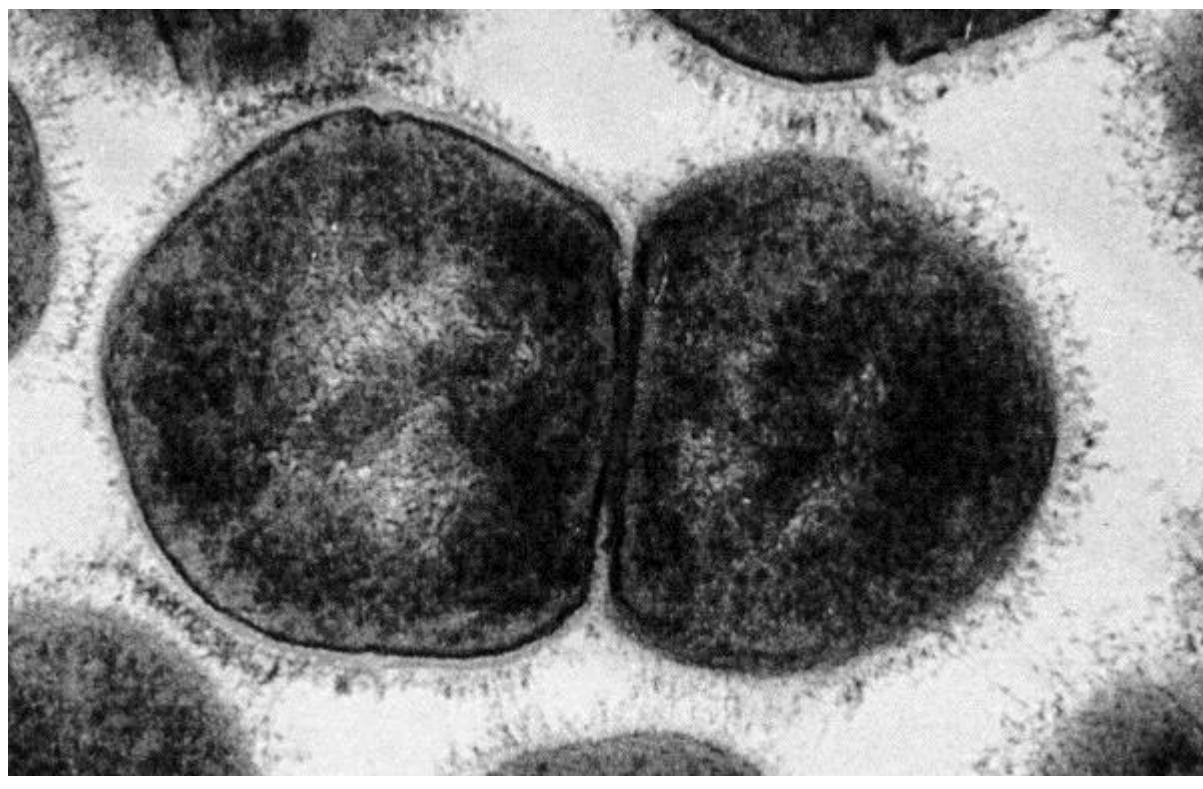
Streptococcus



Electron micrograph of an ultra-thin section of a chain of group A streptococci (20,000X). The cell surface fibrils, consisting primarily of M protein, are clearly 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. Incipient cell division is also indicated by the nascent septum formation (seen as an indentation of the cell wall) near the cell equator. The streptococcal cell diameter is equal to approximately one micron. Electron micrograph of *Streptococcus pyogenes* by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology](#), Rockefeller University.



Negative staining of group A streptococci viewed by TEM 28,000X. The "halo" around the chain of cells (approximately equal in thickness to the cell diameter) is the remnants of the capsule that may be found surrounding the exterior of certain strains of group A streptococci. The septa between pairs of dividing cells may also be seen. Electron micrograph of *Streptococcus pyogenes* by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology](#), Rockefeller University.

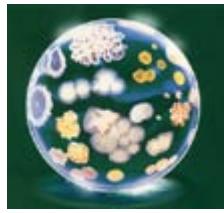


High magnification electron micrograph of an ultra-thin section of a group A streptococcus sibling pair (70,000 X). At this magnification, especially in the cell on the left, the cell wall and cell surface fibrils, consisting primarily of M protein, are well defined. Interdigitation of these fibrils between neighboring cells of different chains is also in plain view. Strain: C126/21/1; M-type 43. Electron micrograph of *Streptococcus pyogenes* by Maria Fazio and Vincent A. Fischetti, Ph.D. with permission. [The Laboratory of Bacterial Pathogenesis and Immunology](#), Rockefeller University.

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Streptococcus pneumoniae: Pneumococcal pneumonia

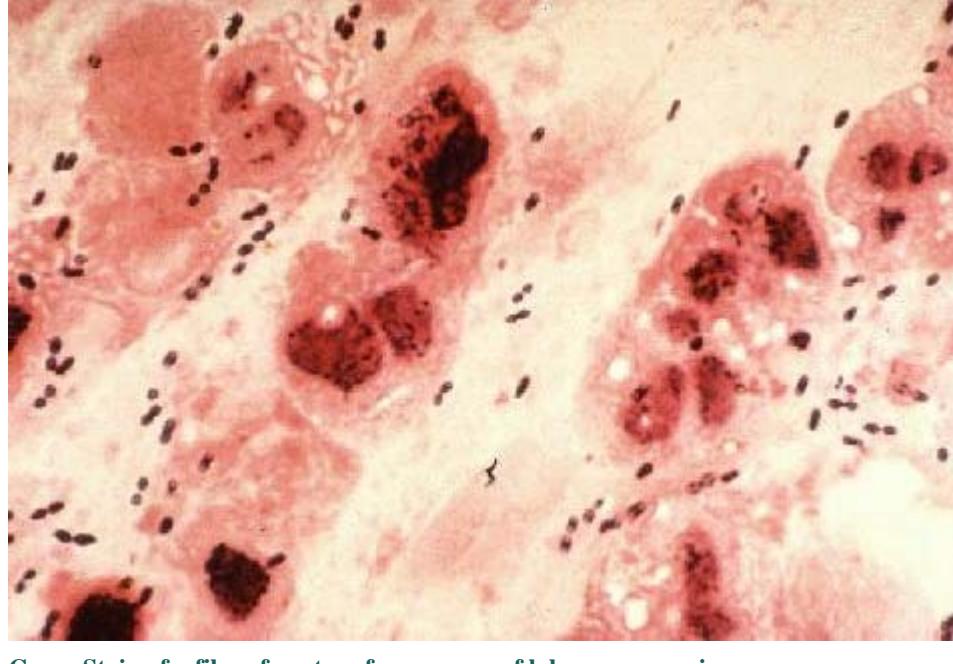
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Introduction

Pneumonia is a disease of the lung that is caused by a variety of bacteria including *Streptococcus*, *Staphylococcus*, *Pseudomonas*, *Haemophilus*, *Chlamydia* and *Mycoplasma*, several viruses, and certain fungi and protozoans. The disease may be divided into two forms, bronchial pneumonia and lobar pneumonia. Bronchial pneumonia is most prevalent in infants, young children and aged adults. It is caused by various bacteria, including *Streptococcus pneumoniae*. Bronchial pneumonia involves the alveoli contiguous to the larger bronchioles of the bronchial tree. Lobar pneumonia is more prone to occur in younger adults. A majority (more than 80%) of the cases of lobar pneumonia are caused by *Streptococcus pneumoniae*. Lobar pneumonia involves all of a single lobe of the lungs (although more than one lobe may be involved), wherein the entire area of involvement tends to become a consolidated mass, in contrast to the spongy texture of normal lung tissue. *Streptococcus pneumoniae* is known in medical microbiology as the **pneumococcus**, referring to its morphology and its consistent involvement in pneumonia. This article deals with pneumonia caused by *Streptococcus pneumoniae*, or **pneumococcal pneumonia**.

Streptococcus pneumoniae

Streptococcus pneumoniae are Gram-positive, lancet-shaped cocci (elongated cocci with a slightly pointed outer curvature). Usually they are seen as pairs of cocci (diplococci), but they may also occur singly and in short chains. When cultured on blood agar, they are alpha hemolytic. Individual cells are between 0.5 and 1.25 micrometers in diameter. They do not form spores, and they are nonmotile. Like other streptococci, they lack catalase and ferment glucose to lactic acid. Unlike other streptococci, they do not display an M protein, they hydrolyze inulin, and their cell wall composition is characteristic both in terms of their peptidoglycan and their teichoic acid).



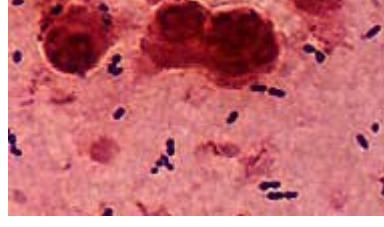
Gram Stain of a film of sputum from a case of lobar pneumonia

Cultivation

Streptococcus pneumoniae is fastidious bacterium, growing best in 5% carbon dioxide. Nearly 20% of fresh clinical isolates require fully anaerobic conditions. In all cases, growth requires a source of catalase (e.g. blood) to neutralize the large amount of hydrogen peroxide produced by the bacteria. In complex media containing blood, at 37°C, the bacterium has a doubling time of 20-30 minutes.

On agar, pneumococci grow as glistening colonies, about 1 mm in diameter. Two serotypes, types 3 and 37, are mucoid. Pneumococci spontaneously undergo a genetically determined, phase variation from opaque to transparent colonies at a rate of 1 in 10^5 . The transparent colony type is adapted to colonization of the nasopharynx, whereas the opaque variant is suited for survival in blood. The chemical basis for the difference in colony appearance is not known, but significant difference in surface protein expression between the two types has been shown.

Streptococcus pneumoniae is a fermentative aerotolerant anaerobe. It is usually cultured in media that contain blood. On blood agar, colonies characteristically produce a zone of alpha (green) hemolysis, which differentiates *S. pneumoniae* from the group A (beta hemolytic) streptococcus, but not from commensal alpha hemolytic (viridans) streptococci which are co-inhabitants of the upper respiratory tract. Special tests such as inulin fermentation, bile solubility, and optochin (an antibiotic) sensitivity must be routinely employed to differentiate the pneumococcus from *Streptococcus viridans*.



Streptococcus pneumoniae Gram-stain of blood broth culture

Streptococcus pneumoniae is a very fragile bacterium and contains within itself the enzymatic ability to disrupt and to disintegrate the cells. The enzyme is called an **autolysin**. The physiological role of this autolysin is to cause the culture to undergo a characteristic autolysis that kills the entire culture when grown to stationary phase. Virtually all clinical isolates of pneumococci harbor this autolysin and undergo lysis usually beginning between 18-24 hours after initiation of growth under optimal conditions. Autolysis is consistent with changes in colony morphology. Colonies initially appear with a plateau-type morphology, then start to collapse in the centers when autolysis begins.

Identification

The minimum criteria for identification and distinction of pneumococci from other streptococci are bile or optochin sensitivity, Gram-positive staining, and hemolytic activity. Pneumococci cause alpha hemolysis on agar containing horse, human, rabbit and sheep erythrocytes. Under anaerobic conditions they switch to beta hemolysis caused by an oxygen-labile hemolysin. Typically, pneumococci form a 16-mm zone of inhibition around a 5 mg optochin disc, and undergo lysis by bile salts (e.g. deoxycholate). Addition of a few drops of 10% deoxycholate at 37°C lyses the entire culture in minutes. The ability of deoxycholate to dissolve the cell wall, depends upon the presence of an autolytic enzyme, LytA. Virtually all clinical isolates of pneumococci harbor the autolysin and undergo deoxycholate lysis.



Streptococcus pneumoniae A mucoid strain on blood agar showing alpha hemolysis (green zone surrounding colonies). Note the zone of inhibition around a filter paper disc impregnated with optochin. Viridans streptococci are not inhibited by optochin.

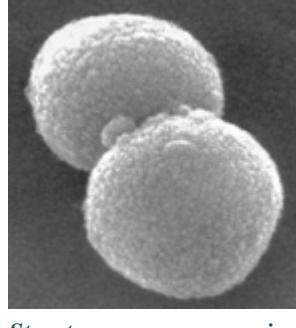
Serotyping

The **quellung reaction** (swelling reaction) forms the basis of serotyping and relies on the swelling of the capsule upon binding of homologous antibody. The test consists of mixing a loopful of colony with equal quantity of specific antiserum and then examining microscopically at 1000X for capsular swelling. Although generally highly specific, cross-reactivity has been observed between capsular types 2 and 5, 3 and 8, 7 and 18, 13 and 30, and with *E. coli*, *Klebsiella*, *H. influenzae* Type b, and certain viridans streptococci.



Streptococcus pneumoniae Quellung (capsular swelling) reaction can be used to demonstrate the presence of a specific capsular type of the bacterium.

Cell Surface Structure



Streptococcus pneumoniae scanning electron micrograph of a pair of diplococci (CDC).

Capsule

A capsule composed of polysaccharide completely envelops the pneumococcal cells. During invasion the capsule is an essential determinant of virulence. The capsule interferes with phagocytosis by preventing C3b opsonization of the bacterial cells. 90 different capsule types of pneumococci have been identified and form the basis of antigenic serotyping of the organism. Anti-pneumococcal vaccines are based on formulations of various capsular (polysaccharide) antigens derived from the highly-prevalent strains.



Streptococcus pneumoniae Fluorescent antibody stain of capsular material (CDC)

Cell Wall

The cell wall of *S. pneumoniae* is roughly six layers thick and is composed of peptidoglycan with teichoic acid attached to approximately every third N-acetylmuramic acid. Lipoteichoic acid is chemically identical to the teichoic acid but is attached to the cell membrane by a lipid moiety. Both the teichoic acid and the lipoteichoic acid contain phosphorylcholine; two choline residues may be covalently added to each carbohydrate repeat. This is an essential element in the biology of *S. pneumoniae* since the choline specifically adheres to choline-binding receptors that are located on virtually all human cells.

Surface Proteins

On the basis of functional genomic analysis, it is estimated that the pneumococcus contains more than 500 surface proteins. Some are membrane-associated lipoproteins, and others are physically associated with the cell wall. The latter includes five penicillin binding proteins (PBPs), two neuraminidases, and an IgA protease. A unique group of proteins on the pneumococcal surface is the family of **choline-binding proteins (CBPs)**. Twelve CBPs are noncovalently bound to the choline moiety of the cell wall and are used to "snap" various different functional elements onto the bacterial surface. The CBPs all share a common C-terminal choline-binding domain while the N-termini of the CBPs are distinct, indicating their functions are different. The CBP family includes such important determinants of virulence such as **PspA** (protective antigen), **LytA, B, and C** (three autolysins), and **CbpA** (an adhesin).

Genetics

S. pneumoniae has a **natural transformation system** as a mechanism for genetic exchange. This process is of medical significance because it clearly underlies the explosion of antibiotic resistance in the bacterium over the past 20 years. For example, penicillin resistance is due to altered penicillin-binding proteins (PBPs) which exhibit a low affinity for beta lactam antibiotics. Comparison of the nucleotide sequences encoding the PBPs in *S. pneumoniae* and *S. mitis* demonstrates that horizontal gene transfer has occurred between these two bacteria. In the laboratory, *S. pneumoniae* can also be transformed with genes from related and unrelated bacteria. As well, in the upper respiratory tract of the host, horizontal exchanges of genetic information could take place between strains of pneumococci that co-habitate or compete for dominance as normal flora.

Streptococcus pneumoniae can also develop antibiotic resistance by the timeless process of mutation and selection. The bacterium has a relatively fast growth rate and achieves large cell densities in an infectious setting. These conditions not only favor the occurrence natural transformation, but also the emergence of spontaneous mutants resistant to the antibiotic.

During transformation the binding, uptake and incorporation of exogenous DNA occur as a sequence of programmed events during a physiologically defined state known as **competence**. Competent bacteria self-aggregate, easily form protoplasts, are prone to autolysis and have an increased H⁺ and Na⁺ content that leads to increased glycolysis and enhanced ATP reserves. A unique set of at least 11 proteins is preferentially expressed during competence. Early in the competent state, a 17 amino acid peptide, known as competence-stimulating peptide (CSP) is released from the growing bacteria. CSP induces competence when it reaches a critical concentration that depends on the cell density, consistent with a quorum-sensing model.

Pneumococcal Pneumonia

Pathogenesis

Streptococcus pneumoniae is a normal inhabitant of the human upper respiratory tract. The bacterium can cause pneumonia, usually of the lobar type, paranasal sinusitis and otitis media, or meningitis which is usually secondary to one of the former infections. *Streptococcus pneumoniae* is currently the leading cause of invasive bacterial disease in children and the elderly.

Pneumococci spontaneously cause disease in humans, monkeys, rabbits, horses, mice and guinea pigs. Nasopharyngeal colonization occurs in approximately 40% of the population. Pneumonia and otitis media are the most common infections, meningitis being much more variable. The rabbit and the mouse have been used

extensively as animal models disease, leading to a reasonable understanding of many of the pneumococcal determinants of virulence.

Colonization

Pneumococci adhere tightly to the nasopharyngeal epithelium by multiple mechanisms that, for most individuals, appears to result in an immune response that generates type-specific immunity. For some people, however, progression into the lungs or middle ear occurs. Passage of pneumococci up the eustachian tube is accompanied by bacterial induced changes in the surface receptors of the epithelial cell, particularly by neuraminidase.

Inflammation in the middle ear is caused by pneumococcal cell wall components, and pneumolysin inflicts major cytotoxicity on ciliated cells of the cochlea.

Upon reaching the lower respiratory tract by aerosol, pneumococci bypass the ciliated upper respiratory epithelial cells unless there is damage to the epithelium. Instead, they progress to the alveolus and associate with specific alveolar cells which produce a choline-containing surfactant.

Experimentally, in healthy tissues, it requires approximately 100,000 bacteria/ml to trigger an inflammatory response. However, if a proinflammatory signal is supplied, inflammation ensues with as few as 10 bacteria. This signal is a cytokine in experimental systems or an intercurrent viral infection in clinical situations. The inflammatory response can cause considerable tissue damage.

Invasion

The bacteria invade and grow primarily due to their resistance to the host phagocytic response. The cell wall components directly activate multiple inflammatory cascades including the alternative pathway of complement activation, the coagulation cascade, and the cytokine cascade, inducing interleukin-1, interleukin-6 and tumor necrosis factor from macrophages and other cells.

In addition, as pneumococci begin to lyse in response to host defensins and antimicrobial agents, they release cell wall components, pneumolysin and other substances that lead to greater inflammation and cytotoxic effects. Pneumolysin and hydrogen peroxide kill cells and induce production of nitric oxide which may play a key role in septic shock.

During invasion, the interaction between the bacterial cell wall choline and the host PAF receptor G-protein contributes to a state of altered vascular permeability. In the lung, this leads to arrival of an inflammatory exudate. At first, a serous exudate forms. This is followed by the arrival of leukocytes, thereby making the switch from a serous to a purulent exudate. Sites of pneumococcal infection are particularly noted for the intensity of the purulent response.

Pneumococci occasionally are able to directly invade endothelial cells. The ligands by which pneumococci bind to activated human cells include choline located on the cell wall teichoic acid that can serve as a direct ligand to the PAF receptor, and the choline-binding protein, CbpA, which binds to a specific carbohydrate on the alveolar cell surface. When bound to the PAF receptor, the pneumococcus enters a vacuole in a receptor-mediated endocytic process and the vacuole moves across the cell expelling the bacteria on the abluminal surface. In vitro, pneumococci will adhere to and traverse an endothelial barrier over approximately 4 hours.

If bacteremia occurs, the risk of meningitis increases. Pneumococci can adhere specifically to cerebral capillaries using the same pairings of choline to PAF receptor and CbpA to carbohydrate receptor. Thus, the bacteria subvert the endocytosis/recycling pathway of the PAF receptor for cellular transmigration. Once in the cerebrospinal fluid, a variety of pneumococcal components, particularly cell wall components, incite the inflammatory response.

Bacterial Determinants of Virulence

Capsule

The bacterial capsule interferes with phagocytosis by leukocytes, a property dependent on its chemical composition. Apparently, resistance to phagocytosis is brought about by interference with binding of complement C3b to the cell surface.

During invasion of the mucosal surface, encapsulated strains are 100,000 times more virulent than unencapsulated strains. The polysaccharide is nontoxic and noninflammatory, and the capsule does not appear to engage any host

defenses except for the induction of antibody-mediated immunity. The pneumococcal capsule is not an antigenic disguise, and it does not impede the activities of underlying components, such as the cell wall and surface proteins, to engage the host defense systems. However, C-reactive protein or antibodies to teichoic acid, both of which bind to the cell wall under the capsule, fail to opsonize encapsulated strains.

Cell Wall Components

The pneumococcal cell wall is a collection of potent inflammatory stimuli. Challenge with cell wall components alone can recreate many of the symptoms of pneumonia, otitis media and meningitis in experimental models. The phosphorylcholine decorating the teichoic acid and the lipoteichoic acid is a key molecule enabling invasion, and acts both as an adhesin and as a docking site for the choline-binding proteins (CBPs). Other respiratory pathogens such as *Haemophilus*, *Pseudomonas*, *Neisseria* and *Mycoplasma* also have phosphorylcholine on lipopolysaccharide, proteins or fimbriae, suggesting a shared mechanism for invasion of the respiratory tract. Two host-derived elements that recognize choline are platelet activating factor (PAF) receptor and the C-reactive protein. Since respiratory pathogens may be recognized and cleared by the C-reactive protein response as part of the constitutive defenses, respiratory pathogens may share this invasive mechanism to subvert the signaling cascade of endogenous PAF.

The peptidoglycan/teichoic acid complex of the pneumococcus is highly inflammatory. Smaller components of peptidoglycan progressively lose specific inflammatory activity. The cell wall directly activates the alternative pathway of the complement cascade, generating chemotaxins for leukocytes, and the coagulation cascade, which promotes a "procoagulant state" favoring thrombosis. In addition, peptidoglycan binds to CD14, a cell surface receptor known to initiate the inflammatory response for endotoxin. This induces a cytokine cascade resulting in production of interleukin-1, interleukin 6 and tumor necrosis factor from human cells.

Choline Binding Proteins

The CBP family includes such important determinants as **PspA** (protective antigen), **LytA, B, and C** (three autolysins), and **CbpA** (an adhesin).

The **protective antigen (PspA)** is a 65 kD protein with 10 choline-binding repeats. PspA appears to inhibit complement-mediated opsonization of pneumococci, and mutants lacking PspA have reduced virulence. Antibodies against PspA confer passive protection in mice.

Autolysin LytA is responsible for pneumococcal lysis in stationary phase as well as in the presence of antibiotics. The protein has two functional domains: a C-terminal domain with six choline-binding repeats that anchor the protein on the cell wall, and an N-terminal domain that provides amidase activity. **Autolysin LytB** is a glucosaminidase involved in cell separation, and **LytC** exhibits lysozyme-like activity.

CbpA is a major pneumococcal adhesin. It has eight choline-binding repeats. The adhesin interacts with carbohydrates on the pulmonary epithelial surface carbohydrates. CbpA-deficient mutants are defective in colonization of the nasopharynx and fail to bind to various human cells in vitro. CbpA also has been reported to bind secretory IgA and complement component C3.

Hemolysins

In addition to surface-associated virulence determinants, pneumococci secrete exotoxins. Two hemolysins have been described, the most potent of which is pneumolysin. **Pneumolysin** is stored intracellularly and is released upon lysis of pneumococci by autolysin. Pneumolysin binds to cholesterol and thus can indiscriminately bind to all cells without restriction to a receptor. This protein assembles into oligomers to form transmembrane pores which ultimately lead to cell lysis. Pneumolysin can also stimulate the production of inflammatory cytokines, inhibit beating of the epithelial cell cilia, inhibit lymphocyte proliferation, decrease the bactericidal activity of neutrophils, and activate complement. A second hemolysin activity has been described but has not been identified. In addition, pneumococci also produce hydrogen peroxide in amounts greater than human leukocytes produce. This small molecule is also a potent hemolysin.

Neuraminidase and IgA protease

Epidemiology

S. pneumoniae is a transient member of the normal flora, colonizing the nasopharynx of 40% of healthy adults and children with no adverse effects. Children carry this pathogen in the nasopharynx asymptotically for about 4-6 weeks, often several serotypes at a time. New serotypes are acquired approximately every 2 months. Serotypes 6, 14, 18, 19, and 23 are the most prevalent accounting for 60-80% of infections depending on the area of the world. Pneumococcal infection accounts for more deaths than any other vaccine-preventable bacterial disease. Those most commonly at risk for pneumococcal infection are children between 6 months and 4 years of age and adults over 60 years of age. Virtually every child will experience pneumococcal otitis media before the age of 5 years. It is estimated that 25% of all community-acquired pneumonia is due to pneumococcus (1,000 per 100,000 inhabitants). The Centers for Disease Control and Prevention (CDC) reported 60,000 cases of invasive pneumococcal disease in 1997 approximately 6,000 deaths. Recently, epidemics of disease have reappeared in settings such as chronic care facilities, military camps and day care centers, a situation not recognized since the pre-antibiotic era.

Also of concern, is the increased emergence of antibiotic resistance, especially in the past decade. Multiple antibiotic resistant strains of *S. pneumoniae* that emerged in the early 1970s in Papua New Guinea and South Africa were thought to be a fluke, but multiple antibiotic resistance now covers the globe and has rapidly increased since 1995. Increases in penicillin resistance have been followed by resistance to cephalosporins and multidrug resistance. The incidence of resistance to penicillin increased from <0.02 in 1987 to 3% in 1994 to 30% in some communities in the United States and 80% in regions of some other countries in 1998. Resistance to other antibiotics has emerged simultaneously: 26% resistant to trimethoprim-sulfa, 9% resistant to cefotaxime, 30% resistant to macrolides, and 25% resistant to multiple drugs. Resistant organisms remain fully virulent but seem to have arisen in less than 10 serotypes. Serotypes 6A, 6B, 9V, 14, 19A and 23F are included in the vast majority of resistant strains.

Vaccines

Given the 90 different capsular types of pneumococci, a comprehensive vaccine based on polysaccharide alone is not feasible. Thus, vaccines based on a subgroup of highly prevalent types have been formulated. The number of serotypes in the vaccine has increased from four in 1945, to 14 in the 1970s, and finally to the current 23-valent formulation (25 mg of each of serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F). These serotypes represent 85-90% of those that cause invasive disease and the vaccine efficacy is estimated at 60%. However, underutilization of the vaccine is so extensive that the pneumococcus remains the most common infectious agent leading to hospitalization in all age groups. This is further complicated by the fact that polysaccharides are not immunogenic in children under the age of 2 years where a significant amount of disease occurs.

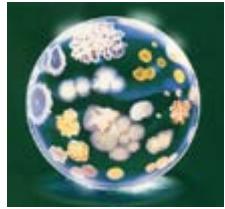
For more Information on *Streptococcus pneumoniae* see

[Streptococcus pneumoniae Disease - Technical Information from CDC](#)

[Streptococcus pneumoniae - Additional Information and Links from CDC](#)

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Listeria monocytogenes and Listeriosis

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Listeria monocytogenes Transmission EM

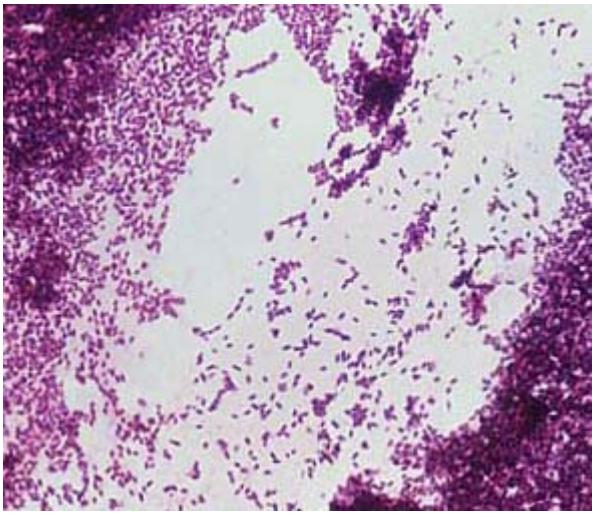
Introduction

Listeria monocytogenes is a Gram-positive rod-shaped bacterium. It is the agent of **listeriosis**, a serious infection caused by eating food contaminated with the bacteria. Listeriosis has recently been recognized as an important public health problem in the United States. The disease affects primarily pregnant women, newborns, and adults with weakened immune systems.

Listeriosis is a serious disease for humans; the **overt form** of the disease has a mortality greater than 25 percent. The two main clinical manifestations are sepsis and meningitis. Meningitis is often complicated by encephalitis, a pathology that is unusual for bacterial infections.

Microscopically *Listeria* species appear as small, Gram-positive rods, which are sometimes arranged in short chains. In direct smears they may be coccoid, so they can be mistaken for streptococci. Longer cells may resemble corynebacteria. (Indeed, as Gram-positive, nonsporeforming, catalase-positive rods, the genus *Listeria* was classified in the family Corynebacteriaceae through the seventh edition of Bergey's Manual). Flagella are produced at room temperature but not at 37° C. Hemolytic activity on blood agar has been used as a marker to distinguish *Listeria monocytogenes* among other *Listeria* species, but it is not an absolutely definitive criterion. Further biochemical characterization may be necessary to distinguish between the different *Listeria* species.

16S rRNA cataloging studies of Stackebrandt et al. (1983) demonstrated that *Listeria monocytogenes* was a distinct taxon within the ***Clostridium-Lactobacillus-Bacillus*** branch of the bacterial phylogeny constructed by Woese (1981). Within this phylogeny, *Listeria* is most closely related to the genus *Brochothrix*.



Listeria monocytogenes Gram Stain

Natural Habitats

Until about 1960, *Listeria monocytogenes* was thought to be associated almost exclusively with infections in animals, and less frequently in humans. However, in the last 30 years, listeriae, including the pathogenic species *L. monocytogenes* and *L. ivanovii* have been isolated from a variety of sources, and they are now recognized to be widely distributed in Nature. In addition to humans, at least 42 species of wild and domestic mammals and 17 avian species, including domestic and game fowl, can harbor listeriae. *Listeria monocytogenes* is reportedly carried in the intestinal tract of 5-10% of the human population without any apparent symptoms of disease. Listeriae have also been isolated from crustaceans, fish, oysters, ticks, and flies.

The term **listeriosis** encompasses a wide variety of disease symptoms that are similar in animals and humans. *Listeria monocytogenes* causes listeriosis in animals and humans; *L. ivanovii* causes the disease in animals only, mainly sheep. Encephalitis is the most common form of the disease in ruminant animals. In young animals, visceral or septicemic infections often occur. Intra-uterine infection of the fetus via the placenta frequently results in abortion in sheep and cattle.

The true incidence of listeriosis in humans is not known, because in the average healthy adult, infections are usually asymptomatic, or at most produce a mild influenza-like disease. Clinical features range from mild influenza-like symptoms to meningitis and/or meningoencephalitis. Illness is most likely to occur in pregnant women, neonates, the elderly and immunocompromised individuals, but apparently healthy individuals may also be affected. In the serious (overt) form of the disease, meningitis, frequently accompanied by septicemia, is the most commonly encountered disease manifestation. In pregnant women, however, even though the most usual symptom is a mild influenza-like illness without meningitis, infection of the fetus is extremely common and can lead to abortion, stillbirth, or delivery of an acutely ill infant.

In humans, overt listeriosis following infection with *L. monocytogenes* is usually sporadic, but outbreaks of epidemic proportions have occurred. In 1981, there was an outbreak that involved over 100 people in Canada. Thirty-four of the infections occurred in pregnant women, among whom there were nine stillbirths, 23 infants born infected, and two live healthy births. Among 77 non pregnant adults who developed overt disease, there was nearly 30% mortality. The source of the outbreak was coleslaw produced by a local manufacturer.

In 1985, in California, 142 people developed overt listeriosis. Of these, 93 cases were perinatal, and among the 49 cases that were in non pregnant individuals, 48 were immunocompromised. Thirty fetuses or newborn infants died and 18 adults died. The source of the bacteria was a certain brand of "pasteurized" soft cheese that apparently had gotten contaminated with non pasteurized (raw) milk during the manufacturing process.

Pathogenesis

Listeria monocytogenes is presumably ingested with raw, contaminated food. An invasin secreted by the pathogenic bacteria enables the listeriae to penetrate host cells of the epithelial lining. The bacterium is widely distributed so this event may occur frequently. Normally, the immune system eliminates the infection before it spreads. Adults with no history of listeriosis have T lymphocytes primed specifically by *Listeria* antigens. However, if the immune

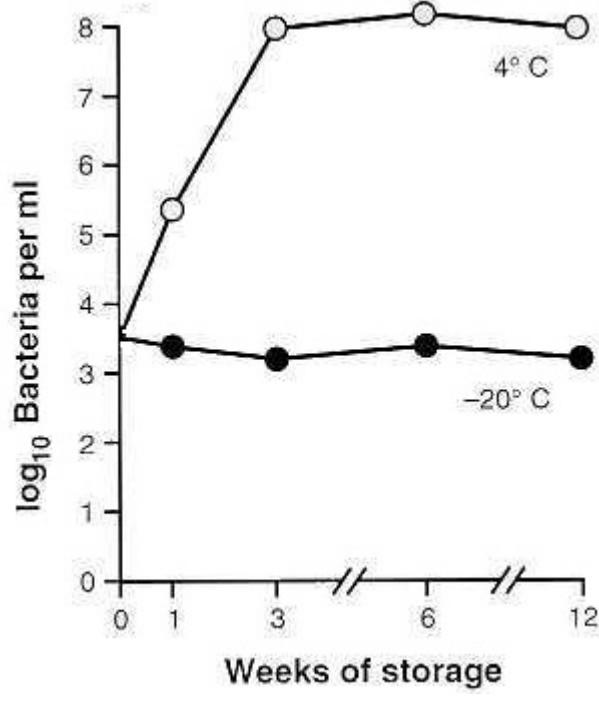
system is compromised, systemic disease may develop. *Listeria monocytogenes* multiplies not only extracellularly but also intracellularly, within macrophages after phagocytosis, or within parenchymal cells which are entered by induced phagocytosis.

In mice infected with *L. monocytogenes*, the bacteria first appear in macrophages and then spread to hepatocytes in the liver. The bacteria stimulate a CMI response that includes the production of TNF, gamma interferon, macrophage activating factors and a cytotoxic T cell response. Possibly, in humans, a failure to control *L. monocytogenes* by means of CMI allows the bacteria to spread systemically. As well, unlike other bacterial pathogens, *Listeria* are able to penetrate the endothelial layer of the placenta and thereby infect the fetus.

Virulence Factors

Growth at low temperatures

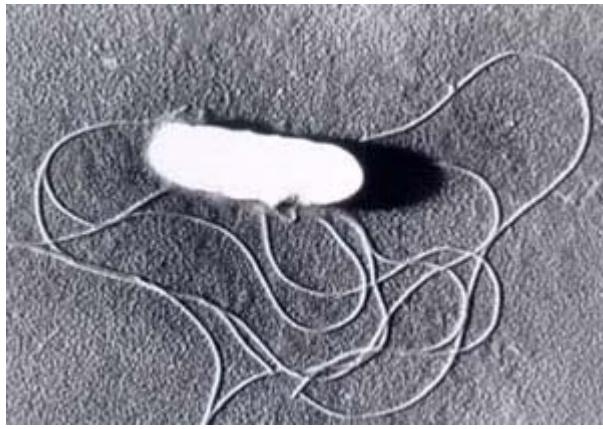
A peculiar property of *L. monocytogenes* that affects its food-borne transmission is the ability to multiply at low temperatures. The bacteria may therefore grow and accumulate in contaminated food stored in the refrigerator. So it is not surprising that listeriosis is usually associated with ingestion of milk, meat or vegetable products that have been held at refrigeration temperatures for a long period of time.



Growth and viability of *Listeria* in certain foods at freezing temperatures (-20 degrees) and refrigeration temperatures (4 degrees) over a 12 week period. Adapted from [Baron's Medical Microbiology, Miscellaneous Pathogenic Bacteria](#) by H Hof.

Motility

As in the case of *Vibrio cholerae*, wherein movement, attachment and penetration of the intestinal mucosa are determinants of infection (if not disease), this was thought to be the situation with *Listeria*, which is also acquired by ingestion and must also find a way to attach to the intestinal mucosa. With cholera, the actively-motile vibrios are thought to use their flagella to swim against the peristaltic movement of the bowel content and to penetrate (by swimming laterally) the mucosal lining of the gut where they adhere. Curiously, although *Listeria* are actively motile by means of peritrichous flagella at room temperature (20-25 degrees), the organisms do not synthesize flagella at body temperatures (37 degrees). Instead, virulence is associated with another type of motility: the ability of the bacteria to move themselves into, within and between host cells by polymerization of host cell actin at one end of the bacterium ("growing actin tails") that can propel the bacteria through cytoplasm. However, one should not totally dismiss the advantage of flagellar motility for existence and spread of the bacteria outside of the immediate host environment.



Listeria monocytogenes Scanning EM showing Flagella

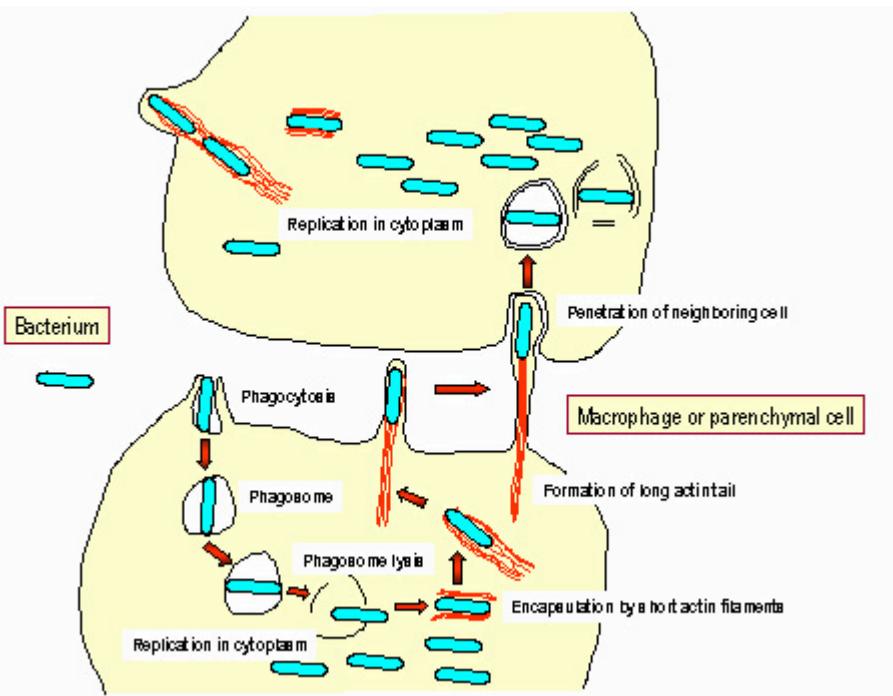
Adherence and Invasion

Listeria can attach to and enter mammalian cells. The bacterium is thought to attach to epithelial cells of the GI tract by means of D-galactose residues on the bacterial surface which adhere to D-galactose receptors on the host cells. If this is correct, it is the opposite of the way that most other bacterial pathogens are known to adhere, i.e., the bacterium displays the protein or carbohydrate ligand on its surface and the host displays the amino acid or sugar residue to which the ligand binds. Having said this, macrophages are well known to have "mannose binding receptors" on their surface whose function presumably is to bind to bacterial surface polysaccharides that terminate in mannose, as a prelude to phagocytic uptake.

The bacteria are then taken up by **induced phagocytosis**, analogous to the situation in *Shigella*. An 80 kDa membrane protein called **internalin** probably mediates invasion. A complement receptor on macrophages has been shown to be the internalin receptor, as well.

After engulfment, the bacterium may escape from the phagosome before phagolysosome fusion occurs mediated by a toxin, which also acts as a hemolysin, **listeriolysin O (LLO)**. This toxin is one of the so-called SH-activated hemolysins, which are produced by a number of other Gram-positive bacteria, such as group A streptococci (streptolysin O), pneumococci (pneumolysin), and *Clostridium perfringens*. The hemolysin gene is located on the chromosome within a cluster of other virulence genes which are all regulated by a common promoter. Survival of the bacterium within the phagolysosome may be aided by its ability to produce catalase and superoxide dismutase which neutralize the effects of the phagocytic oxidative burst.

Additional genetic determinants are necessary for further steps in the intracellular life cycle of *L. monocytogenes*. One particular gene product, **Act A (encoded by actA)** promotes the polymerization of actin, a component of the host cell cytoskeleton, on the bacterial surface. Within the host cell environment, surrounded by a sheet of actin filaments, the bacteria reside and multiply. The growing actin sheet functions as a propulsive force which drives the bacteria across the intracellular pathways until they finally reach the surface. Then, the host cell is induced to form slim, long protrusions containing living *L. monocytogenes*. Those cellular projections are engulfed by adjacent cells, including non-professional phagocytes such as parenchymal cells. By such a mechanism, direct cell-to-cell spread of *Listeria* in an infected tissue may occur without an extracellular stage.



Steps in the invasion of cells and intracellular spread by *L. monocytogenes*. The bacterium apparently invades via the intestinal mucosa. It is thought to attach to intestinal cells by means of D-galactose residues on the bacterial surface which adhere to D-galactose receptors on susceptible intestinal cells. The bacterium is taken up (including by non phagocytic cells) by induced phagocytosis, which is thought to be mediated by a membrane associated protein called internalin. Once ingested the bacterium produces listeriolysin (LLO) to escape from the phagosome. The bacterium then multiplies rapidly in the cytoplasm and moves through the cytoplasm to invade adjacent cells by polymerizing actin to form long tails.

Other Determinants of Virulence

L. monocytogenes produces two other hemolysins besides LLO: **phosphatidylinositol-specific phospholipase C (PI-PLC)** and **phosphatidylcholine-specific phospholipase C (PC-PLC)**. Unlike LLO, which lyses host cells by forming a pore in the cell membrane, these phospholipases disrupt membrane lipids such as phosphatidylinositol and phosphatidylcholine (lecithin).

The bacterium also produces a **Zn⁺⁺ dependent protease** which may act as some sort of exotoxin. Mutations in the encoding gene (*mpl*) reduce virulence in the mouse model.

Finally, an operon called ***ImaBA*** encodes a 20 kDa protein located on the bacterial surface. The protein **LMaA** induces delayed type hypersensitivity and other CMI responses.

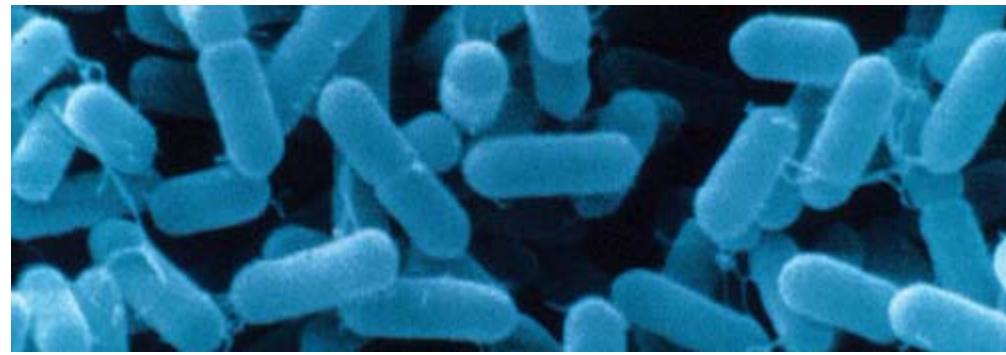
Host Defenses

Because *L. monocytogenes* multiplies intracellularly, it is largely protected against circulating immune factors (AMI) such as antibodies and complement-mediated lysis. The effective host response is cell-mediated immunity (CMI), involving both lymphokines (especially interferon) produced by CD4+ (T_{H1}) cells and direct lysis of infected cells by CD8+ (T_C) cells. Both of these defense mechanisms are expressed in the microenvironment of the infected foci, which are organized as granulomas, characterized by a central accumulation of macrophages with irregularly shaped nuclei, and by peripheral lymphocytes recognizable by rounded nuclei and a narrow border of intensely staining cytoplasm.

Treatment and Prevention

If diagnosed early enough, antibiotic treatment of pregnant women or immunocompromised individuals can prevent serious consequences of the disease. However, early diagnosis is the exception rather than the rule, since the first signs of a case or an outbreak are reports of stillbirth or serious infections resembling listeriosis. By then, any cohorts who have become infected from eating the same food are likely recovered from an inapparent or flu-type infection, or they themselves may have developed serious disease. However, processed foods known to be the source of *Listeria* that may still be in the market place, restaurant or home should obviously not be used, and recalls should be imperative. It must also be constantly recognized that *L. monocytogenes* is able to grow at low

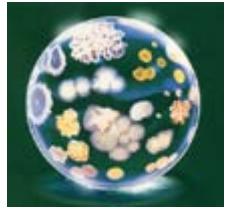
temperatures.



Listeria monocytogenes Scanning EM

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The Pathogenic Neisseriae

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Neisseria meningitidis scanning EM

Introduction

The family *Neisseriaceae* consists of Gram-negative aerobic bacteria from fourteen genera (Bergey's 2001), including *Neisseria*, *Chromobacterium*, *Kingella*, and *Aquaspirillum*. The genus *Neisseria* contains two important human pathogens, *N. gonorrhoeae* and *N. meningitidis*. *N. gonorrhoeae* causes gonorrhea, and *N. meningitidis* is the cause of meningococcal meningitis. *N. gonorrhoeae* infections have a high prevalence and low mortality, whereas *N. meningitidis* infections have a low prevalence and high mortality.

Neisseria gonorrhoeae infections are acquired by sexual contact and usually affect the mucous membranes of the urethra in males and the endocervix and urethra in females, although the infection may disseminate to a variety of tissues. The pathogenic mechanism involves the attachment of the bacterium to nonciliated epithelial cells via pili (fimbriae) and the production of lipopolysaccharide endotoxin. Similarly, the lipopolysaccharide of *Neisseria meningitidis* is highly toxic, as it has an additional virulence factor in the form of its antiphagocytic capsule. Both pathogens produce IgA proteases which promote virulence. Many normal individuals may harbor *Neisseria meningitidis* in the upper respiratory tract, but *Neisseria gonorrhoeae* is never part of the normal flora and is only found after sexual contact with an infected person (or direct contact, in the case of infections in the newborn).

In the vocabulary of the public health and medical microbiologist, *N. gonorrhoeae* is often referred to as the "gonococcus", while *N. meningitidis* is known as the "meningococcus" and one form of the disease it causes is called meningococcemia.

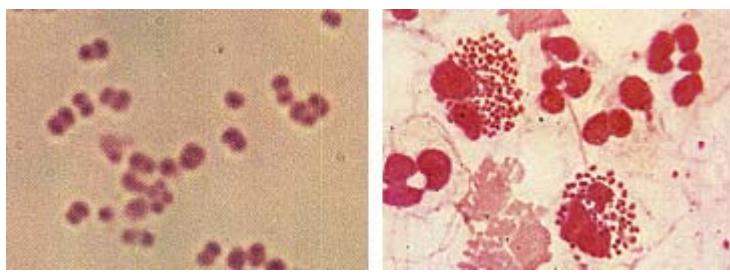


Figure 1. Left: *Neisseria gonorrhoeae* Gram stain of pure culture; **Right:** *Neisseria gonorrhoeae* Gram stain of a pustular exudate.

Neisseria gonorrhoeae

Neisseria gonorrhoeae is a Gram-negative coccus, 0.6 to 1.0 μm in diameter, usually seen in pairs with adjacent flattened sides (Figure 1 Left and Fig 2 below). The organism is frequently found intracellularly in polymorphonuclear leukocytes (neutrophils) of the gonorrhea pustular exudate (Figure 1 Right). Fimbriae, which play a major role in adherence, extend several micrometers from the cell surface (Figure 2 below).



Figure 2. *Neisseria gonorrhoeae*

Neisseria gonorrhoeae possesses a typical Gram-negative outer membrane composed of proteins, phospholipids, and lipopolysaccharide (LPS). However, neisserial LPS is distinguished from enteric LPS by its highly-branched basal oligosaccharide structure and the absence of repeating O-antigen subunits. For these reasons, neisserial LPS is referred to as **lipooligosaccharide (LOS)**. The bacterium characteristically releases outer membrane fragments called "blebs" during growth. These blebs contain LOS and probably have a role in pathogenesis if they are disseminated during the course of an infection.

N. gonorrhoeae is a relatively fragile organism, susceptible to temperature changes, drying, uv light, and other environmental conditions. Strains of *N. gonorrhoeae* are variable in their cultural requirements so that media containing hemoglobin, NAD, yeast extract and other supplements are needed for isolation and growth of the organism. Cultures are grown at 35-36 degrees in an atmosphere of 3-10% added CO₂.

Infections caused by *N. gonorrhoeae*

The disease **gonorrhea** is a specific type of **urethritis** that practically always involves mucous membranes of the urethra, resulting in a copious discharge of pus, more apparent in the male than in the female. The first usage of the term "gonorrhea", by Galen in the second century, implied a "flow of seed". For centuries thereafter, gonorrhea and syphilis were confused, resulting from the fact that the two diseases were often present together in infected individuals. Paracelsus (1530) thought that gonorrhea was an early symptom of syphilis. The confusion was further heightened by the classic blunder of English physician John Hunter, in 1767. Hunter intentionally inoculated himself with pus from a patient with symptoms of gonorrhea and wound up giving himself syphilis! The causative agent of gonorrhea, *Neisseria gonorrhoeae*, was first described by A. Neisser in 1879 in the pustular exudate of a case of gonorrhea. The organism was grown in pure culture in 1885, and its etiological relationship to human disease was later established using human volunteers in order to fulfill the experimental requirements of Koch's postulates.

Gonorrhreal infection is generally limited to superficial mucosal surfaces lined with columnar epithelium. The areas most frequently involved are the urethra, cervix, rectum, pharynx, and conjunctiva. Squamous epithelium, which lines the adult vagina, is not susceptible to infection by the *N. gonorrhoeae*. However, the prepubescent vaginal epithelium, which has not been keratinized under the influence of estrogen, may be infected. Hence, gonorrhea in young girls may present as **vulvovaginitis**. Mucosal infections are usually characterized by a purulent discharge.

Uncomplicated gonorrhea in the adult male is an inflammatory and pyogenic infection of the mucous membranes of the anterior urethra. The most common symptom is a discharge that may range from a scanty, clear or cloudy fluid to one that is copious and purulent. Dysuria (difficulty in urination) is often present. Inflammation of the urethral tissues results in the characteristic redness, swelling, heat, and pain in the region. There is intense burning and pain upon urination.

Endocervical infection is the most common form of uncomplicated gonorrhea in women. Such infections are usually characterized by vaginal discharge and sometimes by dysuria. About 50% of women with cervical infections are asymptomatic. Asymptomatic infections occur in males, as well. Males with asymptomatic urethritis are an important reservoir for transmission and are at increased risk for developing complications. Asymptomatic males and females are a major problem as unrecognized carriers of the disease, which occurs in the U.S. at an estimated rate of over one million cases per year.

In the male, the organism may invade the prostate resulting in **prostatitis**, or extend to the testicles resulting in **orchitis**. In the female, cervical involvement may extend through the uterus to the fallopian tubes resulting in **salpingitis**, or to the ovaries resulting in **ovaritis**. As many as 15% of women with uncomplicated cervical infections may develop **pelvic inflammatory disease (PID)**. The involvement of testicles, fallopian tubes or ovaries may result in sterility. Occasionally, disseminated infections occur. The most common forms of disseminated infection are a **dermatitis-arthritis syndrome, endocarditis and meningitis**.

Rectal infections (**proctitis**) with *N. gonorrhoeae* occur in about one-third of women with cervical infection. They most often result from autoinoculation with cervical discharge and are rarely symptomatic. Rectal infections in homosexual men usually result from anal intercourse and are more often symptomatic. Partners must be treated as well to avoid reinfection.

Ocular infections by *N. gonorrhoeae* can have serious consequences of corneal scarring or perforation. Ocular infections (**ophthalmia neonatorum**) occur most commonly in newborns who are exposed to infected secretions in the birth canal. Part of the intent in adding silver nitrate or an antibiotic to the eyes of the newborn is to prevent ocular infection by *N. gonorrhoeae*.

Pathogenesis

Gonorrhea in adults is almost invariably transmitted by sexual intercourse. The bacteria adhere to columnar epithelial cells, penetrate them, and multiply on the basement membrane. Adherence is mediated through **fimbriae** and **opa (P.II)** proteins. Although nonspecific factors such as surface charge and hydrophobicity may play a role. Fimbriae undergo both phase and antigenic variation. The bacteria attach only to microvilli of nonciliated columnar epithelial cells. Attachment to ciliated cells does not occur.

Most of the information on bacterial invasion comes from studies with tissue culture cells and human fallopian tube organ culture. After the bacteria attach to the nonciliated epithelial cells of the fallopian tube, they are surrounded by the microvilli, which draw them to the surface of the mucosal cell. The bacteria enter the epithelial cells by a process called **parasite-directed endocytosis**. During endocytosis the membrane of the mucosal cell retracts and pinches off a membrane-bound vacuole that contains the bacteria. The vacuole is transported to the base of the cell, where the bacteria are released by exocytosis into the subepithelial tissue. The neisseriae are not destroyed within the endocytic vacuole, but it is not clear whether they actually replicate in the vacuoles as intracellular parasites.

A major porin protein, **P.I (Por)**, in the outer membrane of the bacterium is thought to be the invasin that mediates penetration of a host cell. Each *N. gonorrhoeae* strain expresses only one type of Por; however, there are several variations of Por that partly account for different antigenic types of the bacterium.

Neisseria gonorrhoeae can produce one or several outer membrane proteins called **Opa (P.II)** proteins . These

proteins are subject to phase variation and are usually found on cells from colonies possessing a unique opaque phenotype called **O+**. At any particular time, the bacterium may express zero, one, or several different Opa proteins, and each strain has 10 or more genes for different Opas.

Rmp (P.III) is an outer membrane protein found in all strains of *N. gonorrhoeae*. It does not undergo antigenic variation and is found in a complex with Por and LOS. It shares partial homology with the OmpA protein of *Escherichia coli*. Antibodies to Rmp, induced either by a neisserial infection or by colonization with *E. coli*, tend to block bactericidal antibodies directed against Por and LOS. In fact, anti-Rmp antibodies may increase susceptibility to infection by *N. gonorrhoeae*.

During infection, bacterial lipooligosaccharide (LOS) and peptidoglycan are released by autolysis of cells. Both bacterial polysaccharides activate the host alternative complement pathway, while LOS also stimulates the production of tumor necrosis factor (TNF) that causes cell damage. Neutrophils are immediately attracted to the site and feed on the bacteria. For unknown reasons, many gonococci are able to survive inside of the phagocytes, at least until the neutrophils themselves die and release the ingested bacteria.

Neisserial LOS has a profound effect on the virulence and pathogenesis of *N. gonorrhoeae*. The bacteria can express several antigenic types of LOS and can alter the type of LOS they express by some unknown mechanism. Gonococcal LOS produces mucosal damage in fallopian tube organ cultures and brings about the release of enzymes, such as proteases and phospholipases, that may be important in pathogenesis. Thus, gonococcal LOS appears to have an indirect role in mediating tissue damage. Gonococcal LOS is also involved in the resistance of *N. gonorrhoeae* to the bactericidal activity of normal human serum. Specific LOS oligosaccharide types are known to be associated with a serum-resistant phenotypes of *N. gonorrhoeae*.

N. gonorrhoeae can utilize host-derived N-acetylneuraminic acid (sialic acid) to sialylate the oligosaccharide component of its LOS, converting a serum-sensitive organism to a serum-resistant one. Organisms with nonsialylated LOS are more invasive than those with sialylated LOS but organisms with sialylated LOS are more resistant to bactericidal effects of serum. There is also antigenic similarity between neisserial LOS and antigens present on human erythrocytes. This similarity to "self" may preclude an effective immune response to these LOS antigens by maintaining the immunotolerance of the host.

N. gonorrhoeae is highly efficient at utilizing transferrin-bound iron for in vitro growth; many strains can also utilize lactoferrin-bound iron. The bacteria bind only human transferrin and lactoferrin. This specificity is thought to be, in part, the reason these bacteria are exclusively human pathogens.

Strains of *N. gonorrhoeae* produce two distinct extracellular **IgA1 proteases** which cleave the heavy chain of the human immunoglobulin at different points within the hinge region. Split products of IgA1 have been found in the genital secretions of women with gonorrhea, suggesting that the bacterial IgA1 protease is present and active during genital infection. It is thought that the Fab fragments of IgA1 may bind to the bacterial cell surface and block the Fc-mediated functions other immunoglobulins.

Occasionally, as described above, invading *Neisseria gonorrhoeae* enter the bloodstream causing a Gram-negative bacteremia which may lead to a disseminated bacterial infection. Asymptomatic infections of the urethra or cervix usually serve as focal sources for bacteremia. Strains of *N. gonorrhoeae* that cause disseminated infections are usually resistant to complement and the serum bactericidal reaction. This accounts for their ability to persist in the bacteremia. In Gram-negative bacteremias of this sort, the effect of bacterial endotoxin can be exacerbated by the lysis of bacterial cells which may simply liberate soluble LPS.

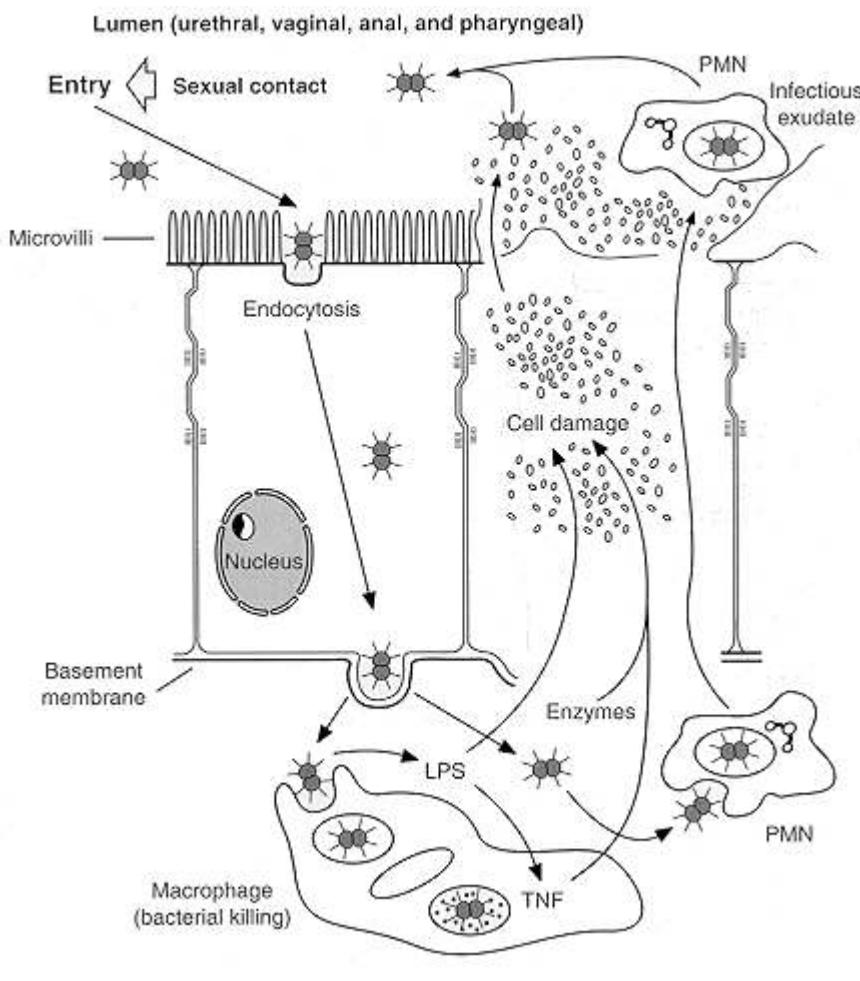


Figure 3. Pathogenesis of uncomplicated gonorrhea according to Morse in Baron, Chapter 14, [Neisseria, Branhamella, Moraxella and Eikenella](#)

Virulence Factors

Like the other pyogenic bacteria, *Neisseria gonorrhoeae* has a wide range of virulence determinants, although it does not produce any exotoxins. The first stages of infection, involving adherence and invasion, are mediated by surface components of the gonococci. The bacterium first attaches to epithelial cells by means of its fimbriae, specifically **N-methylphenylalanine (Type 4) pili**, the main subunit of which is **PilE**. After initial attachment, the bacteria enter a second stage of binding mediated by the outer membrane protein **P.II** (also known as **Opa**) which is needed for tight binding and invasion of epithelial cells. Also, **P.II** from one bacterium will bind to LOS of an adjacent bacterium, which allows for the construction of a microcolony which may be functionally analogous to a biofilm. However, the invasion of a cell involves a single bacterium, not whole microcolonies. *Neisseria gonorrhoeae* also produces an IgA1 protease that probably play a role in the colonization stage.

The outer membrane porin of *N. gonorrhoeae* **P.I** (also known as **Por**) is equivalent to the *ompC* and *ompF* porins of *E. coli* that are involved in the passage of solutes through the outer membrane. However, P.I apparently has a role in virulence that allows the gonococci to survive inside of phagocytes. Purified P.I has been shown to inhibit the ability of phagocytes to kill ingested bacteria.

The lipooligosaccharide (LOS) of the outer membrane is thought to be responsible for most of the symptoms of gonorrhea. Gonococcal LOS triggers an intense inflammatory response. Subsequent activation of complement, attraction and feeding by phagocytes, and the lysis of the phagocytes themselves, contributes to the purulent discharge. The local production of TNF, elicited by LOS, is thought to be the main cause of damage to the fallopian tubes. In addition, in strains that cause systemic infection, LOS binds sialic acid from the serum forming a microcapsule of **sialylated LOS**, which allows the gonococci to resist the host immune response and serum bactericidal reaction.

Nonsialylated LOS and P.I (Por) on the bacterial surface are known to be effective targets for bactericidal antibodies. However, if antibodies produced against **P.III** (also known as **Rmp**) react with their antigenic site on the gonococcal surface, the effect is to block bactericidal antibodies against LOS and P.I and to protect the

bacterium from complement-mediated lysis.

Finally, *Neisseria gonorrhoeae* has a well-developed iron acquisition system that permits it to extract iron from its host during growth, which is necessary to support bacterial invasion. Basically, the bacterium is able to form two transferrin receptors (**Tbp1** and **Tbp2**) and one lactoferrin receptor (**Lbp**) in its outer membrane, which are induced under low-iron conditions, and which are able to directly extract iron from transferrin and lactoferrin, respectively. The proteins can also extract iron from heme and hemoglobin.

Table 1. Surface components of *N. gonorrhoeae* that may play a role in virulence

Designation	Location	Contribution
PilE	major fimbrial protein	initial binding to epithelial cells
P.II (Opa)	outer membrane protein	contributes to invasion
P.I (Por)	outer membrane porin	may prevent phagolysosome formation in neutrophils and/or reduce oxidative burst
LOS	outer membrane lipooligosaccharide	elicits inflammatory response, triggers release of TNF
P.III (Rmp)	outer membrane protein	elicits formation of ineffective antibodies that block bactercidal antibodies against P.I and LOS
Tbp1 and Tbp2	outer membrane receptors for transferrin	iron acquisition for growth
Lbp	outer membrane receptor for lactoferrin	iron acquisition for growth

Host Defenses

Infection stimulates inflammation and a local immune (IgA) response. Inflammation focuses the host defenses but also becomes the pathology of the disease. It is not known whether the secretory immune response is protective. Serum antibodies also appear, and IgG and complement may be components of the inflammatory exudate. But whether the immune defenses provide much protection against reinfection has not been clearly shown. In any case, immunity is expected to be strain specific so that reinfection may occur.

Not everyone exposed to *N. gonorrhoeae* acquires the disease. This may be due to variations in the size or virulence of the inoculum, to natural resistance, or to specific immunity. A 50% infective dose (ID50) of about 1,000 bacteria has been determined based on experimental urethral inoculation of male volunteers. No data is available for females.

Nonspecific factors have been implicated in natural resistance to gonococcal infection. In women, changes in the genital pH and hormones may increase resistance to infection at certain times of the menstrual cycle. Urine contains bactericidal and bacteriostatic components against *N. gonorrhoeae*. Factors in urine that may be important are pH, osmolarity, and the concentration of urea. The variability in the susceptibility of gonococcal strains to the bactericidal and bacteriostatic properties of urine is thought to be one of the reasons some males apparently do not develop a gonorrhea infection when exposed.

Most uninfected individuals have serum antibodies that react with gonococcal antigens. These antibodies probably result from colonization or infection by various Gram-negative bacteria that possess cross-reactive antigens. Such "natural antibodies" may be important in individual natural resistance or susceptibility to infection, but this has not been clearly demonstrated.

Infection with *N. gonorrhoeae* stimulates both mucosal and systemic antibodies to a variety of gonococcal antigens. Mucosal antibodies are primarily IgA and IgG. In genital secretions, antibodies have been identified that react with Por, Opa, Rmp and LOS. Vaccine trials have suggested that specific anti-fimbrial antibodies inhibit the fimbrial-mediated attachment of the homologous gonococcal strain. In general, the IgA response is brief and declines

rapidly after treatment; IgG levels decline more slowly. Anti-Por antibodies apparently are bactericidal for the gonococcus. IgG that reacts with Rmp blocks the bactericidal activity of antibodies directed against Por and LOS. Genital infection with *N. gonorrhoeae* stimulates a serum antibody response against the LOS of the infecting strain. Disseminated gonococcal infection results in much higher levels of anti-LOS antibody than do genital infections.

Strains that cause uncomplicated genital infections usually are killed by normal human serum and are termed **serum sensitive**. This bactericidal activity is mediated by IgM and IgG antibodies that recognize sites on the LOS. Strains that cause disseminated infections are not killed by most normal human serum and are referred to as **serum resistant**. Resistance is mediated, in part, by IgA that blocks the IgG-mediated bactericidal activity of the serum. Serum from convalescent patients with disseminating infections contains bactericidal IgG to the LOS of the infecting strain.

Individuals with inherited complement deficiencies have a markedly increased risk of acquiring systemic neisserial infections and are subject to recurring episodes of systemic gonococcal **and** meningococcal infections, indicating that the complement system is important in host defense. Gonococci activate complement by both the classic and alternative pathways. Complement activation by gonococci leads to the formation of the C5b-9 complex (membrane attack complex) on the outer membrane. In normal human serum, similar numbers of C5b-9 complexes are deposited on serum-sensitive and serum-resistant organisms, but the membrane attack complex is not functional on serum-resistant organisms.

Treatment

The recommended treatment for uncomplicated infections is a third-generation cephalosporin or a fluoroquinolone plus an antibiotic (e.g., doxycycline or erythromycin) effective against possible coinfection with *Chlamydia trachomatis*. Sex partners should be referred and treated. The current **CDC Treatment Guidelines** recommend treatment of all gonococcal infections with antibiotic regimens effective against resistant strains. The recommended antimicrobial agents are ceftriaxone, cefixime, ciprofloxacin, or oflaxacin.

Control

There is no effective vaccine to prevent gonorrhea. Candidate vaccines consisting of **PilE** protein or **Por** are of little benefit. The development of an effective vaccine has been hampered by the lack of a suitable animal model and the fact that an effective immune response has never been demonstrated. Condoms are effective in preventing the transmission of gonorrhea.

The evolution of antimicrobial resistance in *N. gonorrhoeae* may ultimately affect the control of gonorrhea. Strains with multiple chromosomal resistance to penicillin, tetracycline, erythromycin, and cefoxitin have been identified in the United States and most other parts of the world. Sporadic high-level resistance to spectinomycin and fluoroquinolones has been reported. Penicillinase-producing strains of *N. gonorrhoeae* were first described in 1976. Five related β-lactamase plasmids of different sizes have been identified. Their prevalence penicillin-resistant strains has increased dramatically in the United States since 1984.

Plasmid-mediated resistance of *N. gonorrhoeae* to tetracycline was first described in 1986 and has now been reported in most parts of the world. This resistance is due to the presence of the streptococcal tetM determinant on a gonococcal conjugative plasmid.

Tailpiece

The only natural host for *N. gonorrhoeae* is humans. Gonorrhea has all but disappeared in Scandinavia and several other European countries. However, the disease is very common in the United States. CDC estimates that more than 700,000 persons in the U.S. get new gonorrheal infections each year. Only about half of these infections are reported to CDC. In 2002, 351,852 cases of gonorrhea were reported to CDC. In the period from 1975 to 1997, the national gonorrhea rate declined, following the implementation of the national gonorrhea control program in the mid-1970s. After a small increase in 1998, the gonorrhea rate has decreased slightly since 1999. In 2002, the rate of reported gonorrheal infections was 125.0 per 100,000 persons.

Gonorrhea is transmitted almost exclusively by sexual contact. Any sexually active person can be infected with

gonorrhea. In the United States, the highest reported rates of infection are among sexually active teenagers, young adults, and African Americans. Persons who have multiple sex partners are at highest risk. Rates of gonorrhea are higher in males and in minority and inner-city populations.

Gonorrhea is usually contracted from a sex partner who is either asymptomatic or has only minimal symptoms. It is estimated that the efficiency of transmission after one exposure is about 35 percent from an infected woman to an uninfected man and 50 to 60 percent from an infected man to an uninfected woman. More than 90 percent of men with urethral gonorrhea will develop symptoms within 5 days; fewer than 50 percent of women with genital gonorrhea will do so. Women with asymptomatic infections are at higher risk of developing pelvic inflammatory disease and disseminated gonococcal infection.

Neisseria meningitidis

The bacterium *Neisseria meningitidis*, the **meningococcus**, is identical in its staining and morphological characteristics to *Neisseria gonorrhoeae*. However, at the ultrastructural level, *N. meningitidis* has a prominent antiphagocytic polysaccharide capsule. *N. meningitidis* strains are grouped on the basis of their capsular polysaccharides, into 12 serogroups, some of which are subdivided according to the presence of outer membrane protein and lipopolysaccharide antigens.

Neisseria meningitidis is usually cultivated in a peptone-blood base medium in a moist chamber containing 5-10% CO₂. All media must be warmed to 37 degrees prior to inoculation as the organism is extremely susceptible to temperatures above or below 37 degrees. This trait is rather unique among bacteria. Also, the organism tends to undergo rapid autolysis after death, both in vitro and in vivo. This accounts for the dissemination of lipopolysaccharide (endotoxin) during septicemia and meningitis.

The organism tends to colonize the posterior nasopharynx of humans, and humans are the only known host. Individuals who are colonized are carriers of the pathogen who can transmit disease to nonimmune individuals. The bacterium also colonizes the posterior nasopharynx in the early stages of infection prior to invasion of the meninges. Most individuals in close contact with a case of meningococcal meningitis become carriers of the organism. This carrier rate can reach 20 percent of the contact group before the first case is recognized, and may reach as high as 80 percent at the height of an epidemic.

Structure and Classification

The only distinguishing structural feature between *N. meningitidis* and *N. gonorrhoeae* is the presence of a polysaccharide capsule in the former. The capsule is antiphagocytic and is an important virulence factor.

Meningococcal capsular polysaccharides provide the basis for grouping the organism. Twelve serogroups have been identified (A, B, C, H, I, K, L, X, Y, Z, 29E, and W135). The most important serogroups associated with disease in humans are A, B, C, Y, and W135. The chemical composition of these capsular polysaccharides is known. The prominent outer membrane proteins of *N. meningitidis* have been designated class 1 through class 5. The **class 2 and 3 proteins function as porins and are analogous to gonococcal Por**. The **class 4 and 5 proteins are analogous to gonococcal Rmp and Opa, respectively**. Serogroup B and C meningococci have been further subdivided on the basis of serotype determinants located on the class 2 and 3 proteins. A handful of serotypes are associated with most cases of meningococcal disease, whereas other serotypes within the same serogroup rarely cause disease. All known group A strains have the same protein serotype antigens in the outer membrane. Another serotyping system exists based on the antigenic diversity of meningococcal LOS.

Meningitis

The term **meningitis** refers to inflammation the meninges of the brain or spinal cord. Meninges are any of the three membranes that envelope the brain and spinal cord. The disease **meningitis** is caused by a number of different bacteria and viruses. Bacterial causes include *Haemophilus influenzae*, *Escherichia coli*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Neisseria meningitidis*. Although a variety of cocci cause meningitis, the term **meningococcus** is reserved for the Gram-negative, bean-shaped diplococcus, *Neisseria meningitidis*. Like its relative *N. gonorrhoeae*, the organism tends to occur intracellularly in the cytoplasm of neutrophils which are attracted to the site of inflammation in the meninges, so this type of infection is

called pyogenic (pus-forming).

Marchiafava and Celli were the first to report observing Gram-negative diplococci in cerebrospinal fluid of a fatal case of meningitis in 1884. In 1887, Weichselbaum isolated the bacterium from six cases of meningitis and established the isolates as a distinct species and proven to be the cause of meningitis.

Pathogenesis

Infection with *N. meningitidis* has two presentations, **meningococcemia**, characterized by skin lesions, and **acute bacterial meningitis**. The fulminant form of disease (with or without meningitis) is characterized by multisystem involvement and high mortality.

Infection is by aspiration of infective bacteria, which attach to epithelial cells of the nasopharyngeal and oropharyngeal mucosa, cross the mucosal barrier, and enter the bloodstream. If not clear whether blood-borne bacteria may enter the central nervous system and cause meningitis.

The mildest form of disease is a transient bacteremic illness characterized by a fever and malaise; symptoms resolve spontaneously in 1 to 2 days. The most serious form is the fulminant form of disease complicated by meningitis. The manifestations of meningococcal meningitis are similar to acute bacterial meningitis caused by other bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *E. coli*. Chills, fever, malaise, and headache are the usual manifestations of infection. Signs of meningeal inflammation are also present.

Clinical manifestations of *N. meningitidis* infection

The onset of meningococcal meningitis may be abrupt or insidious. Infants with meningococcal meningitis rarely display signs of meningeal irritation. Irritability and refusal to take food are typical; vomiting occurs early in the disease and may lead to dehydration. Fever is typically absent in children younger than 2 months of age.

Hypothermia is more common in neonates. As the disease progresses, apnea, seizures, disturbances in motor tone, and coma may develop.

In older children and adults, specific symptoms and signs are usually present, with fever and altered mental status the most consistent findings. Headache is an early, prominent complaint and is usually very severe. Nausea, vomiting, and photophobia are also common symptoms.

Neurologic signs are common; approximately one-third of patients have convulsions or coma when first seen by a physician. Signs of meningeal irritation such as spinal rigidity, hamstring spasms and exaggerated reflexes are common.

Petechiae (minute hemorrhagic spots in the skin) or purpura (hemorrhages into the skin) occurs from the first to the third day of illness in 30 to 60% of patients with meningococcal disease, with or without meningitis. The lesions may be more prominent in areas of the skin subjected to pressure, such as the axillary folds, the belt line, or the back.

Fulminant meningococcemia occurs in 5 to 15% of patients with meningococcal disease and has a high mortality rate. It begins abruptly with sudden high fever, chills, myalgias, weakness, nausea, vomiting, and headache. Apprehension, restlessness, and delirium occur within the next few hours. Widespread purpuric and ecchymotic skin lesions appear suddenly. Typically, no signs of meningitis are present. Pulmonary insufficiency develops within a few hours, and many patients die within 24 hours of being hospitalized despite appropriate antibiotic therapy and intensive care.



Figure 4. The characteristic skin rash (purpura) of meningococcal septicemia, caused by *Neisseria meningitidis*

Virulence Factors

For a time, the virulence of *Neisseria meningitidis* was attributed to the production of an "exotoxin" that could be recovered from culture filtrates of the organism. But when studies revealed that antitoxin reacted equally well with washed cells as culture filtrate, it was realized that the bacteria underwent autolysis during growth and released parts of their cell walls in a soluble form. Hence, the major toxin of *N. meningitidis* is its **lipooligosaccharide, LOS**, and its mechanism is endotoxic. The other important determinant of virulence of *N. meningitidis* is its **antiphagocytic polysaccharide capsule**.

The human nasopharynx is the only known reservoir of *N. meningitidis*. Meningococci are spread via respiratory droplets, and transmission requires aspiration of infective particles. Meningococci attach to the nonciliated columnar epithelial cells of the nasopharynx. Attachment is mediated by **fimbriae** and possibly by other outer membrane components. Invasion of the mucosal cells occurs by a mechanism similar to that observed with gonococci. Events involved after bloodstream invasion are unclear and how the meningococcus enters the central nervous system is not known.

Purified meningococcal LOS is highly toxic and is as lethal for mice as the LOS from *E. coli* or *Salmonella typhimurium*; however, meningococcal LOS is 5 to 10 times more effective than enteric LPS in eliciting a dermal Shwartzman phenomenon (a characteristic type of inflammatory reaction) in rabbits. Meningococcal LOS has been shown to suppress leukotriene B4 synthesis in human polymorphonuclear leukocytes. The loss of leukotriene B4 deprives the leukocytes of a strong chemokinetic and chemotactic factor.

Host Defenses

N. meningitidis establishes systemic infections only in individuals who lack serum bacterial antibodies directed against the capsular or noncapsular (cell wall) antigens of the invading strain, or in patients deficient in the late-acting complement components.

The integrity of the pharyngeal and respiratory epithelium appears to be important in protection from invasive disease. Chronic irritation of the mucosa due to dust or low humidity, or damage to the mucosa resulting from a concurrent upper respiratory infection, may be predisposing factors for invasive disease.

The presence of serum bactericidal IgG and IgM is probably the most important host factor in preventing invasive disease. These antibodies are directed against both capsular and noncapsular surface antigens. The antibodies are produced in response to colonization with carrier strains of *N. meningitidis*, as well as *N. lactamica*, and other nonpathogenic *Neisseria* species that are normal inhabitants of the upper respiratory tract. Protective antibodies are also stimulated by cross-reacting antigens on other bacterial species such as *Escherichia coli*. The role of bactericidal antibodies in prevention of invasive disease explains why high attack rates are seen in infants from 6 to 9 months old, the time at which maternal antibodies are being lost. Individuals with complement deficiencies (C5, C6, C7, or C8) may develop meningococcemia despite protective antibody. This emphasizes the importance of the

complement system in defense against meningococcal disease.

Epidemiology

The meningococcus usually inhabits the human nasopharynx without causing detectable disease. This carrier state may last for a few days to months and is important because it not only provides a reservoir for meningococcal infection but also stimulates host immunity. Between 5 and 30% of normal individuals are carriers at any given time, yet few develop meningococcal disease. Carriage rates are highest in older children and young adults. Attack rates highest in infants 3 months to 1 year old. Meningococcal meningitis occurs both sporadically (mainly groups B and C meningococci) and in epidemics (mainly group A meningococci), with the highest incidence during late winter and early spring. Whenever group A strains become prevalent in the population, the incidence of meningitis increases markedly.

Treatment

Penicillin is the drug of choice to treat meningococcemia and meningococcal meningitis. Although penicillin does not penetrate the normal blood-brain barrier, it readily penetrates the blood-brain barrier when the meninges are acutely inflamed. Either chloramphenicol or a third-generation cephalosporin such as cefotaxime or ceftriaxone is used in persons allergic to penicillins.

Meningococcal disease is contracted through association with infected individuals, as evidenced by the 500- to 800-fold greater attack rate among household contacts than among the general population. Because such household members are at high risk, they require chemoprophylaxis. Sulfonamides were the chemoprophylactic agent of choice until the emergence of sulfonamide-resistant meningococci. At present, approximately 25 percent of clinical isolates of *N. meningitidis* in the United States are resistant to sulfonamides; nowadays, rifampin is the chemoprophylactic agent of choice.

Control

Groups A, C, AC, and ACYW135 capsular polysaccharide vaccines are available. However, the polysaccharide vaccines are ineffective in young children (in children under 1 year old, antibody levels decline rapidly after immunization) and the duration of protection is limited in children vaccinated at 1 to 4 years of age. Routine vaccination is not currently recommended because the risk of infection is low. The group B capsular polysaccharide is a homopolymer of sialic acid and is not immunogenic in humans. A group B meningococcal vaccine consisting of outer membrane protein antigens has recently been developed, but is not licensed in the United States.

Tailpiece

Search for a universal vaccine for meningococcal meningitis

There is an obvious need for a universal vaccine for meningococcal meningitis, but the development of an effective vaccine against all forms of *N. meningitidis* has been hampered by the high degree of variation in the proteins on the surface of the bacterium which leads to the occurrence of many different antigenic types.

More than 10% of the population may be carrying the bacterium at any one time on the mucosal surfaces of the nose and throat. The majority of these carriers will not have any symptoms of the disease, but this continual exposure to the immune system puts pressure on the bacterium to mutate its surface components in order to survive. Thus, natural selection is the driving force for the emergence of new antigenic variants.

Among the class 2 and 3 outer membrane proteins of *N. meningitidis*, Por A has been considered a primary target for a vaccine-induced antibody. PorA is a major component of the outer membrane of *N. meningitidis*, and anti-PorA antibodies are thought to be a critical component in immunity. Interactions between antibodies and PorA have been studied. Different strains of the bacterium have different PorA amino acid sequences within the region of the protein that specifically binds to antibody molecules. PorA has several large amino acid "loop" regions that protrude from the surface, and it is these loops that are targets for antibody binding.

In the laboratory, the antigen-binding fragment (Fab) of anti-PorA antibodies can be crystallized and reacted with

the antigenic loop regions of PorA in order to determine the specificity of binding between antigen and antibody. Slight changes in PorA amino acid sequence have been shown to cause loss in the ability to bind to antibody molecules. In nature, the bacterium mutates to insert new amino acid residues into the tip of the loop, which alters or eliminates many of the interactions with antibody and allows the bacterium to bypass previous immune responses.

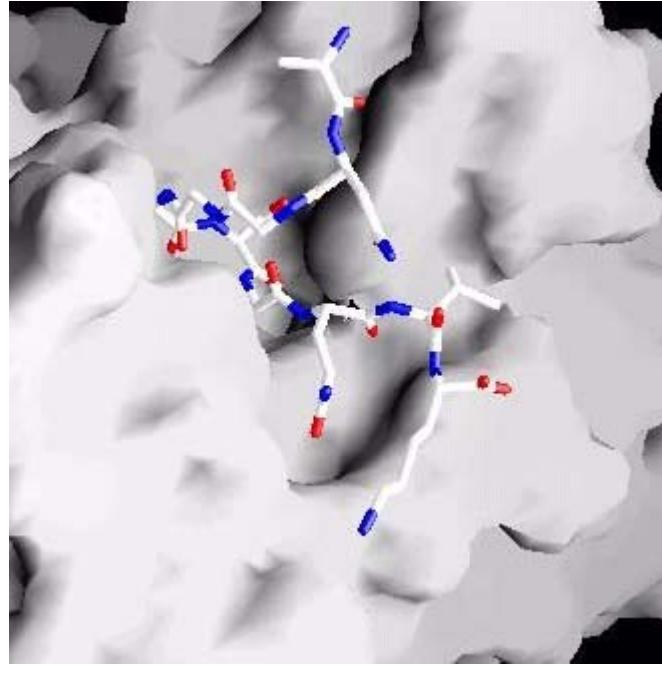
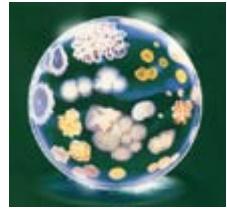


Figure 5. Image of the antibody (Fab) molecular surface, with the PorA antigen superimposed. The dark colored groove on the surface of the antibody matches precisely the shape of the PorA antigen; hence any changes in the sequence of PorA in this region can disrupt antibody binding. Jeremy Derrick, UMIST. [SRS ANNUAL REPORT 1999-2000](#)

Hence, by introducing changes into portions of the PorA protein that are exposed at the surface, the bacterium can evade the attention of the immune system. These alterations are apparently introduced without compromising the biological function of PorA, as a pore-forming protein. Designing vaccines that are able to take into account these changes is a huge challenge, but as more information of this type becomes known, it leads to a more rational approach to design of a universal vaccine for meningococcal meningitis.

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Haemophilus influenzae

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Introduction

Haemophilus influenzae is a small, nonmotile Gram-negative bacterium in the family *Pasteurellaceae*, on the level with the *Vibrionaceae* and the *Enterobacteriaceae*. The family also includes *Pasteurella* and *Actinobacillus*, two other genera of bacteria that are parasites of animals. Encapsulated strains of *Haemophilus influenzae* isolated from cerebrospinal fluid are coccobacilli, 0.2 to 0.3 to 0.5 to 0.8 μm , similar in morphology to *Bordetella pertussis*, the agent of whooping cough. Non encapsulated organisms from sputum are pleomorphic and often exhibit long threads and filaments. The organism may appear Gram-positive unless the Gram stain procedure is very carefully carried out. Furthermore, elongated forms from sputum may exhibit bipolar staining, leading to an erroneous diagnosis of *Streptococcus pneumoniae*.



Figure 1. Gram stain of *Haemophilus influenzae* from sputum

H. influenzae is highly adapted to its human host. It is present in the nasopharynx of approximately 75 percent of healthy children and adults. It is rarely encountered in the oral cavity and it has not been detected in any other animal species. It is usually the non encapsulated strains that are harbored as normal flora, but a minority of healthy individuals (3-7 percent) intermittently harbor *H. influenzae* type b (Hib) encapsulated strains in the upper respiratory tract. Pharyngeal carriage of Hib is important in the transmission of the bacterium. The success of current vaccination programs against Hib is due in part to the effect of vaccination on decreasing carriage of the organism.

What's in a name?

Haemophilus influenzae is widespread in its distribution among the human population. It was first isolated by Pfeiffer during the influenza pandemic of 1890. It was mistakenly thought to be the cause of the disease influenza, and it was named accordingly. Probably, *H. influenzae* was an important secondary invader to the influenza virus in the 1890 pandemic, as it has been during many subsequent influenza epidemics. In pigs, a synergistic association between swine influenza virus and *Haemophilus. suis* is necessary for swine influenza. Similar situations between human influenza virus and *H. influenzae* have been observed in chick embryos and infant rats.

Haemophilus "loves heme", more specifically it requires a precursor of heme in order to grow. Nutritionally, *Haemophilus influenzae* prefers a complex medium and requires preformed growth factors that are present in blood, specifically **X factor** (i.e., hemin) and **V factor** (NAD or NADP). In the laboratory it is usually grown on chocolate blood agar which is prepared by adding blood to an agar base at 80 degrees. The heat releases X and V factors from the RBCs and turns the medium a chocolate brown color. The bacterium grows best at 35-37 degrees and has an optimal pH of 7.6. *Haemophilus influenzae* is generally grown in the laboratory under aerobic conditions or under slight CO₂ tension (5% CO₂), although it is capable of glycolytic growth and of respiratory growth using

nitrate as a final electron acceptor.

In 1995, *Haemophilus influenzae* was the first free-living organism to have its entire chromosome sequenced, sneaking in just ahead of *Escherichia coli* in that race, mainly because its genome is smaller in size than *E. coli*'s. For a relatively obscure bacterium, there was already a good understanding of its genetic processes, especially transformation.

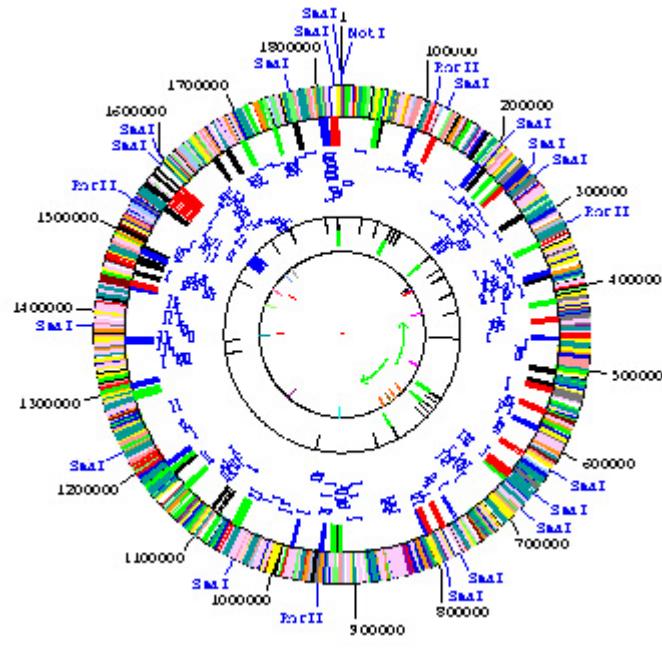


Figure 2. A map of the circular chromosome of *Haemophilus influenzae* illustrating the location of known genes and predicted coding regions

Observations of genetic transformation in *Haemophilus* have included drug resistance and synthesis of specific capsular antigens. The latter is thought to be the main determinant of *H. influenzae*.

Transformation in *Haemophilus influenzae* occurs by several different mechanisms and is more efficient than in enteric bacteria. When developing competence, the bacterium develops membranous "blebs" in the outer membrane that contain a specific DNA-binding protein. This outer membrane protein recognizes a specific 11-base pair sequence of DNA nucleotides that appears in *Haemophilus* DNA with much higher frequency than in other genera of bacteria. There is some evidence that *Haemophilus* is able to undergo both interspecies and intraspecies transformation *in vivo* (in host tissues). The restriction endonucleases from *Haemophilus*, e.g. **Hind III**, are widely used in biotechnology and in the analysis and cloning of DNA.

Pathogenesis

The pathogenesis of *H. influenzae* infections is not completely understood, although the presence of the **type b polysaccharide capsule** is known to be the major factor in virulence. Encapsulated organisms can penetrate the epithelium of the nasopharynx and invade the blood capillaries directly. Their capsule allows them to resist phagocytosis and complement-mediated lysis in the nonimmune host. Nontypable (non encapsulated) strains are less invasive, but they are apparently able to induce an inflammatory response that causes disease. Outbreaks of *H. influenzae* type b infection may occur in nurseries and child care centers, and prophylactic administration of antibiotics is warranted. Vaccination with type b polysaccharide (in the form of **Hib conjugate vaccines**) is effective in preventing infection, and several vaccines are now available for routine use.

Naturally-acquired disease caused by *H. influenzae* seems to occur in humans only. In infants and young children (under 5 years of age), *H. influenzae* type b causes **bacteremia** and acute bacterial **meningitis**. Occasionally, it causes **epiglottitis** (obstructive laryngitis), **cellulitis**, **osteomyelitis**, and **joint infections**. Nontypable *H. influenzae* causes ear infections (**otitis media**) and **sinusitis** in children, and is associated with **respiratory tract infections** (pneumonia) in infants, children and adults.

Haemophilus influenzae infections

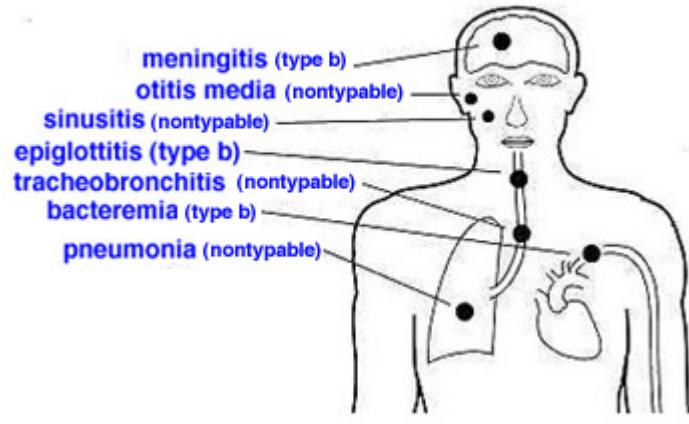


Figure 3. Tissues infected by type b and nontypable strains of *Haemophilus influenzae*

Seven serotypes of the bacterium have been identified on the basis of capsular polysaccharides. Until the implementation of widespread vaccination programs, type b *H. influenzae* was the most common cause of meningitis in children between the ages of 6 months and 2 years (see Figure 4 below), resulting in 12,000 to 20,000 cases annually in the U.S. It would be interesting to view comparative data since the era of vaccination against *H. influenzae* meningitis, which began in 1985. Certainly, there are fewer than 100 cases annually of bacterial meningitis caused by *H. influenzae* type b.

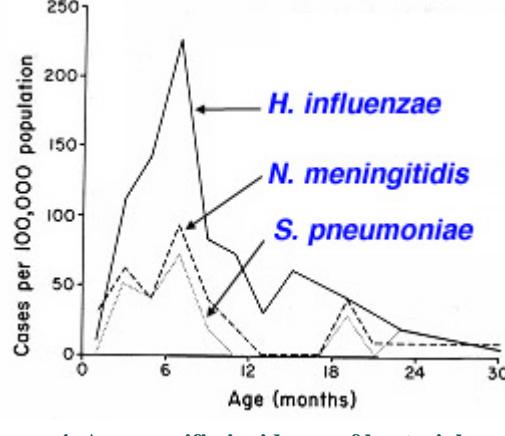


Figure 4. Age-specific incidence of bacterial meningitis caused by *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* prior to 1985

Disease caused by *H. influenzae* usually begins in the upper respiratory tract as nasopharyngitis and may be followed by sinusitis and otitis, possibly leading to pneumonia. In severe cases, bacteremia may occur which frequently results in joint infections or meningitis.

Virulence

H. influenzae does not produce any demonstrable exotoxins. The direct role of **endotoxin** in meningitis or bacteremia is unclear, although the Gram-negative bacterium's outer membrane **lipooligosaccharide** is thought to play a role in inflammation associated with otitis media. All virulent strains produce **neuraminidase** and an **IgA protease**, but the role of these extracellular enzymes in invasion is unproven. **Fimbriae** increase the adherence of bacteria to human mucosal cells in vitro, and they are required for successful colonization of the nasopharynx. The Anton antigen, as defined in red blood cells, appears to be the receptor.

Virulence, at least in the case of bacteremia and meningitis, is directly related to capsule formation. Virtually all of these infections are caused by the type b serotype, and its capsular polysaccharide, containing ribose, ribitol and phosphate, is the proven determinant of virulence. The capsule material is antiphagocytic, and it is ineffective in inducing the alternative complement pathway, so that the bacterium can invade the blood or cerebrospinal fluid without attracting phagocytes or provoking an inflammatory response and complement-mediated bacteriolysis. For this reason, anticapsular antibody, which promotes both phagocytosis and bacteriolysis, is the main factor in

immune defense against *H. influenzae* infections (below).

The **polyribosyl ribitol phosphate (PRP) capsule** is the most important virulence factor because it renders type b *H. influenzae* resistant to phagocytosis by polymorphonuclear leukocytes in the absence of specific anticapsular antibody, and it reduces the bacterium's susceptibility to the bactericidal effect of serum. However, susceptibility to the bactericidal effect of serum depends on the presence of antibodies to a number of other antigenic sites, including the **lipooligosaccharide** and **outer membrane proteins** designated as **P1** and **P2**.

Type b *H. influenzae* is plainly the most virulent of the *Haemophilus* species; 95 percent of bloodstream and meningeal *Haemophilus* infections in children are due to this bacterium. In contrast, in adults, nontypable strains of *H. influenzae* are the most common cause of *Haemophilus* infection, presumably because most adults have naturally acquired antibody to PRP.

Immunity

The age incidence of *H. influenzae* meningitis is inversely proportional to the titer of bactericidal antibody in the blood, whether passively acquired from the mother or actively formed (see Figure 5 below). Without artificial immunization, in children aged 2 months to 3 years, antibody levels are minimal; thereafter antibody levels increase and the disease becomes much less common. From this curve, it is obvious that artificial active immunization should begin at 2 months of age, when nearly all passive immunity has waned, and the child enters a vulnerable non immune period of life.

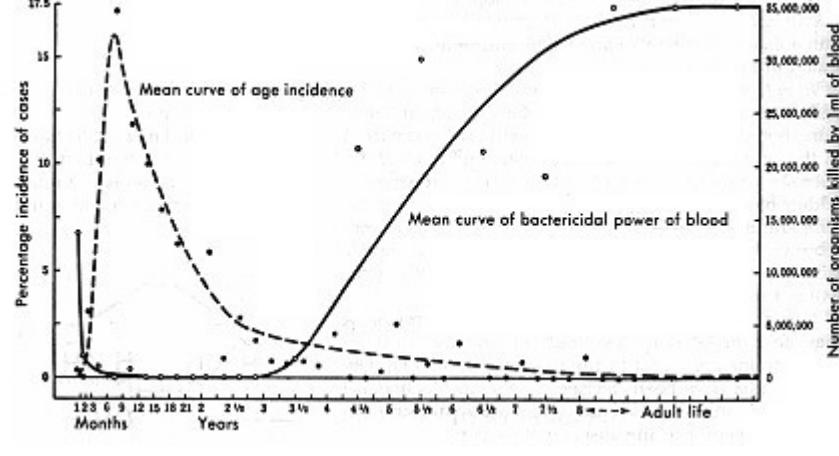


Figure 5. Relation of the age incidence of bacterial meningitis caused by *Haemophilus influenzae* to bactericidal antibody titers in the blood (data pre 1985)

H. influenzae is susceptible to lysis by antibody and complement. Furthermore, anticapsular antibodies promote phagocytosis, as well as bacteriolysis. Thus, serum antibody, complement, lysozyme and phagocytes can work in concert during a bacteremia. During meningitis, phagocytosis is probably the main host defense mechanism since complement rarely occurs in the cerebrospinal fluid.

For many years it was believed that bactericidal antibody directed against PRP capsule of *H. influenzae* type b was entirely responsible for host resistance to infection. However, some recent studies have stressed a role for antibody to somatic (cell wall) antigens as well. For example, antibody to PRP can often be detected in the sera of children on admission to the hospital with sepsis due to *H. influenzae* type b. Adsorption of immune serum with PRP alone does not remove its protective capabilities, whereas adsorption with whole organisms does. Separation of the outer membrane of type b *H. influenzae* into its many protein constituents reveals several individual membrane proteins that may be associated with immunity. Bactericidal antibodies that react with individual outer membrane proteins or with lipooligosaccharide constituents have been identified. These findings support indicate the potential importance of antibody to noncapsular antigens in immunity to *H. influenzae* type b infection. In addition, opsonizing antibodies, which also play a role in protection, may be directed against PRP or somatic constituents (see Figure 6 below).

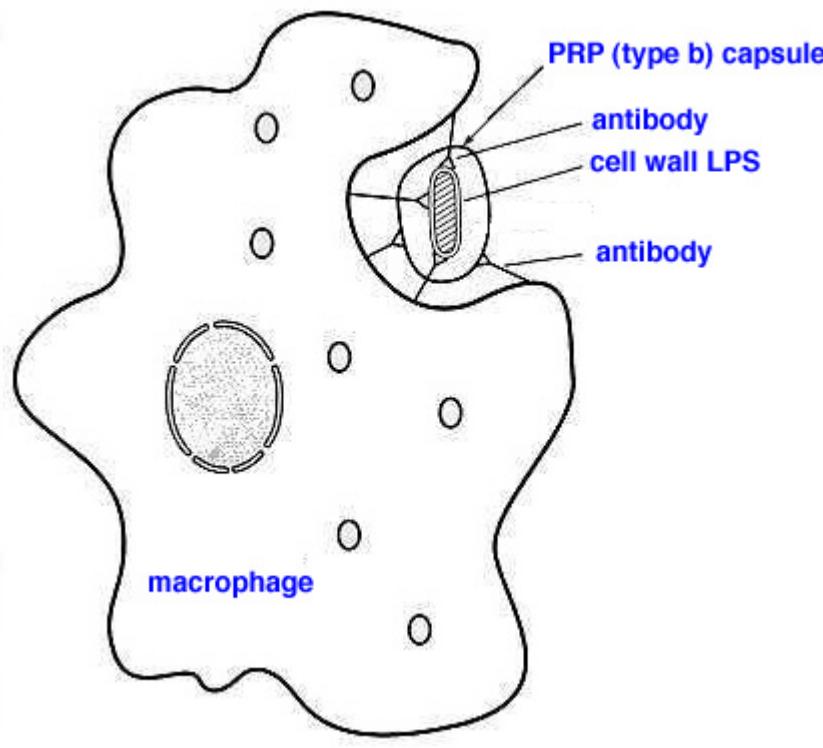


Figure 6. Phagocytic engulfment of *H. influenzae* bacterium opsonized by antibodies specific for the capsule and somatic (cell wall) antigen

Recent studies of nontypable *H. influenzae* have shown that bactericidal antibody to outer membrane proteins develop in infants in response to otitis media caused by the organism. Normal adults generally have both bactericidal and opsonizing antibodies directed against nontypable *H. influenzae*.

Treatment and Prevention

Virtually all patients treated early in the course of *H. influenzae* meningitis are cured. The mortality rate of treated infections is less than 10 percent, but nearly 30 percent of the children who recover have residual neurologic effects. Ampicillin has been the drug of choice, but presently over 20 percent of all strains of *H. influenzae* are resistant to ampicillin because of plasmid-mediated β -lactamase production.

The recommended treatment for *H. influenzae* meningitis is ampicillin for strains of the bacterium that do not make β -lactamase, and a third-generation cephalosporin or chloramphenicol for strains that do. Amoxicillin, together with a substance such as clavulanic acid, that blocks the activity of β -lactamase, has been unreliable in treatment of meningitis, although it is effective in treatment of sinusitis, otitis media and respiratory infections. Chloramphenicol was long considered the drug of choice for meningitis caused by penicillin-resistant *H. influenzae*, and it is still highly effective, but not without potential toxic side effects. Third-generation cephalosporins, such as ceftriaxone or cefotaxime, are effective against *H. influenzae* and penetrate the meninges well. Tetracyclines and sulfa drugs remain effective in treating sinusitis or respiratory infection caused by nontypable *H. influenzae*. Amoxicillin plus clavulanic acid (Augmentin) is effective against β -lactamase producing strains. Erythromycin is ineffective in treatment of *H. influenzae* infections.

The use of polyribosyl ribitol phosphate (PRP) vaccine and, more recently, protein-conjugated PRP, has vastly reduced the frequency of infection due to type b *H. influenzae*. The **PRP vaccine** consists of the type b capsular polysaccharide. Like most bacterial polysaccharides, it elicits a strong primary antibody response, but with little induction of memory. *H. influenzae* type b **Hib conjugate vaccines**, which couple the polysaccharide to a protein, induce memory type antibody responses in children and are effective in younger infants who are at higher risk for the disease.

There are **several types of Hib conjugate vaccines** available for use. All of the vaccines are approved for use in children 15 months of age and older and some are approved for use in children beginning at 2 months of age. All of the vaccines are considered effective. The vaccines are given by injections. More than 90% of infants obtain long term immunity with 2-3 doses of the vaccine.

All children should have a vaccine approved for infants beginning at 2 months. Depending on the type used, the recommended schedule for infants will vary. All unvaccinated children 15 - 59 months old should receive a single dose of conjugate vaccine. Children 60 months of age or older and adults normally do not need to be immunized.

Whether the vaccine provides protection against ear infections is not known. It also does not protect against diseases caused by other types of *Haemophilus*, nor does it protect against meningitis caused by other types of bacteria.

Specific characteristics of the four conjugate vaccines available for infants and children vary based on the type of protein carrier, the size of the polysaccharide, and the chemical linkage between the polysaccharide and carrier (see Table 1 below).

Current recommendations for vaccination of infants require parenteral administration of three different vaccines, diphtheria-tetanus-pertussis (DTP), Hib conjugate, and hepatitis B, during two or three different visits to a health-care provider. TETRAMUNE (see table footnote below) is the first licensed combination vaccine that provides protection against diphtheria, tetanus, pertussis, and Hib disease.

Table 1. Hib conjugate vaccines licensed for use among children

Vaccine	Trade name (manufacturer)	Polysaccharide	Linkage	Protein carrier
PRP-D	ProHIBiT (Connaught)	Medium	6-carbon	Diphtheria toxoid
HbOC*	HibTITER (Lederle-Praxis)	Small	None	CRM197 mutant <i>Corynebacterium diphtheriae</i> toxin protein
PRP-OMP	PedvaxHIB (Merck Sharp and Dohme)	Medium	Thioether	<i>Neisseria meningitidis</i> outer membrane protein complex
PRP-T	ActHIB OmniHIB (Pasteur Mérieux Vaccins)	Large	6-carbon	Tetanus toxoid

* TETRAMUNE consists of HbOC and DTP vaccine (TRI-IMMUNOL), also manufactured by Lederle-Praxis.

Tailpiece

Before 1985, *Haemophilus influenzae* type b (Hib) was the most common cause of bacterial meningitis in children under 5 years of age (approximately 12,000 cases per year, most in children younger than 18 months). Approximately 5% of affected children died, and neurologic sequelae developed in 15% to 30% of the surviving children. An additional estimated 7,500 cases of other invasive Hib infections also occurred annually in young children. The cumulative risk for Hib invasive disease before the age of 5 was one in 200 children, similar to the risk for poliomyelitis during the 1950s.

In 1985, the first Hib polysaccharide vaccines were licensed for use in the United States. These vaccines contained purified polyribosylribitol phosphate (PRP) capsular material from the type b serovar. Antibody against PRP was shown to be the primary component of serum bactericidal activity against the organism. PRP vaccines were ineffective in children less than 18 months of age because of the T-cell-independent nature of the immune response to PRP polysaccharide.

Conjugation of the PRP polysaccharide with protein carriers confers T-cell-dependent characteristics to the vaccine and substantially enhances the immunologic response to the PRP antigen. In 1989, the first Hib conjugate vaccines were licensed for use among children 15 months of age or older. In 1990, two new vaccines were approved for use among infants.

The incidence of Hib invasive disease among children aged 4 years or younger has declined by 98% since the introduction of Hib conjugate vaccines. One goal of the **Childhood Immunization Initiative** was to eliminate invasive Hib disease among children aged 4 years or younger by 1996. However, approximately 300 cases of *Haemophilus influenzae* invasive disease per year continue to be reported in the U.S., mainly in non immunized children. Most cases are caused by nontypable *Haemophilus influenzae*. The bar graph below (Figure 7) shows the age distribution of cases in 1996 and is comparable to Figure 5, which displays results from the pre-immunization era.

Number of cases

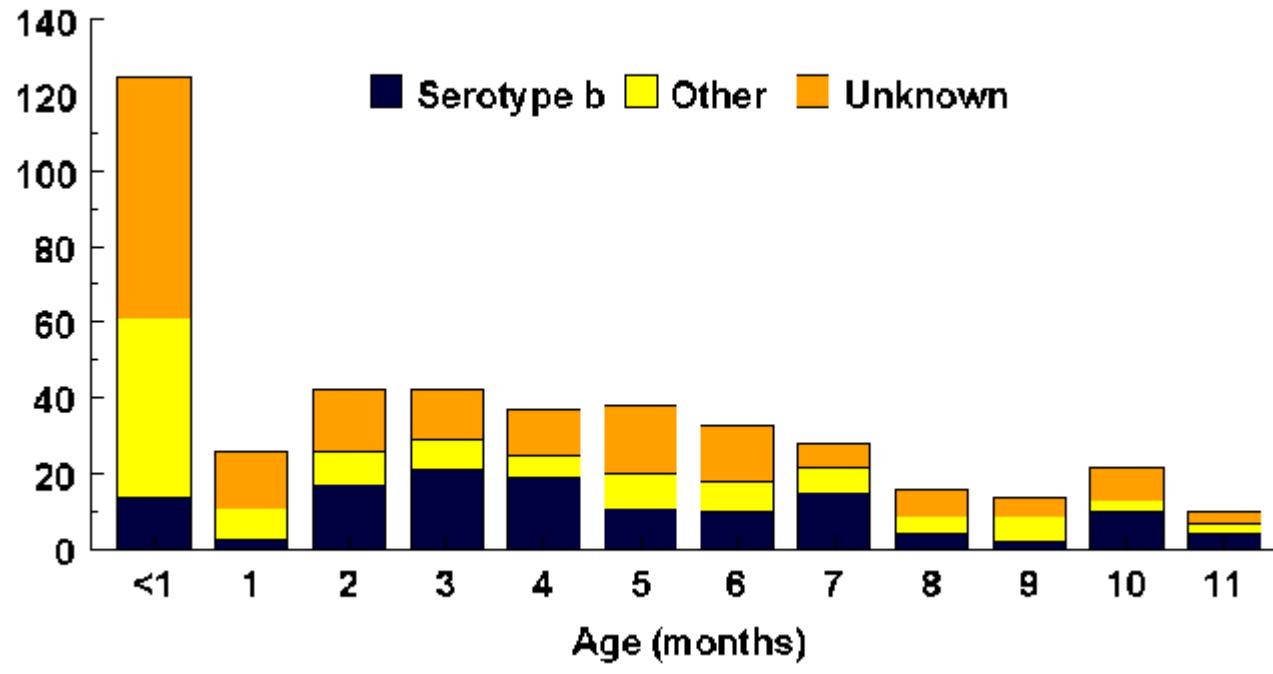
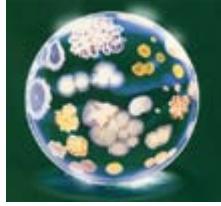


Figure 7. Age-

specific incidence of bacterial meningitis in children caused by *Haemophilus influenzae* in 1996

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Pseudomonas aeruginosa

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Gram stain of *Pseudomonas aeruginosa* cells

Pseudomonas aeruginosa is the epitome of an opportunistic pathogen of humans. The bacterium almost never infects uncompromised tissues, yet there is hardly any tissue that it cannot infect if the tissue defenses are compromised in some manner.

Pseudomonas aeruginosa is a Gram-negative, aerobic rod belonging to the bacterial family ***Pseudomonadaceae***. The family includes other genera, which, together with certain other organisms, constitute the bacteria informally known as **pseudomonads**. These bacteria are common inhabitants of soil and water. They occur regularly on the surfaces of plants and occasionally on the surfaces of animals. The pseudomonads are well known to plant microbiologists because they are one of the few groups of bacteria that are true pathogens of plants. In fact, *Pseudomonas aeruginosa* is occasionally a pathogen of plants. But *Pseudomonas aeruginosa* and two former *Pseudomonas* species (now reclassified as *Burkholderia*) are pathogens of humans. A general treatment of the pseudomonads is presented in [The Genus *Pseudomonas*](#). This chapter deals specifically with *Pseudomonas aeruginosa* as a pathogen of humans.

Pseudomonas aeruginosa is an **opportunistic pathogen**, meaning that it exploits some break in the host defenses to initiate an infection. It causes **urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections** and a variety of **systemic infections**, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed. *Pseudomonas aeruginosa* infection is a serious problem in patients hospitalized with cancer, cystic fibrosis, and burns. The case fatality rate in these patients is 50 percent.

Pseudomonas aeruginosa is primarily a **nosocomial pathogen**. According to the CDC, the overall incidence of *P. aeruginosa* infections in US hospitals averages about 0.4 percent (4 per 1000 discharges), and the bacterium is the fourth most commonly-isolated nosocomial pathogen accounting for 10.1 percent of all hospital-acquired infections.

Characteristics

Pseudomonas aeruginosa is a Gram-negative rod measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm . Almost all strains are motile by means of a single polar flagellum.

The bacterium is ubiquitous in soil and water, and on surfaces in contact with soil or water. Its metabolism is respiratory and never fermentative, but it will grow in the absence of O_2 if NO_3^- is available as a respiratory electron acceptor.

The typical *Pseudomonas* bacterium in nature might be found in a **biofilm**, attached to some surface or substrate, or in a **planktonic form**, as a unicellular organism, actively swimming by means of its flagellum. *Pseudomonas* is one of the most vigorous, fast-swimming bacteria seen in hay infusions and pond water samples.

In its natural habitat *Pseudomonas aeruginosa* is not particularly distinctive as a pseudomonad, but it does have a combination of physiological traits that are noteworthy and may relate to its pathogenesis.

--*Pseudomonas aeruginosa* has very simple nutritional requirements. It is often observed "growing in distilled water" which is evidence of its minimal nutritional needs. In the laboratory, the simplest medium for growth of *Pseudomonas aeruginosa* consists of acetate for carbon and ammonium sulfate for nitrogen.

--*P. aeruginosa* possesses the metabolic versatility for which pseudomonads are so renowned. Organic growth factors are not required, and it can use more than seventy-five organic compounds for growth.

--Its optimum temperature for growth is 37 degrees, and it is able to grow at temperatures as high as 42 degrees.

--It is tolerant to a wide variety of physical conditions, including temperature. It is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics.

--*Pseudomonas aeruginosa* has a predilection for growth in moist environments, which is probably a reflection of its natural existence in soil and water.

These natural properties of the bacterium undoubtedly contribute to its ecological success as an opportunistic pathogen. They also help explain the ubiquitous nature of the organism and its prominence as a nosocomial pathogen.

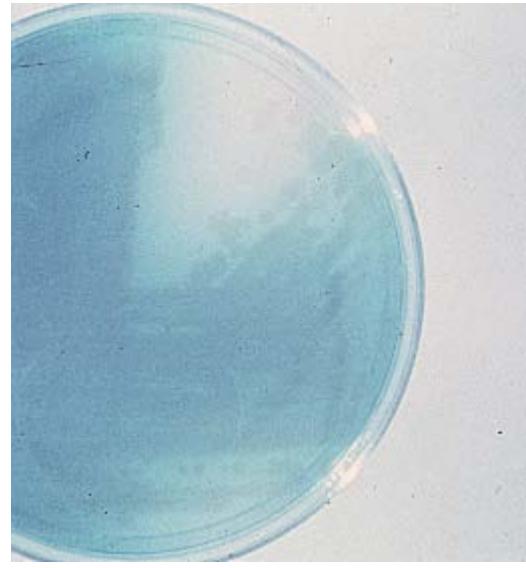
P. aeruginosa isolates may produce three **colony types**. Natural isolates from soil or water typically produce a small, **rough** colony. Clinical samples, in general, yield one or another of two smooth colony types. One type has a fried-egg appearance which is large, **smooth**, with flat edges and an elevated appearance. Another type, frequently obtained from respiratory and urinary tract secretions, has a **mucoid** appearance, which is attributed to the production of **alginate slime**. The smooth and mucoid colonies are presumed to play a role in colonization and virulence.



Pseudomonas aeruginosa colonies on agar

P. aeruginosa strains produce two types of soluble pigments, the fluorescent pigment **pyoverdin** and the blue pigment **pyocyanin**. The latter is produced abundantly in media of low-iron content and functions in iron metabolism in the bacterium. Pyocyanin (from "pyocyaneus") refers to "blue pus" which is a characteristic of

suppurative infections caused by *Pseudomonas aeruginosa*.



The soluble blue pigment pyocyanin is produced by many, but not all, strains of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is notorious for its **resistance to antibiotics** and is, therefore, a particularly dangerous and dreaded pathogen. The bacterium is naturally resistant to many antibiotics due to the permeability barrier afforded by its outer membrane LPS. Also, its tendency to colonize surfaces in a biofilm form makes the cells impervious to therapeutic concentrations antibiotics. Since its natural habitat is the soil, living in association with the bacilli, actinomycetes and molds, it has developed resistance to a variety of their naturally-occurring antibiotics. Moreover, *Pseudomonas* maintains **antibiotic resistance plasmids**, both R-factors and RTFs, and it is able to transfer these genes by means of the bacterial processes of transduction and conjugation.

Only a few antibiotics are effective against *Pseudomonas*, including fluoroquinolones, gentamicin and imipenem, and even these antibiotics are not effective against all strains. The futility of treating *Pseudomonas* infections with antibiotics is most dramatically illustrated in cystic fibrosis patients, virtually all of whom eventually become infected with a strain that is so resistant that it cannot be treated.

Diagnosis

Diagnosis of *P. aeruginosa* infection depends upon isolation and laboratory identification of the bacterium. It grows well on most laboratory media and commonly is isolated on blood agar or eosin-methylthionine blue agar. It is identified on the basis of its Gram morphology, inability to ferment lactose, a positive oxidase reaction, its fruity odor, and its ability to grow at 42° C. Fluorescence under ultraviolet light is helpful in early identification of *P. aeruginosa* colonies. Fluorescence is also used to suggest the presence of *P. aeruginosa* in wounds.

Pathogenesis

For an opportunistic pathogen such as *Pseudomonas aeruginosa*, the disease process begins with some alteration or circumvention of normal host defenses. The pathogenesis of *Pseudomonas* infections is multifactorial, as suggested by the number and wide array of virulence determinants possessed by the bacterium. Multiple and diverse determinants of virulence are expected in the wide range of diseases caused, which include **septicemia, urinary tract infections, pneumonia, chronic lung infections, endocarditis, dermatitis, and osteochondritis**.

Most *Pseudomonas* infections are both invasive and toxinogenic. The ultimate *Pseudomonas* infection may be seen as composed of three distinct stages: (1) bacterial attachment and colonization; (2) local invasion; (3) disseminated systemic disease. However, the disease process may stop at any stage. Particular bacterial determinants of virulence mediate each of these stages and are ultimately responsible for the characteristic syndromes that accompany the disease.

Colonization

Although colonization usually precedes infections by *Pseudomonas aeruginosa*, the exact source and mode of

transmission of the pathogen are often unclear because of its ubiquitous presence in the environment. It is sometimes present as part of the normal flora of humans, although the prevalence of colonization of healthy individuals outside the hospital is relatively low (estimates range from 0 to 24 percent depending on the anatomical locale).

The fimbriae of *Pseudomonas* will adhere to the epithelial cells of the upper respiratory tract and, by inference, to other epithelial cells as well. These adhesins appear to bind to specific galactose or mannose or sialic acid receptors on epithelial cells. Colonization of the respiratory tract by *Pseudomonas* requires **fimbrial adherence** and may be aided by production of a protease enzyme that degrades fibronectin in order to expose the underlying fimbrial receptors on the epithelial cell surface. Tissue injury may also play a role in colonization of the respiratory tract since *P. aeruginosa* will adhere to tracheal epithelial cells of mice infected with Influenza virus but not to normal tracheal epithelium. This has been called **opportunistic adherence**, and it may be an important step in *Pseudomonas* keratitis and urinary tract infections, as well as infections of the respiratory tract.

The receptor on tracheal epithelial cells for *Pseudomonas* pili is probably sialic acid (N-acetylneurameric acid). Mucoid strains, which produce an **exopolysaccharide (alginate)** have an additional or alternative adhesin which attaches to the tracheobronchial mucin (N-acetylgalactosamine). Besides pili and the mucoid polysaccharide, there are possibly two other cell surface adhesins utilized by *Pseudomonas* to colonize the respiratory epithelium or mucin. Also, it is likely that surface-bound **exoenzyme S** could serve as an adhesin for glycolipids on respiratory cells.

The mucoid exopolysaccharide produced by *P. aeruginosa* is a repeating polymer of mannuronic and glucuronic acid referred to as **alginate**. Alginate slime forms the matrix of the *Pseudomonas* **biofilm** which anchors the cells to their environment and, in medical situations, it protects the bacteria from the host defenses such as lymphocytes, phagocytes, the ciliary action of the respiratory tract, antibodies and complement. Biofilm mucoid strains of *P. aeruginosa* are also less susceptible to antibiotics than their planktonic counterparts. Mucoid strains of *P. aeruginosa* are most often isolated from patients with cystic fibrosis and they are usually found in post mortem lung tissues from such individuals.

Invasion

The ability of *Pseudomonas aeruginosa* to invade tissues depends upon production of extracellular enzymes and toxins that break down physical barriers and damage host cells, as well as resistance to phagocytosis and the host immune defenses. As mentioned above, the bacterial capsule or slime layer effectively protects cells from opsonization by antibodies, complement deposition, and phagocyte engulfment.

Two extracellular **proteases** have been associated with virulence that exert their activity at the invasive stage: **elastase** and **alkaline protease**. **Elastase** has several activities that relate to virulence. The enzyme cleaves collagen, IgG, IgA, and complement. It also lyses fibronectin to expose receptors for bacterial attachment on the mucosa of the lung. Elastase disrupts the respiratory epithelium and interferes with ciliary function. **Alkaline protease** interferes with fibrin formation and will lyse fibrin. Together, elastase and alkaline protease destroy the ground substance of the cornea and other supporting structures composed of fibrin and elastin. Elastase and alkaline protease together are also reported to cause the inactivation of gamma Interferon (IFN) and Tumor Necrosis Factor (TNF).

P. aeruginosa produces three other soluble proteins involved in invasion: a **cytotoxin** (mw 25 kDa) and two **hemolysins**. The cytotoxin is a pore-forming protein. It was originally named **leukocidin** because of its effect on neutrophils, but it appears to be cytotoxic for most eukaryotic cells. Of the two hemolysins, one is a **phospholipase** and the other is a **lecithinase**. They appear to act synergistically to break down lipids and lecithin. The cytotoxin and hemolysins contribute to invasion through their cytotoxic effects on eukaryotic cells.

One *Pseudomonas* pigment is probably a determinant of virulence for the pathogen. The blue pigment, **pyocyanin**, impairs the normal function of human nasal cilia, disrupts the respiratory epithelium, and exerts a proinflammatory effect on phagocytes. A derivative of pyocyanin, **pyochelin**, is a **siderophore** that is produced under low-iron conditions to sequester iron from the environment for growth of the pathogen. No role in virulence is known for the fluorescent pigments.

Dissemination

Blood stream invasion and dissemination of *Pseudomonas* from local sites of infection is probably mediated by the same cell-associated and extracellular products responsible for the localized disease, although it is not entirely clear how the bacterium produces systemic illness. *P. aeruginosa* is resistant to phagocytosis and the serum bactericidal response due to its mucoid capsule and possibly LPS. The proteases inactivate complement, cleave IgG antibodies, and inactivate IFN, TNF and probably other cytokines. The Lipid A moiety of *Pseudomonas* LPS (endotoxin) mediates the usual pathologic aspects of Gram-negative septicemia, e.g. fever, hypotension, intravascular coagulation, etc. It is also assumed that *Pseudomonas***Exotoxin A** exerts some pathologic activity during the dissemination stage (see below).

Toxinogenesis

P. aeruginosa produces two extracellular protein toxins, **Exoenzyme S** and **Exotoxin A**. **Exoenzyme S** is probably an exotoxin. It has the characteristic subunit structure of the A-component of a bacterial toxin, and it has ADP-ribosylating activity (for a variety of eukaryotic proteins) characteristic of exotoxins. Exoenzyme S is produced by bacteria growing in burned tissue and may be detected in the blood before the bacteria are. It has been suggested that exoenzyme S may act to impair the function of phagocytic cells in the bloodstream and internal organs to prepare for invasion by *P. aeruginosa*.

Exotoxin A has exactly the same mechanism of action as the **diphtheria toxin**, it causes the ADP ribosylation of eukaryotic elongation factor 2. It is partially-identical to diphtheria toxin, but it is antigenically-distinct. It utilizes a different receptor on host cells, but otherwise it enters cells in the same manner as the diphtheria toxin and it has the exact enzymatic mechanism. The production of Exotoxin A is regulated by exogenous iron, but the details of the regulatory process are distinctly different in *C. diphtheriae* and *P. aeruginosa*.

Exotoxin A appears to mediate both local and systemic disease processes caused by *Pseudomonas aeruginosa*. It has necrotizing activity at the site of bacterial colonization and is thereby thought to contribute to the colonization process. Toxinogenic strains cause a more virulent form of pneumonia than nontoxinogenic strains. In terms of its systemic role in virulence, purified Exotoxin A is highly lethal for animals including primates. Indirect evidence involving the role of exotoxin A in disease is seen in the increased chance of survival in patients with *Pseudomonas* septicemia that is correlated with the titer of anti-exotoxin A antibodies in the serum. Also, tox⁻ mutants show a reduced virulence in some models.

Table 1 (below) is a summary of the virulence determinants of *Pseudomonas aeruginosa*. **Table 2** is a brief description of the diseases caused by *Pseudomonas aeruginosa*.

Table 1. Summary of the Virulence Determinants of Pathogenic *Pseudomonas aeruginosa*

Adhesins

- fimbriae (N-methyl-phenylalanine pili)
- polysaccharide capsule (glycocalyx)
- alginate slime (biofilm)

Invasins

- elastase
- alkaline protease
- hemolysins (phospholipase and lecithinase)
- cytotoxin (leukocidin)
- siderophores and siderophore uptake systems
- pyocyanin diffusible pigment

Motility/chemotaxis

- flagella

Toxins

Exoenzyme S
Exotoxin A
Lipopopolysaccharide

Antiphagocytic surface properties

capsules, slime layers
LPS

Defense against serum bactericidal reaction

slime layers, capsules
LPS
protease enzymes

Defense against immune responses

capsules, slime layers
protease enzymes

Genetic attributes

genetic exchange by transduction and conjugation
inherent (natural) drug resistance
R factors and drug resistance plasmids

Ecologic criteria

adaptability to minimal nutritional requirements
metabolic diversity
widespread occurrence in a variety of habitats



Pseudomonas aeruginosa Scanning electron micrograph. CDC

Table 2. Diseases caused by *Pseudomonas aeruginosa*

Endocarditis. *Pseudomonas aeruginosa* infects heart valves of IV drug users and prosthetic heart valves. The

organism establishes itself on the endocardium by direct invasion from the blood stream.

Respiratory infections. Respiratory infections caused by *Pseudomonas aeruginosa* occur almost exclusively in individuals with a compromised lower respiratory tract or a compromised systemic defense mechanism. Primary pneumonia occurs in patients with chronic lung disease and congestive heart failure. Bacteremic pneumonia commonly occurs in neutropenic cancer patients undergoing chemotherapy. Lower respiratory tract colonization of cystic fibrosis patients by mucoid strains of *Pseudomonas aeruginosa* is common and difficult, if not impossible, to treat.

Bacteremia and Septicemia. *Pseudomonas aeruginosa* causes bacteremia primarily in immunocompromised patients. Predisposing conditions include hematologic malignancies, immunodeficiency relating to AIDS, neutropenia, diabetes mellitus, and severe burns. Most *Pseudomonas* bacteremia is acquired in hospitals and nursing homes. *Pseudomonas* accounts for about 25 percent of all hospital acquired Gram-negative bacteremias.

Central Nervous System infections. *Pseudomonas aeruginosa* causes meningitis and brain abscesses. The organism invades the CNS from a contiguous structure such as the inner ear or paranasal sinus, or is inoculated directly by means of head trauma, surgery or invasive diagnostic procedures, or spreads from a distant site of infection such as the urinary tract.

Ear infections including external otitis. *Pseudomonas aeruginosa* is the predominant bacterial pathogen in some cases of external otitis including "swimmer's ear". The bacterium is infrequently found in the normal ear, but often inhabits the external auditory canal in association with injury, maceration, inflammation, or simply wet and humid conditions.

Eye infections. *Pseudomonas aeruginosa* can cause devastating infections in the human eye. It is one of the most common causes of bacterial keratitis, and has been isolated as the etiologic agent of neonatal ophthalmia. *Pseudomonas* can colonize the ocular epithelium by means of a fimbrial attachment to sialic acid receptors. If the defenses of the environment are compromised in any way the bacterium can proliferate rapidly and, through the production of enzymes such as elastase, alkaline protease and exotoxin A, cause a rapidly destructive infection that can lead to loss of the entire eye.

Bone and joint infections. *Pseudomonas* infections of bones and joints result from direct inoculation of the bacteria or the hematogenous spread of the bacteria from other primary sites of infection. Blood-borne infections are most often seen in IV drug users, and in conjunction with urinary tract or pelvic infections. *Pseudomonas aeruginosa* has a particular tropism for fibrocartilagenous joints of the axial skeleton. *Pseudomonas aeruginosa* causes chronic contiguous osteomyelitis, usually resulting from direct inoculation of bone, and is the most common pathogen implicated in osteochondritis after puncture wounds of the foot.

Urinary tract infections. Urinary tract infections (UTI) caused by *Pseudomonas aeruginosa* are usually hospital-acquired and related to urinary tract catheterization, instrumentation or surgery. *Pseudomonas aeruginosa* is the third leading cause of hospital-acquired UTIs, accounting for about 12 percent of all infections of this type. The bacterium appears to be among the most adherent of common urinary pathogens to the bladder uroepithelium. As in the case of *E. coli* urinary tract infection can occur via an ascending or descending route. In addition, *Pseudomonas* can invade the bloodstream from the urinary tract, and this is the source of nearly 40 percent of *Pseudomonas* bacteremias.

Gastrointestinal infections. *Pseudomonas aeruginosa* can produce disease in any part of the gastrointestinal tract from the oropharynx to the rectum. As in other forms of *Pseudomonas* disease, those involving the GI tract occur primarily in immunocompromised individuals. The organism has been implicated in perirectal infections, pediatric diarrhea, typical gastroenteritis, and necrotizing enterocolitis. The GI tract is also an important portal of entry in *Pseudomonas* septicemia.

Skin and soft tissue infections, including wound infections, pyoderma and dermatitis. *Pseudomonas aeruginosa* can cause a variety of skin infections, both localized and diffuse. The common predisposing factors are breakdown of the integument which may result from burns, trauma or dermatitis; high moisture conditions such as those found in the ear of swimmers and the toe webs of athletes and combat troops, in the perineal region and under diapers of infants, and on the skin of whirlpool and hot tub users. Individuals with AIDS are easily infected. *Pseudomonas* has also been implicated in folliculitis and unmanageable forms of acne vulgaris.

Host Defenses

Most strains of *P. aeruginosa* are resistant to killing in serum alone, but the addition of polymorphonuclear leukocytes results in bacterial killing. Killing is most efficient in the presence of type-specific opsonizing antibodies, directed primarily at the antigenic determinants of LPS. This suggests that phagocytosis is an important defense and that opsonizing antibody is the principal functioning antibody in protecting from *P. aeruginosa* infections.

Once *P. aeruginosa* infection is established, other antibodies, such as antitoxin, may be important in controlling disease. The observation that patients with diminished antibody responses (caused by underlying disease or associated therapy) have more frequent and more serious *P. aeruginosa* infections underscores the importance of antibody-mediated immunity in controlling infections. Cystic fibrosis is the exception. Most cystic fibrosis patients have high levels of circulating antibodies to bacterial antigens, but are unable to clear *P. aeruginosa* efficiently from their lungs. Cell-mediated immunity does not seem to play a major role in resistance or defense against *Pseudomonas* infections.

Epidemiology and Control of *P. aeruginosa* Infections

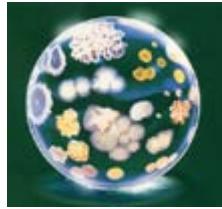
Pseudomonas aeruginosa is a common inhabitant of soil, water, and vegetation. It is found on the skin of some healthy persons and has been isolated from the throat (5 percent) and stool (3 percent) of nonhospitalized patients. The gastrointestinal carriage rates increase in hospitalized patients to 20 percent within 72 hours of admission.

Within the hospital, *P. aeruginosa* finds numerous reservoirs: disinfectants, respiratory equipment, food, sinks, taps, and mops. Furthermore, it is constantly reintroduced into the hospital environment on fruits, plants, vegetables, as well by visitors and patients transferred from other facilities. Spread occurs from patient to patient on the hands of hospital personnel, by direct patient contact with contaminated reservoirs, and by the ingestion of contaminated foods and water.

The spread of *P. aeruginosa* can best be controlled by observing proper isolation procedures, aseptic technique, and careful cleaning and monitoring of respirators, catheters, and other instruments. Topical therapy of burn wounds with antibacterial agents such as silver sulfadiazine, coupled with surgical debridement, dramatically reduces the incidence of *P. aeruginosa* sepsis in burn patients.

Pseudomonas aeruginosa is frequently resistant to many commonly used antibiotics. Although many strains are susceptible to gentamicin, tobramycin, colistin, and amikacin, resistant forms have developed. The combination of gentamicin and carbenicillin is frequently used to treat severe *Pseudomonas* infections. Several types of vaccines are being tested, but none is currently available for general use.

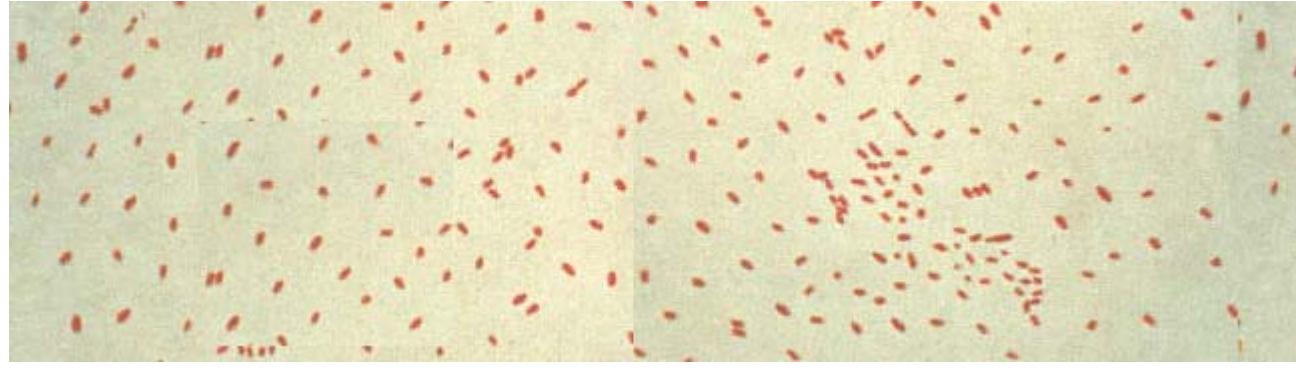
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Bordetella pertussis and Whooping Cough

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Bordetella pertussis, the agent of pertussis or whooping cough. Gram stain. (CDC)

Bordetella pertussis

Whooping cough (pertussis) is caused by the bacterium *Bordetella pertussis*. *B. pertussis* is a very small Gram-negative aerobic coccobacillus that appears singly or in pairs. Its metabolism is respiratory, never fermentative, and taxonomically, *Bordetella* is placed among the "Gram-negative Aerobic Rods and Cocci" in Bergey's Manual. *Bordetella* is not assigned to any family. The bacteria are nutritionally fastidious and are usually cultivated on rich media supplemented with blood. They can be grown in synthetic medium, however, which contains buffer, salts, an amino acid energy source, and growth factors such as nicotinamide (for which there is a strict requirement). Even on blood agar the organism grows slowly and requires 3-6 days to form pinpoint colonies.

Bordetella pertussis colonizes the cilia of the mammalian respiratory epithelium (Figure 1). Generally, it is thought that *B. pertussis* does not invade the tissues, but some recent work has shown the bacterium in alveolar macrophages. The bacterium is a pathogen for humans and possibly for higher primates, and no other reservoir is known. Whooping cough is a relatively mild disease in adults but has a significant mortality rate in infants. Until immunization was introduced in the 1930s, whooping cough was one of the most frequent and severe diseases of infants in the United States.

Pathogenesis

The disease pertussis has two stages. The first stage, colonization, is an upper respiratory disease with fever, malaise and coughing, which increases in intensity over about a 10-day period. During this stage the organism can be recovered in large numbers from pharyngeal cultures, and the severity and duration of the disease can be reduced by antimicrobial treatment. Adherence mechanisms of *B. pertussis* involve a "**filamentous hemagglutinin**" (FHA), which is a fimbrial-like structure on the bacterial surface, and **cell-bound pertussis toxin (PTx)**. Short range effects of soluble toxins play a role as well in invasion during the colonization stage.

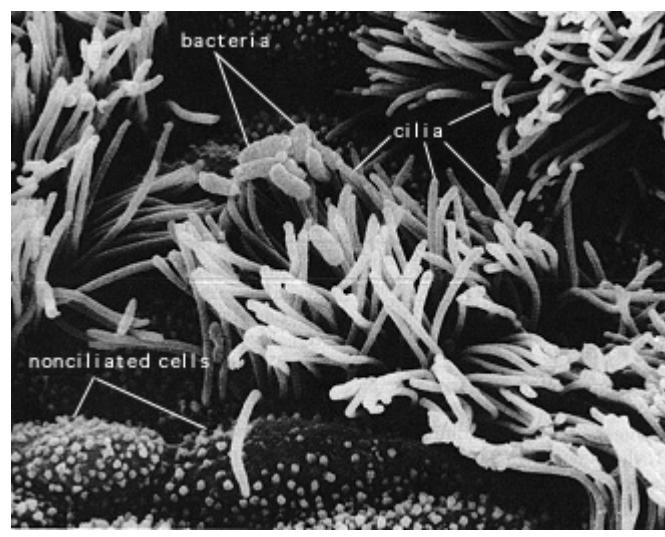


Figure 1. Colonization of tracheal epithelial cells by *Bordetella pertussis*

The second or toxemic stage of pertussis follows relatively nonspecific symptoms of the colonizaton stage. It begins gradually with prolonged and paroxysmal coughing that often ends in a characteristic inspiratory gasp (whoop). To hear the characteristic sound of whooping cough click [whoop.wav](#) (whoop.wav is copyright of Dr Doug Jenkinson, Nottingham, England. www.whoopingcough.net). During the second stage, *B. pertussis* can rarely be recovered, and antimicrobial agents have no effect on the progress of the disease. As described below, this stage is mediated by a variety of soluble toxins.

Colonization

Studies of *B. pertussis* and its adhesins have focused on cultured mammalian cells that lack most of the features of ciliated epithelial cells. However, some generalities have been drawn. The two most important colonization factors are the filamentous hemagglutinin (FHA) and the pertussis toxin (PTx). Filamentous hemagglutinin is a large (220 kDa) protein that forms filamentous structures on the cell surface. FHA binds to galactose residues on a sulfated glycolipid called sulfatide which is very common on the surface of ciliated cells. Mutations in the FHA structural gene reduce the ability of the organism to colonize, and antibodies against FHA provide protection against infection. However, it is unlikely that FHA is the only adhesin involved in colonization. The structural gene for FHA has been cloned and expressed in *E. coli*, raising the possibility of its production for use in a component vaccine.

One of the toxins of *B. pertussis*, the pertussis toxin (PTx), is also involved in adherence to the tracheal epithelium. Pertussis toxin is a 105 kDa protein composed of six subunits: S1, S2, S3, (2)S4, and S5. The toxin is both secreted into the extracellular fluid and cell bound. Some components of the cell-bound toxin (S2 and S3) function as adhesins, and appear to bind the bacteria to host cells. S2 and S3 utilize different receptors on host cells. S2 binds specifically to a glycolipid called lactosylceramide, which is found primarily on the ciliated epithelial cells. S3 binds to a glycoprotein found mainly on phagocytic cells.

The S1 subunit of pertussis toxin is the A component with ADP ribosylating activity, and the function of S2 and S3 is presumed to be involved in binding the intact (extracellular) toxin to its target cell surface. Antibodies against PTx components prevent colonization of ciliated cells by the bacteria and provide effective protection against infection. Thus, pertussis toxin is clearly an important virulence factor in the initial colonization stage of the infection.

Since the S3 subunit of pertussis toxin is able to bind to the surface of phagocytes, and since FHA will attach to integrin CR3 on phagocyte surfaces (the receptor for complement C3b), it has been speculated that the bacterium might bind preferentially to phagocytes in order to facilitate its own engulfment. The role of such self-initiated phagocytosis is not clear. Bacteria taken up by this abnormal route may avoid stimulating the oxidative burst that normally accompanies phagocytic uptake of bacterial cells which are opsonized by antibodies or complement C3b. Once inside of cells the bacteria might utilize other toxins (i.e. adenylate cyclase toxin) to compromise the bactericidal activities of phagocytes. In any case, there is some evidence that *Bordetella pertussis* can use this mechanism to get into and to persist in phagocytes as an intracellular parasite. If *B. pertussis* is an intracellular

parasite it would explain why immunity to pertussis correlates better with the presence of specific cytotoxic T cells than it does with the presence of antibodies to bacterial products.

B. pertussis produces at least two other types of adhesins, two types of fimbriae and a nonfimbrial surface protein called pertactin, but their role in adherence and pathogenesis is not well established.

Toxins Produced by *B. pertussis*

B. pertussis produces a variety of substances with toxic activity in the class of exotoxins and endotoxins.

It secretes its own **invasive adenylate cyclase** which enters mammalian cells (*Bacillus anthracis* produces a similar enzyme, EF). This toxin acts locally to reduce phagocytic activity and probably helps the organism initiate infection. This toxin is a 45 kDa protein that may be cell-associated or released into the environment. Mutants of *B. pertussis* in the adenylate cyclase gene have reduced virulence in mouse models. The organisms can still colonize but cannot produce the lethal disease. The adenylate cyclase toxin is a single polypeptide with an enzymatic domain (i.e., adenylate cyclase activity) and a binding domain that will attach to host cell surfaces. The adenylate cyclase was originally identified as a hemolysin because it will lyse red blood cells. In fact, it is responsible for hemolytic zones around colonies of *Bordetella pertussis* growing on blood agar. Probably it inserts into the erythrocyte membrane which causes hemolysis. An interesting feature of the adenylate cyclase toxin is that it is active only in the presence of a eukaryotic regulatory molecule called calmodulin, which up-regulates the activity of the eukaryotic adenylate cyclase. The adenylate cyclase toxin is only active in the eukaryotic cell since no similar regulatory molecule exists in prokaryotes. Thus, the molecule seems to have evolved specifically to parasitize eukaryotic cells. Anthrax EF (edema factor) is also a calmodulin-dependent adenylate cyclase.

It produces a highly **lethal toxin** (formerly called dermonecrotic toxin) which causes inflammation and local necrosis adjacent to sites where *B. pertussis* is located. The lethal toxin is a 102 kDa protein composed of four subunits, two with a mw of 24kDa and two with mw of 30 kDa. It causes necrotic skin lesions when low doses are injected subcutaneously in mice and is lethal in high doses. The role of the toxin in whooping cough is not known.

It produces a substance called the **tracheal cytotoxin** which is toxic for ciliated respiratory epithelium and which will stop the ciliated cells from beating. This substance is not a classic bacterial exotoxin since it is not composed of protein. The tracheal cytotoxin is a peptidoglycan fragment, which appears in the extracellular fluid where the bacteria are actively growing. The toxin kills ciliated cells and causes their extrusion from the mucosa. It also stimulates release of cytokine IL-1, and so causes fever.

It produces the **pertussis toxin, PTx**, a protein that mediates both the colonization and toxemic stages of the disease. PTx is a two component, A+B bacterial exotoxin. The A subunit (S1) is an ADP ribosyl transferase. The B component, composed of five polypeptide subunits (S2 through S5), binds to specific carbohydrates on cell surfaces. The role of PTx in invasion has already been discussed. PTx is transported from the site of growth of the *Bordetella* to various susceptible cells and tissues of the host. Following binding of the B component to host cells, the A subunit is inserted through the membrane and released into the cytoplasm in a mechanism of direct entry. The A subunit gains enzymatic activity and transfers the ADP ribosyl moiety of NAD to the membrane-bound regulatory protein Gi that normally inhibits the eukaryotic adenylate cyclase. The Gi protein is inactivated and cannot perform its normal function to inhibit adenylate cyclase. The conversion of ATP to cyclic AMP cannot be stopped and intracellular levels of cAMP increase. This has the effect to disrupt cellular function, and in the case of phagocytes, to decrease their phagocytic activities such as chemotaxis, engulfment, the oxidative burst, and bactericidal killing. Systemic effects of the toxin include lymphocytosis and alteration of hormonal activities that are regulated by cAMP, such as increased insulin production (resulting in hypoglycemia) and increased sensitivity to histamine (resulting in increased capillary permeability, hypotension and shock). PTx also affects the immune system in experimental animals. B cells and T cells that leave the lymphatics show an inability to return. This alters both AMI and CMI responses and may explain the high frequency of secondary infections that accompany pertussis (the most frequent secondary infections during whooping cough are pneumomia and otitis media).

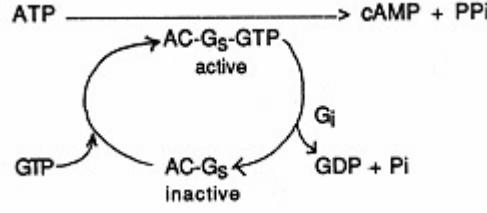
Although the effects of the pertussis toxin are dependent on ADP ribosylation, it has been shown that mere binding of the B oligomer can elicit a response on the cell surface such as lymphocyte mitogenicity, platelet activation, and production of insulin effects.

The pertussis toxin gene has been cloned and sequenced and the subunits expressed in *E. coli*. The toxin can be

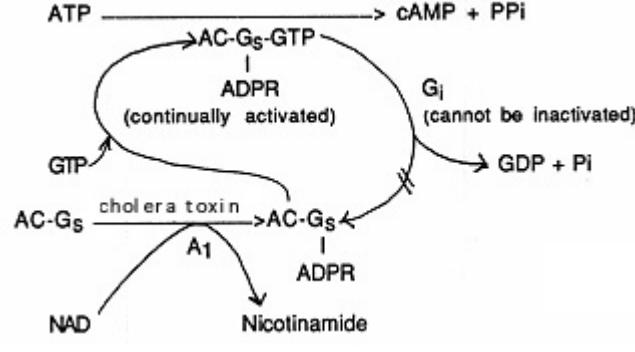
inactivated and converted to toxoid for use in component vaccines.

Figure 2. Comparison between cholera toxin and pertussis toxin (ptx) in their ability to interfere with the regulation of the eukaryotic adenylate cyclase complex.

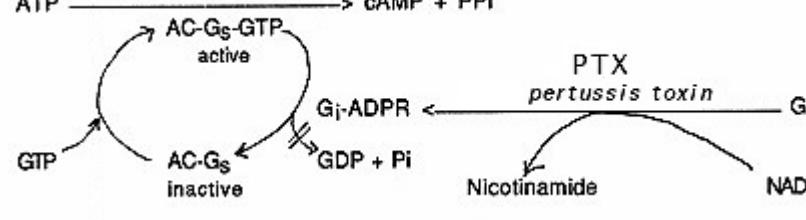
Normal regulation of adenylate cyclase activity in mammalian cells (Adenylate cyclase (AC) is activated normally by a stimulatory regulatory protein (Gs) and guanosine triphosphate (GTP); however the activation is normally brief because an inhibitory regulatory protein (Gi) hydrolyzes the GTP.



Adenylate cyclase activated by cholera toxin (The cholera toxin A1 fragment catalyzes the attachment of ADP-Ribose (ADPR) to the regulatory protein Gs, forming Gs-ADPR from which GTP cannot be hydrolyzed. Since GTP hydrolysis is the event that inactivates adenylate cyclase (AC), the enzyme remains continually activated.



Adenylate cyclase activated by pertussis toxin (The pertussis A subunit transfers the ADP ribosyl moiety of NAD to the membrane-bound regulatory protein Gi that normally inhibits the eukaryotic adenylate cyclase. The Gi protein is inactivated and cannot perform its normal function to inhibit adenylate cyclase. The conversion of ATP to cyclic AMP cannot be stopped.)



Lipopolysaccharide. As a Gram-negative bacterium *Bordetella pertussis* possesses lipopolysaccharide (endotoxin) in its outer membrane, but its LPS is unusual. It is heterogeneous, with two major forms differing in the phosphate content of the lipid moiety. The alternative form of Lipid A is designated Lipid X. The unfractionated material elicits the usual effects of LPS (i.e., induction of IL-1, activation of complement, fever, hypotension, etc.), but the distribution of those activities is different in the two forms of LPS. For example, Lipid X, but not Lipid A, is pyrogenic, and its O-side chain is a very powerful immune adjuvant. Furthermore, *Bordetella* LPS is more potent in the limulus assay than LPS from other Gram-negative bacteria, so it is not reliable to apply knowledge of the biological activity of LPS in the *Enterobacteriaceae* to the LPS of *Bordetella*. The role of this unusual LPS in the pathogenesis of whooping cough has not been investigated.

Regulation of Virulence Factors in *B. pertussis*

The production of virulence factors in *B. pertussis* is regulated in several different ways. Expression of virulence factors is regulated by the bvg operon.

First, the organisms undergo an event called phase variation resulting in the loss of most virulence factors and some undefined outer membrane proteins. Phase variation has been shown to occur at a genetic frequency of 10⁻⁴ - 10⁻⁶ generations and results from a specific DNA frame shift that comes about after the insertion of a single nucleotide into the bvg (also known as vir) operon.

A similar process called phenotypic modulation, occurs in response to environmental signals such as temperature or chemical content, and is reversible. This is an adaptive process mediated by the products of the bvg operon, and is an example of a two-component environmental-sensing (regulatory) system used by other bacteria. The expression of these regulatory proteins is itself regulated by environmental signals, such that entry into a host might induce components required for survival and production of disease.

The Whooping Cough Vaccine

The development of the whooping cough vaccine in the 1950s has made whooping cough an uncommon disease in developed countries. In countries where the vaccine is not used whooping cough is an important cause of mortality in children, with an estimated 51,000,000 cases and 600,000 deaths annually.

Historically, the whooping cough vaccine has been administered as a merthiolate-killed bacterial cell suspension which is part of the DTP vaccine (The P in DTP stands for Pertussis cells). Unfortunately, about 20% of the children that receive the **whole cell vaccine** experience mild side effects. About 0.1% of infants experience convulsions soon after receiving the vaccine and in a very small number of cases (1 in 150,000?) severe or irreversible brain damage may occur. In the absence of the disease in an immune population, parents have begun to wonder if the risk of vaccinating children outweighs the risk of the disease, and the value of the whole cell vaccine has been questioned.

Several new **acellular vaccines** have been developed from purified components of *B. pertussis*. Demonstration of the protective effects of anti-PTx and anti-FHA antibodies in the mouse model, focused vaccine production on combinations of inactivated pertussis toxin (toxoid) and filamentous hemagglutinin. Multicomponent acellular vaccines containing combinations of pertussis toxoid, filamentous hemagglutinin, pertactin, and the two types of fimbriae, are now being used in several countries including the U.S. The new vaccine, known as **acellular pertussis** has fewer side effects than the whole cell vaccine and is currently recommended for use under the conditions described below.

For decades, the pertussis vaccine has been given in combination with vaccines against diphtheria and tetanus. The combination is known as the **DTP** vaccine. Recently, infants have been able to receive a vaccine that combines the DTP vaccine with the vaccine against *Haemophilus influenzae* type b meningitis (Hib). This vaccine is called **DTPH**. The diphtheria-tetanus-pertussis vaccine using acellular pertussis is known as **DTaP**. The diphtheria-tetanus-pertussis vaccination is given in five doses: at 2, 4, 6, 12-18 months and 4-6 years of age. Previously, DTaP had been recommended only for the fourth and fifth doses. Following FDA licensure of DTaP for infants, the Advisory Committee on Immunization Practices of the United States Public Health Service now recommends that DTaP be used for the first four doses and that DTaP still be used for the fourth and fifth doses for children who received DTP in their first three doses. The Committee is awaiting study results before making a recommendation for the fifth dose for children who now will receive DTaP in their first four doses. The recommendation still permits the use of DTP and DTPH--the combination that includes the vaccine against *Haemophilus influenzae* type b meningitis.

Whooping Cough In Wisconsin 2004

There were more than 4,800 cases of whooping cough were reported in Wisconsin in 2004, an increase of more than 690 percent over the previous year, when there were 716. In the mid-1980s, when whooping cough outbreaks were considered particularly bad, there were 400 to 500 reported cases per year.

Dane County reported over 150 cases. The Public Health Department of the City of Madison saw over 100 cases in 2004, even though the disease is undoubtedly under-reported. The University Health Services saw a rise in incidence on the UW campus. Last semester UHS confirmed several student cases each week, with several additional unconfirmed occurrences.

Since the development of the pertussis vaccine, the incidence of whooping cough in the U.S. steadily declined until

the past two decades when it began to rise. According to the Center for Disease Control, Wisconsin currently ranks second in the nation for disease incidence rate at 27.7 cases per 100,000 individuals.

It is difficult to draw conclusions by comparing the 2004 outbreak with those in past years. The increase in whooping cough numbers can partially be explained by new testing procedures that became available last year. The new test is quicker and more sensitive than previous tests, and physicians are putting more emphasis on diagnosing the illness.

Also, whooping cough infections tend to run on a 2-5 year cycle, and 2004 could be a high point in the cycle.

Like flu viruses, the bacterium is highly contagious and tends to pass quickly from person to person through coughing and sneezing. If increasing numbers of individuals have the illness, then the risk of infection increases in the general population.

Many young children are vaccinated against whooping cough with the pertussis vaccine. However, the vaccine is only approved for children under seven years of age. Antibody-mediated immunity wanes in approximately ten years, leaving older individuals more susceptible to the disease. Adults get infected, often to a lesser degree, but they are still able to spread the disease to unimmunized children.

For more information on whooping cough, see the CDC listings under

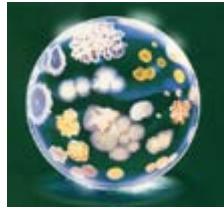
[Pertussis - Technical Information](#)

vaccine: <http://www.cdc.gov/nip/publications/VIS/vis-dtp.pdf>.

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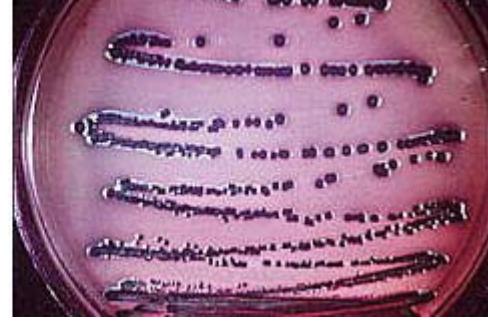




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Pathogenic *E. coli*

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

Escherichia coli

The GI tract of most warm-blooded animals is colonized by *E. coli* within hours or a few days after birth. The bacterium is ingested in foods or water or obtained directly from other individuals handling the infant. The human bowel is usually colonized within 40 hours of birth. *E. coli* can adhere to the mucus overlying the large intestine. Once established, an *E. coli* strain may persist for months or years. Resident strains shift over a long period (weeks to months), and more rapidly after enteric infection or antimicrobial chemotherapy that perturbs the normal flora. The basis for these shifts and the ecology of *Escherichia coli* in the intestine of humans are poorly understood despite the vast amount of information on almost every other aspect of the organism's existence. The entire DNA base sequence of the *E. coli* genome has been known since 1997.

E. coli is the head of the large bacterial family, ***Enterobacteriaceae***, the **enteric bacteria**, which are facultatively anaerobic Gram-negative rods that live in the intestinal tracts of animals in health and disease. The *Enterobacteriaceae* are among the most important bacteria medically. A number of genera within the family are human intestinal pathogens (e.g. *Salmonella*, *Shigella*, *Yersinia*). Several others are normal colonists of the human gastrointestinal tract (e.g. *Escherichia*, *Enterobacter*, *Klebsiella*), but these bacteria, as well, may occasionally be associated with diseases of humans.

The *Enterobacteriaceae* are distinguished from the *Pseudomonadaceae* in a number of ways known reflexively to competent microbiologists. The pseudomonads are respiratory, never fermentative, oxidase-positive, and motile by means of polar flagella. The enterics ferment glucose producing acid and gas, are typically oxidase-negative, and when motile, produce peritrichous flagella.

Physiologically, *E. coli* is versatile and well-adapted to its characteristic habitats. It can grow in media with glucose as the sole organic constituent. Wild-type *E. coli* has no growth factor requirements, and metabolically it can transform glucose into all of the macromolecular components that make up the cell. The bacterium can grow in the presence or absence of O₂. Under anaerobic conditions it will grow by means of fermentation, producing characteristic "mixed acids and gas" as end products. However, it can also grow by means of anaerobic respiration, since it is able to utilize NO₃, NO₂ or fumarate as final electron acceptors for respiratory electron transport

processes. In part, this adapts *E. coli* to its intestinal (anaerobic) and its extraintestinal (aerobic or anaerobic) habitats.

E. coli can respond to environmental signals such as chemicals, pH, temperature, osmolarity, etc., in a number of very remarkable ways considering it is a single-celled organism. For example, it can sense the presence or absence of chemicals and gases in its environment and swim towards or away from them. Or it can stop swimming and grow fimbriae that will specifically attach it to a cell or surface receptor. In response to change in temperature and osmolarity, it can vary the pore diameter of its outer membrane porins to accommodate larger molecules (nutrients) or to exclude inhibitory substances. With its complex mechanisms for regulation of metabolism the bacterium can survey the chemical contents its environment in advance of synthesizing any enzymes necessary to use these compounds. It does not wastefully produce enzymes for degradation of carbon sources unless they are available, and it does not produce enzymes for synthesis of metabolites if they are available as nutrients in the environment.

E. coli is a consistent inhabitant of the human intestinal tract, and it is the **predominant facultative organism in the human GI tract**; however, it makes up a very small proportion of the total bacterial content. The anaerobic *Bacteroides* species in the bowel outnumber *E. coli* by at least 20:1. However, the regular presence of *E. coli* in the human intestine and feces has led to tracking the bacterium in nature as an indicator of fecal pollution and water contamination. As such, it is taken to mean that, wherever *E. coli* is found, there may be fecal contamination by intestinal parasites of humans.

Pathogenesis of *E. coli*

Over 700 antigenic types (**serotypes**) are recognized based on **O, H, and K antigens**. Serotyping is still important in distinguishing the small number of strains that actually cause disease.

E. coli is responsible for three types of infections in humans: **urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (gastroenteritis)**. These three diseases depend on a specific array of pathogenic (virulence) determinants. The virulence determinants of various strains of pathogenic *E. coli* are summarized in Table 1.

Table 1. Summary of the Virulence Determinants of Pathogenic *E. coli* Adhesins

- CFAI/CFAII
- Type 1 fimbriae
- P fimbriae
- S fimbriae
- Intimin (non-fimbrial adhesin)

Invasins

- hemolysin
- siderophores and siderophore uptake systems
- Shigella-like "invasins" for intracellular invasion and spread

Motility/chemotaxis

- flagella

Toxins

- LT toxin
- ST toxin
- Shiga-like toxin
- cytotoxins
- endotoxin LPS)

Antiphagocytic surface properties

capsules
K antigens
LPS

Defense against serum bactericidal reactions

LPS
K antigens

Defense against immune responses

capsules
K antigens
LPS
antigenic variation

Genetic attributes

genetic exchange by transduction and conjugation
transmissible plasmids
R factors and drug resistance plasmids
toxin and other virulence plasmids

Urinary tract infections

Uropathogenic *E. coli* cause 90% of the urinary tract infections (UTI) in anatomically-normal, unobstructed urinary tracts. The bacteria colonize from the feces or perineal region and ascend the urinary tract to the bladder. Bladder infections are 14-times more common in females than males by virtue of the shortened urethra. The typical patient with uncomplicated cystitis is a sexually-active female who was first colonized in the intestine with a uropathogenic *E. coli* strain. The organisms are propelled into the bladder from the periurethral region during sexual intercourse. With the aid of specific adhesins they are able to colonize the bladder.

The adhesin that has been most closely associated with uropathogenic *E. coli* is the **P fimbria** (or **pyelonephritis-associated pili [PAP] pili**). The letter designation is derived from the ability of P fimbriae to bind specifically to the P blood group antigen which contains a D-galactose-D-galactose residue. The fimbriae bind not only to red cells but to a specific galactose disaccharide that is found on the surfaces uroepithelial cells in approximately 99% of the population.

The frequency of the distribution of this host cell receptor plays a role in susceptibility and explains why certain individuals have repeated UTI caused by *E. coli*. Uncomplicated *E. coli* UTI virtually never occurs in individuals lacking the receptors.

Uropathogenic strains of *E. coli* possess other determinants of virulence in addition to P fimbriae. *E. coli* with P fimbriae also possess the gene for Type 1 fimbriae, and there is evidence that P fimbriae are derived from Type 1 fimbriae by insertion of a new fimbrial tip protein to replace the mannose-binding domain of Type 1 fimbria. In any case, **Type 1 fimbriae** could provide a supplementary mechanism of adherence or play a role in aggregating the bacteria to a specific mannosyl-glycoprotein that occurs in urine.

Uropathogenic strains of *E. coli* usually produce **siderophores** that probably play an essential role in iron acquisition for the bacteria during or after colonization. They also produce hemolysins which are cytotoxic due to formation of transmembranous pores in host cells. One strategy for obtaining iron and other nutrients for bacterial growth may involve the lysis of host cells to release these substances. The activity of **hemolysins** is not limited to red cells since the alpha-hemolysins of *E. coli* also lyse lymphocytes, and the beta-hemolysins inhibit phagocytosis and chemotaxis of neutrophils.

Another factor thought to be involved in the pathogenicity of the uropathogenic strains of *E. coli* is their resistance to the complement-dependent bactericidal effect of serum. The presence of K antigens is associated with upper urinary tract infections, and antibody to the **K antigen** has been shown to afford some degree of protection in experimental infections. The K antigens of *E. coli* are "capsular" antigens that may be composed of proteinaceous organelles associated with colonization (e.g., CFA antigens), or made of polysaccharides. Regardless of their chemistry, these capsules may be able to promote bacterial virulence by decreasing the ability of antibodies and/or complement to bind to the bacterial surface, and the ability of phagocytes to recognize and engulf the bacterial cells. The best studied K antigen, K-1, is composed of a polymer of N-acetyl neuraminic acid (sialic acid), which besides being antiphagocytic, has the additional property of being an antigenic disguise.

Neonatal Meningitis

Neonatal meningitis affects 1/2,000-4,000 infants. Eighty percent of *E. coli* strains involved synthesize K-1 capsular antigens (K-1 is only present 20-40% of the time in intestinal isolates).

E. coli strains invade the blood stream of infants from the nasopharynx or GI tract and are carried to the meninges.

The **K-1 antigen** is considered the major determinant of virulence among strains of *E. coli* that cause neonatal meningitis. K-1 is a homopolymer of sialic acid. It inhibits phagocytosis, complement, and responses from the host's immunological mechanisms. K-1 may not be the only determinant of virulence, however, as **siderophore** production and **endotoxin** are also likely to be involved.

Epidemiologic studies have shown that pregnancy is associated with increased rates of colonization by K-1 strains and that these strains become involved in the subsequent cases of meningitis in the newborn. Probably, the infant GI tract is the portal of entry into the bloodstream. Fortunately, although colonization is fairly common, invasion and the catastrophic sequelae are rare.

Neonatal meningitis requires antibiotic therapy that usually includes ampicillin and a third-generation cephalosporin.

Intestinal Diseases Caused by *E. coli*

As a pathogen, *E. coli*, of course, is best known for its ability to cause intestinal diseases. Five classes (virotypes) of *E. coli* that cause diarrheal diseases are now recognized: **enterotoxigenic *E. coli* (ETEC)**, **enteroinvasive *E. coli* (EIEC)**, **enterohemorrhagic *E. coli* (EHEC)**, **enteropathogenic *E. coli* (EPEC)**, and **enteroaggregative *E. coli* (EAEC)**. Each class falls within a serological subgroup and manifests distinct features in pathogenesis.

Enterotoxigenic *E. coli* (ETEC)

ETEC are an important cause of diarrhea in infants and travelers in underdeveloped countries or regions of poor sanitation. The diseases vary from minor discomfort to a severe cholera-like syndrome. ETEC are acquired by ingestion of contaminated food and water, and adults in endemic areas evidently develop immunity. The disease requires colonization and elaboration of one or more enterotoxins. Both traits are plasmid-encoded.

ETEC adhesins are **fimbriae** which are species-specific. For example, the K-88 fimbrial Ag is found on strains from piglets; K-99 Ag is found on strains from calves and lambs; CFA I, and CFA II, are found on strains from humans. These fimbrial adhesins adhere to specific receptors on enterocytes of the proximal small intestine.

Enterotoxins produced by ETEC include the **LT(heat-labile) toxin** and/or the **ST (heat-stable) toxin**, the genes for which may occur on the same or separate plasmids. The **LT enterotoxin** is very similar to **cholera toxin** in both structure and mode of action. It is an 86kDa protein composed of an enzymatically active (A) subunit surrounded by 5 identical binding (B) subunits. It binds to the same identical ganglioside receptors that are recognized by the cholera toxin (i.e., GM1), and its enzymatic activity is identical to that of the cholera toxin.

The **ST enterotoxin** is actually a family of toxins which are peptides of molecular weight about 2,000 daltons. Their small size explains why they are not inactivated by heat. ST causes an increase in cyclic GMP in host cell cytoplasm leading to the same effects as an increase in cAMP. **Sta** is known to act by binding to a guanylate cyclase that is located on the apical membranes of host cells, thereby activating the enzyme. This leads to secretion of fluid and electrolytes resulting in diarrhea.

Symptoms ETEC infections include diarrhea without fever. The bacteria colonize the GI tract by means of a fimbrial adhesin, e.g. CFA I and CFA II, and are noninvasive, but produce either the LT or ST toxin.

Enteroinvasive *E. coli* (EIEC)

EIEC closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. EIEC penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes a dysentery-like diarrhea with fever. EIEC apparently lack fimbrial adhesins but do possess a specific adhesin that, as in *Shigella*, is thought to be an outer membrane protein. Also, like *Shigella*, **EIEC are invasive organisms**. They do not produce LT or ST toxin and, unlike *Shigella*, they do not produce the shiga toxin.

Enteropathogenic *E. coli* (EPEC)

EPEC induce a watery diarrhea similar to ETEC, but they do not possess the same colonization factors and do not produce ST or LT toxins. They produce a **non fimbrial adhesin** designated **intimin**, an outer membrane protein, that mediates the final stages of adherence. Although they do not produce LT or ST toxins, there are reports that they produce an **enterotoxin** similar to that of *Shigella*. Other virulence factors may be related to those in *Shigella*.

Adherence of EPEC strains to the intestinal mucosa is a very complicated process and produces dramatic effects in the ultrastructure of the cells resulting in rearrangements of actin in the vicinity of adherent bacteria. The phenomenon is sometimes called "**attaching and effacing**" of cells. EPEC strains are said to be "**moderately-invasive**" meaning they are not as invasive as *Shigella*, and unlike ETEC or EAggEC, they cause an inflammatory response. The diarrhea and other symptoms of EPEC infections probably are caused by bacterial invasion of host cells and interference with normal cellular signal transduction, rather than by production of toxins.

Some types of EPEC are referred to as **Enteroadherent *E. coli* (EAEC)**, based on specific patterns of adherence. They are an important cause of traveler's diarrhea in Mexico and in North Africa.

Enteroaggregative *E. coli* (EAggEC)

The distinguishing feature of **EAggEC** strains is their ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent diarrhea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non-bloody diarrhea without invading or causing inflammation. This suggests that the organisms produce a toxin of some sort. Recently, a distinctive heat-labile plasmid-encoded toxin has been isolated from these strains, called the **EAST (Enterobacteriophage ST) toxin**. They also produce a **hemolysin** related to the hemolysin produced by *E. coli* strains involved in urinary tract infections. The role of the toxin and the hemolysin in virulence has not been proven. The significance of EAggEC strains in human disease is controversial.

Enterohemorrhagic *E. coli* (EHEC)

EHEC are represented by a single strain (**serotype O157:H7**), which causes a diarrheal syndrome distinct from EIEC (and *Shigella*) in that there is copious bloody discharge and no fever. A frequent life-threatening situation is its toxic effects on the kidneys (hemolytic uremia).

EHEC has recently been recognized as a cause of serious disease often associated with ingestion of inadequately

cooked hamburger meat. **Pediatric diarrhea** caused by this strain can be fatal due to acute **kidney failure (hemolytic uremic syndrome [HUS])**. EHEC are also considered to be "**moderately invasive**". Nothing is known about the colonization antigens of EHEC but **fimbriae** are presumed to be involved. The bacteria do not invade mucosal cells as readily as *Shigella*, but EHEC strains produce a toxin that is virtually identical to the **Shiga toxin**. The toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability of EHEC strains to cause HUS. The toxin is phage encoded and its production is enhanced by iron deficiency.

Table 2. Pathogenic *E. coli*: Summary of Virulence Characteristics of Intestinal Pathogens

ETEC

fimbrial adhesins e.g. CFA I, CFAII, K88, K99
non invasive
produce LT and/or ST toxin
watery diarrhea in infants and travelers; no inflammation, no fever

EIEC

nonfimbrial adhesins, possibly outer membrane protein
invasive (penetrate and multiply within epithelial cells)
does not produce shiga toxin
dysentery-like diarrhea (mucous, blood), severe inflammation, fever

EPEC

non fimbrial adhesin (intimin)
moderately invasive (not as invasive as *Shigella* or EIEC)
does not produce LT or ST; some reports of shiga-like toxin
usually infantile diarrhea; watery diarrhea similar to ETEC, some inflammation, no fever; symptoms probably result mainly from invasion rather than toxigenesis

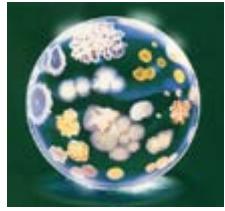
EAggEC

adhesins not characterized
non invasive
produce ST-like toxin (EAST) and a hemolysin
persistent diarrhea in young children without inflammation, no fever

EHEC

adhesins not characterized, probably fimbriae
moderately invasive
does not produce LT or ST but does produce shiga toxin
pediatric diarrhea, copious bloody discharge (hemorrhagic colitis), intense inflammatory response, may be complicated by hemolytic uremia

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Vibrio cholerae and Asiatic Cholera

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Introduction

The genus **Vibrio** consists of Gram-negative straight or curved rods, motile by means of a single polar flagellum. Vibrios are capable of both respiratory and fermentative metabolism. O₂ is a universal electron acceptor; they do not denitrify. Most species are oxidase-positive. In most ways vibrios are related to enteric bacteria, but they share some properties with pseudomonads as well. The Family **Vibrionaceae** is found in the "Facultatively Anaerobic Gram-negative Rods" in Bergey's Manual (1986), on the level with the Family **Enterobacteriaceae**. In the revisionist taxonomy of 2001 (Bergey's Manual), based on phylogenetic analysis, **Vibrionaceae**, **Pseudomonadaceae** and **Enterobacteriaceae** are all landed in the **Gammaproteobacteria**. Vibrios are distinguished from enterics by being oxidase-positive and motile by means of polar flagella. Vibrios are distinguished from pseudomonads by being fermentative as well as oxidative in their metabolism. Of the vibrios that are clinically significant to humans, *Vibrio cholerae*, the agent of cholera, is the most important.

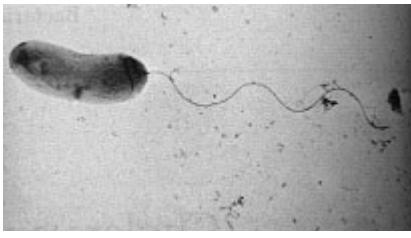
Most vibrios have relatively simple growth factor requirements and will grow in synthetic media with glucose as a sole source of carbon and energy. However, since vibrios are typically marine organisms, most species require 2-3% NaCl or a sea water base for optimal growth. Vibrios vary in their nutritional versatility, but some species will grow on more than 150 different organic compounds as carbon and energy sources, occupying the same level of metabolic versatility as *Pseudomonas*. In liquid media vibrios are motile by polar flagella that are enclosed in a sheath continuous with the outer membrane of the cell wall. On solid media they may synthesize numerous lateral flagella which are not sheathed.

Vibrios are one of the most common organisms in surface waters of the world. They occur in both marine and freshwater habitats and in associations with aquatic animals. Some species are bioluminescent and live in mutualistic associations with fish and other marine life. Other species are pathogenic for fish, eels, and frogs, as well as other vertebrates and invertebrates.

V. cholerae and *V. parahaemolyticus* are pathogens of humans. Both produce diarrhea, but in ways that are entirely different. *V. parahaemolyticus* is an invasive organism affecting primarily the colon; *V. cholerae* is noninvasive, affecting the small intestine through secretion of an enterotoxin. *Vibrio vulnificus* is an emerging pathogen of humans. This organism causes wound infections, gastroenteritis, or a syndrome known as "primary septicemia."

Campylobacter jejuni (formerly *Vibrio fetus*), is now moved to the class **Epsilonproteobacteria** in the family **Campylobacteraceae**. *Campylobacter jejuni* has been associated with dysentery-like gastroenteritis, as well as with other types of infection, including bacteremic and central nervous system infections in humans. Another vibrio-like organism, *Helicobacter pylori* causes duodenal and gastric ulcers and gastric cancer. It is also reclassified into the class **Epsilonproteobacteria** family **Helicobacteraceae**.

Cholera

*Vibrio cholerae*

Cholera (frequently called **Asiatic cholera** or **epidemic cholera**) is a severe diarrheal disease caused by the bacterium ***Vibrio cholerae***. Transmission to humans is by water or food. The natural reservoir of the organism is not known. It was long assumed to be humans, but some evidence suggests that it is the aquatic environment.

V. cholerae produces **cholera toxin**, the model for enterotoxins, whose action on the mucosal epithelium is responsible for the characteristic diarrhea of the disease cholera. In its extreme manifestation, cholera is one of the most rapidly fatal illnesses known. A healthy person may become hypotensive within an hour of the onset of symptoms and may die within 2-3 hours if no treatment is provided. More commonly, the disease progresses from the first liquid stool to shock in 4-12 hours, with death following in 18 hours to several days.

The **clinical description** of cholera begins with sudden onset of massive diarrhea. The patient may lose gallons of protein-free fluid and associated electrolytes, bicarbonates and ions within a day or two. This results from the activity of the cholera enterotoxin which activates the adenylate cyclase enzyme in the intestinal cells, converting them into pumps which extract water and electrolytes from blood and tissues and pump it into the lumen of the intestine. This loss of fluid leads to dehydration, anuria, acidosis and shock. The watery diarrhea is speckled with flakes of mucus and epithelial cells ("rice-water stool") and contains enormous numbers of vibrios. The loss of potassium ions may result in cardiac complications and circulatory failure. Untreated cholera frequently results in high (50-60%) mortality rates.

Treatment of cholera involves the rapid intravenous replacement of the lost fluid and ions. Following this replacement, administration of isotonic maintenance solution should continue until the diarrhea ceases. If glucose is added to the maintenance solution it may be administered orally, thereby eliminating the need for sterility and iv. administration. By this simple treatment regimen, patients on the brink of death seem to be miraculously cured and the mortality rate of cholera can be reduced more than ten-fold. Most antibiotics and chemotherapeutic agents have no value in cholera therapy, although a few (e.g. tetracyclines) may shorten the duration of diarrhea and reduce fluid loss.

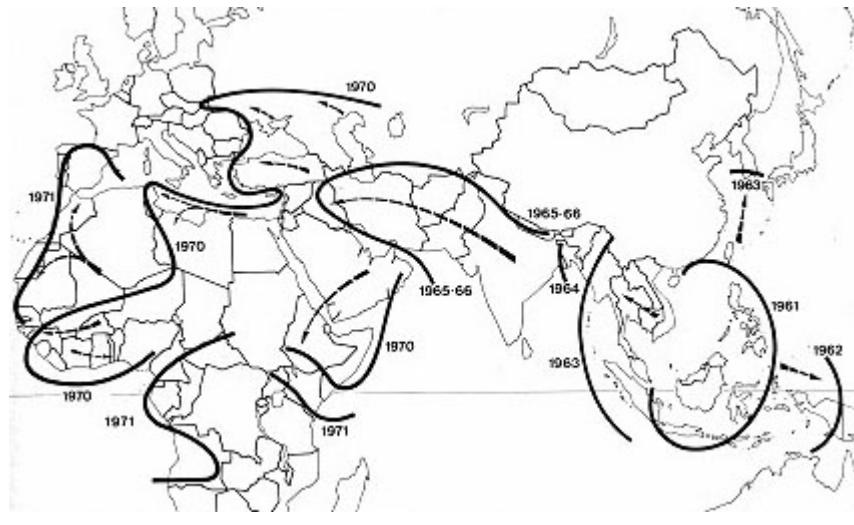
History and spread of epidemic cholera

Cholera has smoldered in an endemic fashion on the Indian subcontinent for centuries. There are references to deaths due to dehydrating diarrhea dating back to Hippocrates and Sanskrit writings. Epidemic cholera was described in 1563 by Garcia del Huerto, a Portuguese physician at Goa, India. The mode of transmission of cholera by water was proven in 1849 by John Snow, a London physician. In 1883, Robert Koch successfully isolated the cholera vibrio from the intestinal discharges of cholera patients and proved conclusively that it was the agent of the disease.

The first long-distance spread of cholera to Europe and the Americas began in 1817, such that by the early 20th century, six waves of cholera had spread across the world in devastating epidemic fashion. Since then, until the 1960s, the disease contracted, remaining present only in southern Asia. In 1961, the "**El Tor**" biotype (distinguished from classic biotypes by the production of hemolysins) reemerged and produced a major epidemic in the Philippines to initiate a **seventh global pandemic** (See map below). Since then, this biotype has spread across Asia, the Middle East, Africa, and parts of Europe.

There are several characteristics of the El Tor strain that confer upon it a high degree of "epidemic virulence" allowing it to spread across the world as previous strains have done. First, the ratio of cases to carriers is much less than in cholera due to classic biotypes (1: 30-100 for El Tor vs. 1: 2 - 4 for "classic" biotypes). Second, the duration of carriage after infection is longer for the El Tor strain than the classic strains. Third, the El Tor strain survives for longer periods in the extraintestinal environment. Between 1969 and 1974, El Tor replaced the classic

strains in the heartland of endemic cholera, the Ganges River Delta of India.



The global spread of cholera during the seventh pandemic, 1961-1971. (CDC)

El Tor broke out explosively in Peru in 1991 (after an absence of cholera there for 100 years), and spread rapidly in Central and South America, with recurrent epidemics in 1992 and 1993. From the onset of the epidemic in January 1991 through September 1, 1994, a total of 1,041,422 cases and 9,642 deaths (overall case-fatality rate: 0.9%) were reported from countries in the Western Hemisphere to the Pan American Health Organization. In 1993, the numbers of reported cases and deaths were 204,543 and 2362, respectively.

In 1982, in Bangladesh, a classic biotype resurfaced with a new capacity to produce more severe illness, and it rapidly replaced the El Tor strain which was thought to be well-entrenched. This classic strain has not yet produced a major outbreak in any other country.

In December, 1992, a large epidemic of cholera began in Bangladesh, and large numbers of people have been involved. The organism has been characterized as *V. cholerae* O139 "Bengal". It is derived genetically from the El Tor pandemic strain but it has changed its antigenic structure such that there is no existing immunity and all ages, even in endemic areas, are susceptible. The epidemic has continued to spread, and *V. cholerae* O139 has affected at least 11 countries in southern Asia. Specific totals for numbers of *V. cholerae* O139 cases are unknown because affected countries do not report infections caused by O1 and O139 separately.

In April 1997, a cholera outbreak occurred among 90,000 Rwandan refugees residing in temporary camps in the Democratic Republic of Congo. During the first 22 days of the outbreak, 1521 deaths were recorded, most of which occurred outside of health-care facilities.

In the United States, cholera was prevalent in the 1800s but has been virtually eliminated by modern sewage and water treatment systems. However, as a result of improved transportation, more persons from the United States travel to parts of Latin America, Africa, or Asia where epidemic cholera is occurring. U.S. travelers to areas with epidemic cholera may be exposed to the bacterium. In addition, travelers may bring contaminated seafood back to the United States. A few foodborne outbreaks have been caused by contaminated seafood brought into this country by travelers. Greater than 90 percent of the cases of cholera in the U.S. have been associated with foreign travel.

V. cholerae may also live in the environment in brackish rivers and coastal waters. Shellfish eaten raw have been a source of cholera, and a few persons in the United States have contracted cholera after eating raw or undercooked shellfish from the Gulf of Mexico.

Antigenic Variation and LPS Structure in *Vibrio cholerae*

Antigenic variation plays an important role in the epidemiology and virulence of cholera. The emergence of the Bengal strain, mentioned above, is an example. The flagellar antigens of *V. cholerae* are shared with many water vibrios and therefore are of no use in distinguishing strains causing epidemic cholera. O antigens, however, do

distinguish strains of *V. cholerae* into 139 known serotypes. Almost all of these strains of *V. cholerae* are nonvirulent. Until the emergence of the Bengal strain (which is "non-O1") a single serotype, designated O1, has been responsible for epidemic cholera. However, there are three distinct **O1 biotypes**, named **Ogawa**, **Inaba** and **Hikojima**, and each biotype may display the "classical" or El Tor phenotype. The Bengal strain (O139) is a new serological strain with a unique O-antigen which partly explains the lack of residual immunity.

Antigenic Determinants of *Vibrio cholerae*

Serotype	O Antigens
Ogawa	A, B
Inaba	A, C
Hikojima	A, B, C

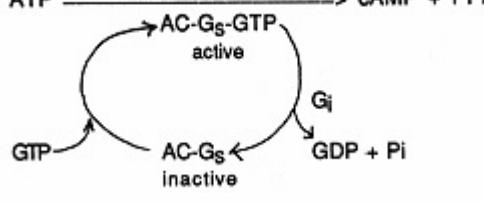
Endotoxin is present in *Vibrio cholerae* as in other Gram-negative bacteria. Fewer details of the chemical structure of *Vibrio cholerae* LPS are known than in the case of *E. coli* and *Salmonella*, but some unique properties have been described. Most importantly, variations in LPS occur in vivo and in vitro, which may be correlated with reversion in nature of nonepidemic strains to classic epidemic strains and vice versa.

Cholera Toxin

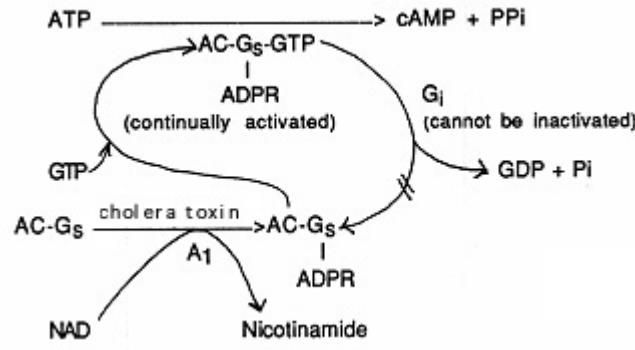
Cholera toxin **activates the adenylate cyclase enzyme** in cells of the intestinal mucosa leading to increased levels of intracellular cAMP, and the secretion of H₂O, Na⁺, K⁺, Cl⁻, and HCO₃⁻ into the lumen of the small intestine. The effect is dependent on a specific receptor, monosialosyl ganglioside (GM1 ganglioside) present on the surface of intestinal mucosal cells. The bacterium produces an invasin, neuraminidase, during the colonization stage which has the interesting property of degrading gangliosides to the monosialosyl form, which is the specific receptor for the toxin.

The toxin has been characterized and contains **5 binding (B) subunits** of 11,500 daltons, an active **(A1) subunit** of 23,500 daltons, and a **bridging piece (A2)** of 5,500 daltons that links A1 to the 5B subunits. Once it has entered the cell, the A1 subunit enzymatically transfers ADP ribose from NAD to a protein (called G_s or N_s), that regulates the adenylate cyclase system which is located on the inside of the plasma membrane of mammalian cells.

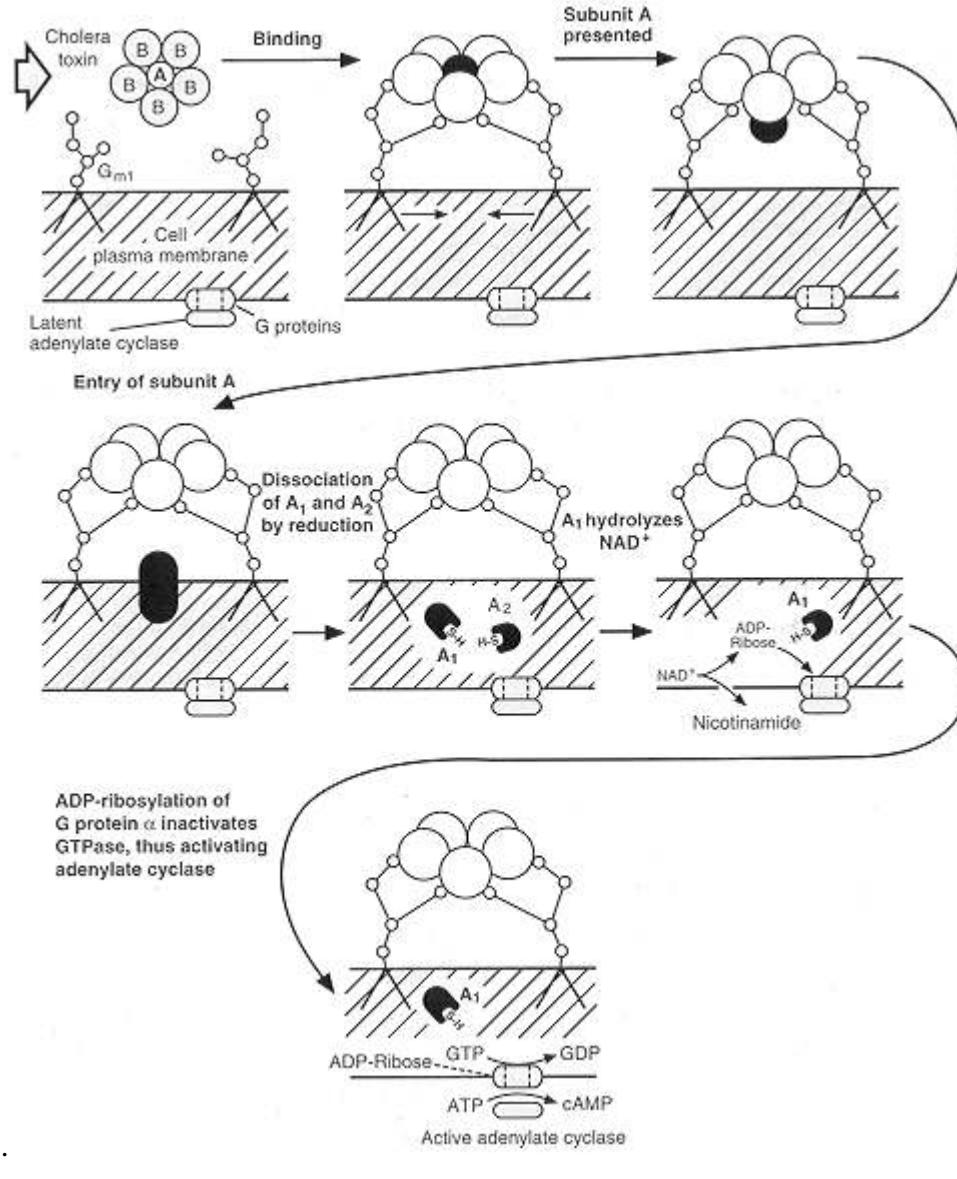
Enzymatically, fragment A1 catalyzes the transfer of the ADP-ribosyl moiety of NAD to a component of the adenylate cyclase system. The process is complex. Adenylate cyclase (AC) is activated normally by a regulatory protein (G_s) and GTP; however activation is normally brief because another regulatory protein (G_i), hydrolyzes GTP. The normal situation is described as follows.



The A1 fragment catalyzes the attachment of ADP-Ribose (ADPR) to the regulatory protein forming G_s-ADPR from which GTP cannot be hydrolyzed. Since GTP hydrolysis is the event that inactivates the adenylate cyclase, the enzyme remains continually activated. This situation can be illustrated as follows.



Thus, the net effect of the toxin is to cause cAMP to be produced at an abnormally high rate which stimulates mucosal cells to pump large amounts of Cl⁻ into the intestinal contents. H₂O, Na⁺ and other electrolytes follow due to the osmotic and electrical gradients caused by the loss of Cl⁻. The lost H₂O and electrolytes in mucosal cells are replaced from the blood. Thus, the toxin-damaged cells become pumps for water and electrolytes causing the diarrhea, loss of electrolytes, and dehydration that are characteristic of cholera.



Mechanism of action of cholera enterotoxin according to Finkelstein in Baron, Chapter 24. Cholera toxin approaches target cell surface. B subunits bind to oligosaccharide of GM1 ganglioside. Conformational alteration of holotoxin occurs, allowing the presentation of the A subunit to cell surface. The A subunit enters the cell. The disulfide bond of the A subunit is reduced by intracellular glutathione, freeing A1 and A2. NAD is hydrolyzed by A1, yielding ADP-ribose and nicotinamide. One of the G proteins of adenylate cyclase is ADP-ribosylated, inhibiting the action of GTPase and locking adenylate cyclase in the "on" mode.

Colonization of the Small Intestine

There are several characteristics of pathogenic *V. cholerae* that are important **determinants of the colonization** process. These include **adhesins**, **neuraminidase**, **motility**, chemotaxis and **toxin** production. If the bacteria are able to survive the gastric secretions and low pH of the stomach, they are well adapted to survival in the small intestine. *V. cholerae* is resistant to bile salts and can penetrate the mucus layer of the small intestine, possibly aided by secretion of neuraminidase and proteases (mucinases). They withstand propulsive gut motility by their own swimming ability and chemotaxis directed against the gut mucosa.

Specific adherence of *V. cholerae* to the intestinal mucosa is probably mediated by long filamentous fimbriae that form bundles at the poles of the cells. These fimbriae have been termed **Tcp pili** (for **toxin coregulated pili**), because expression of these pili genes is coregulated with expression of the cholera toxin genes. Not much is known about the interaction of Tcp pili with host cells, and the host cell receptor for these fimbriae has not been identified. Tcp pili share amino acid sequence similarity with N-methylphenylalanine pili of *Pseudomonas* and *Neisseria*.

Two other possible adhesins in *V. cholerae* are a surface protein that agglutinates red blood cells (**hemagglutinin**) and a group of outer membrane proteins which are products of the **acf** (**accessory colonization factor**) genes. acf mutants have been shown to have reduced ability to colonize the intestinal tract. It has been suggested that *V. cholerae* might use these nonfimbrial adhesins to mediate a tighter binding to host cells than is attainable with fimbriae alone.

V. cholerae produces a protease originally called **mucinase** that degrades different types of protein including fibronectin, lactoferrin and cholera toxin itself. Its role in virulence is not known but it probably is not involved in colonization since mutations in the mucinase gene (designated hap for **hemagglutinin protease**) do not exhibit reduced virulence. It has been suggested that the mucinase might contribute to detachment rather than attachment. Possibly the vibrios would need to detach from cells that are being sloughed off of the mucosa in order to reattach to newly formed mucosal cells.

Genetic Organization and Regulation of Virulence Factors in *Vibrio cholerae*

In *Vibrio cholerae*, the production of virulence factors is regulated at several levels. Regulation of genes at the transcriptional level, especially the genes for toxin production and fimbrial synthesis, has been studied in the greatest detail.

V. cholerae enterotoxin is a product of *ctx* genes. *ctxA* encodes the A subunit of the toxin, and *ctxB* encodes the B subunit. The genes are part of the same operon. The transcript (mRNA) of the *ctx* operon has two ribosome binding sites (rbs), one upstream of the A coding region and another upstream of the B coding region. The rbs upstream of the B coding region is at least seven-times stronger than the rbs of the A coding region. In this way the organism is able to translate more B proteins than A proteins, which is required to assemble the toxin in the appropriate 1A: 5B proportion. The components are assembled in the periplasm after translation. Any extra B subunits can be excreted by the cell, but A must be attached to 5B in order to exit the cell. Intact A subunit is not enzymatically active, but must be nicked to produce fragments A1 and A2 which are linked by a disulfide bond. Once the cholera toxin has bound to the GM1 receptor on host cells, the A1 subunit is released from the toxin by reduction of the disulfide bond that links it to A2, and enters the cell by an unknown translocation mechanism. One hypothesis is that the 5 B subunits form a pore in the host cell membrane through which the A1 unit passes.

Transcription of the *ctxAB* operon is **regulated by a number of environmental signals**, including temperature, pH, osmolarity, and certain amino acids. Several other *V. cholerae* genes are coregulated in the same manner including the *tcp* operon, which is concerned with fimbrial synthesis and assembly. Thus the *ctx* operon and the *tcp* operon are part of a regulon, the expression of which is controlled by the same environmental signals.

The proteins involved in control of this regulon expression have been identified as **ToxR**, **ToxS** and **ToxT**. **ToxR** is a transmembranous protein with about two-thirds of its amino terminal part exposed to the cytoplasm. ToxR dimers, but not ToxR monomers, will bind to the operator region of *ctxAB* operon and activate its transcription. **ToxS** is a periplasmic protein. It is thought that ToxS can respond to environmental signals, change conformation, and somehow influence dimerization of ToxR which activates transcription of the operon. ToxR and ToxS appear

to form a standard two-component regulatory system with ToxS functioning as a sensor protein that phosphorylates and thus converts ToxR to its active DNA binding form. **ToxT** is a cytoplasmic protein that is a transcriptional activator of the tcp operon. Expression of ToxT is activated by ToxR, while ToxT, in turn, activates transcription of tcp genes for synthesis of tcp pili.

Thus, the **ToxR** protein is a **regulatory protein** which functions as an **inducer** in a system of **positive control**. Tox R is thought to interact with ToxS in order to sense some change in the environment and transmit a molecular signal to the chromosome which induces the transcription of genes for attachment (pili formation) and toxin production. It is reasonable to expect that the environmental conditions that exist in the GI tract (i.e., 37° temperature, low pH, high osmolarity, etc.), as opposed to conditions in the extraintestinal (aquatic) environment of the vibrios, are those that are necessary to induce formation of the virulence factors necessary to infect. However, there is conflicting experimental evidence in this regard, which leads to speculation of the ecological function of the toxin during human infection.

Immunity to Cholera

Infection with *V. cholerae* results in a spectrum of responses ranging from life-threatening secretory diarrhea to mild or unapparent infections of no manifestation except a serologic response. The reasons for these differences are not known. One idea is that individuals differ in the availability of intestinal receptors for cholera vibrios or for their toxin, but this has not been proven. Prior immunologic experience is certainly a major factor. For example, in heavily endemic regions such as Bangladesh, the attack rate is relatively low among adults in comparison with children.

After natural infection by *V. cholerae*, circulating antibodies can be detected against several cholera antigens including the toxin, somatic (O) antigens, and flagellar (H) antigens. These antibodies are also raised by parenteral injection of antigens as vaccine components. Antibodies directed against *Vibrio* O antigens are considered "vibriocidal" antibodies because they will lyse *V. cholerae* cells in the presence of complement and serum components. Vibriocidal antibodies reach a peak 8-10 days after the onset of clinical illness, and then decrease, returning to the baseline 2 - 7 months later. Their presence correlates with resistance to infection, but they may not be the mediators of this protection, and the role of circulating antibodies in natural infection is unclear.

After natural infection, people also develop toxin-neutralizing antibodies but there is no correlation between antitoxic antibody levels and the incidence of disease in cholera zones.

Since cholera is essentially a topical disease of the small intestine, it would seem that topical defense might be a main determinant of protection against infection by *V. cholerae*. Recurrent infections of cholera are in fact, rare, and this is probably due to local immune defense mediated by antibodies secreted onto the surfaces of the intestinal mucosa. Moreover, in children who are nursing cholera is less likely to occur, presumably due to protection afforded by secretory antibody in mother's milk.

Secretory IgA, as well as IgG and IgM in serum exudate, can be detected in the intestinal mucosa of immune individuals. Although these antibodies presumably have to function in the absence of complement they still bring about protective immunity. Motility is important in pathogenesis, and antibodies against flagella could immobilize the vibrios. Antibodies against flagella or somatic O antigens could cause clumping and arrested motion of cells. Antitoxic antibodies could react with toxin at the epithelial cell surface and block binding or activity of the toxin. Since the process by which the vibrios attach to the intestinal epithelium is highly specific, antibodies against *Vibrio* fimbriae or other surface components (LPS?) could block attachment.

The observation that natural infection confers effective and long-lasting immunity against cholera has led to efforts to develop a vaccine which will elicit protective immunity. The first attempts at a vaccine in 1960s were directed at whole cell preparations injected parenterally. At best, 90% protection was achieved and this immunity waned rapidly to the baseline within one year. Purified LPS fractions from different biotypes have also been given as vaccines with variable success. The cholera toxin can be converted to toxoid in the presence of formalin and glutaraldehyde. The toxoid is a poor antigen, however, and it elicits a very low level of protection.

At the present time, the manufacture and sale of the only licensed cholera vaccine in the United States has been discontinued. Two recently developed oral vaccines for cholera are licensed and available in other countries (Dukoral®, Biotec AB and Mutacol®, Berna). Both vaccines appear to provide somewhat better immunity and

fewer side-effects than the previously available vaccine. However, neither of these two vaccines is recommended for travelers nor are they available in the United States. Nor are the vaccines recommended for inhabitants of regions where cholera is entrenched, since their use may render complacency with regard to individual susceptibility to disease. One of the vaccines also advertises protection against enterotoxigenic *E. coli* (ETEC) which produces a toxin (LT) identical to cholera toxin, and which is an important cause of traveller's diarrhea.

The oral vaccines are made from a live attenuated strains of *V. cholerae*. The ideal properties of such a "vaccine strain" of the bacterium would be to possess all the pathogenicity factors required for colonization of the small intestine (e.g. motility, fimbriae, neuraminidase, etc.) but not to produce a complete toxin molecule. Ideally it should produce only the B subunit of the toxin which would stimulate formation of antibodies that could neutralize the binding of the native toxin molecule to epithelial cells.

A new vaccine has been developed to combat the *Vibrio cholerae* Bengal strain that has started spreading in epidemic fashion in the Indian subcontinent and Southeast Asia. The Bengal strain differs from previously isolated epidemic strains in that it is serogroup 0139 rather than 01, and it expresses a distinct polysaccharide capsule. Since previous exposure to 01 *Vibrio cholerae* does not provide protective immunity against 0139, there is no residual immunity in the indigenous population to the Bengal form of cholera.

The noncellular vaccine is relatively nontoxic and contains little or no LPS and other impurities. The vaccine will be used for active immunization against *Vibrio cholerae* O139 and other bacterial species expressing similar surface polysaccharides. In addition, human or other antibodies induced by this vaccine could be used to identify *Vibrio cholerae* Bengal for the diagnosis of the infection and for environmental monitoring of the bacterium.

Cholera References and Links

Baron Medical Microbiology Textbook

[Cholera, Vibrio cholerae O1 and O139, and Other Pathogenic Vibrios](#) by Richard A. Finkelstein

World Health Organization

[Cholera](#)

CDC

[Cholera](#)

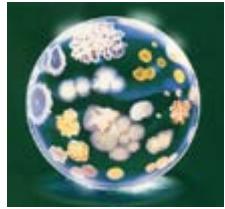
[Travelers' Health Information on Cholera](#)

FDA Bad Bug Book

[Vibrio cholerae Serogroup O1](#)

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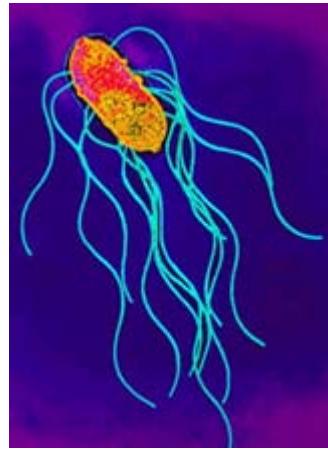
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Salmonella and salmonellosis

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Salmonella enterica

Salmonella is a Gram-negative facultative rod-shaped bacterium in the same proteobacterial family as *Escherichia coli*, the family **Enterobacteriaceae**, trivially known as "enteric" bacteria. *Salmonella* is nearly as well-studied as *E. coli* from a structural, biochemical and molecular point of view, and as poorly understood as *E. coli* from an ecological point of view. Salmonellae live in the intestinal tracts of warm and cold blooded animals. Some species are ubiquitous. Other species are specifically adapted to a particular host. In humans, *Salmonella* are the cause of two diseases called **salmonellosis: enteric fever (typhoid)**, resulting from bacterial invasion of the bloodstream, and **acute gastroenteritis**, resulting from a foodborne infection/intoxication.

Discovery of the Typhoid Bacillus

At the beginning of the 19th century, typhoid was defined on the basis of clinical signs and symptoms and pathological (anatomical) changes. However, at this time, all sorts of enteric fevers were characterized as "typhoid".

In 1880s, the typhoid bacillus was first observed by Eberth in spleen sections and mesenteric lymph nodes from a patient who died from typhoid. Robert Koch confirmed a related finding by Gaffky and succeeded in cultivating the bacterium in 1881. But due to the lack of differential characters, separation of the typhoid bacillus from other enteric bacteria was uncertain.

In 1896, it was demonstrated that the serum from an animal immunized with the typhoid bacillus agglutinated (clumped) the typhoid bacterial cells, and it was shown that the serum of patients afflicted with typhoid likewise agglutinated the typhoid bacillus. Serodiagnosis of typhoid was thus made possible by 1896.

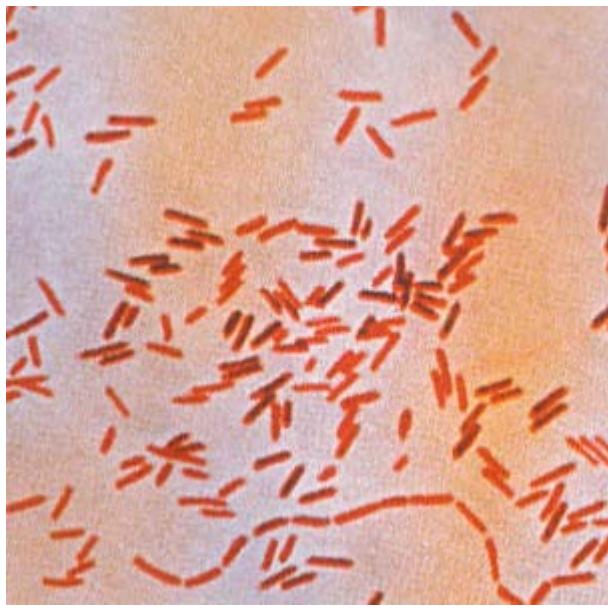


Figure 1. *Salmonella typhi*, the agent of typhoid. Gram stain. (CDC)

Salmonella Nomenclature

The genus *Salmonella* is a member of the family *Enterobacteriaceae*. It is composed of bacteria related to each other both phenotypically and genotypically. *Salmonella* DNA base composition is 50-52 mol% G+C, similar to that of *Escherichia*, *Shigella*, and *Citrobacter*. The bacteria of the genus *Salmonella* are also related to each other by DNA sequence. The genera with DNA most closely related to *Salmonella* are *Escherichia*, *Shigella*, and *Citrobacter*. Similar relationships were found by numerical taxonomy and 16S ssRNA analysis.

Salmonella nomenclature has been controversial since the original taxonomy of the genus was not based on DNA relatedness, rather names were given according to clinical considerations, e.g., *Salmonella typhi*, *Salmonella cholerae-suis*, *Salmonella abortus-ovis*, and so on. When serological analysis was adopted into the Kauffmann-White scheme in 1946, a *Salmonella* species was defined as "a group of related fermentation phage-type" with the result that each *Salmonella* serovar was considered as a species. Since the host-specificity suggested by some of these earlier names does not exist (e.g., *S. typhi-murium*, *S. cholerae-suis* are in fact ubiquitous), names derived from the geographical origin of the first isolated strain of the newly discovered serovars were next chosen, e.g., *S. london*, *S. panama*, *S. stanleyville*.

Subsequently it was found that all *Salmonella* serovars form a single DNA hybridization group, i.e., a single species composed of seven subspecies, and the nomenclature had to be adapted. To avoid confusion with the familiar names of serovars, the species name *Salmonella enterica* was proposed with the following names for the subspecies:

- enterica I
- salamae II
- arizonae IIIa
- diarizonae IIIb
- houtenae IV
- bongori V
- indica VI

Each subspecies contains various serovars defined by a characteristic antigenic formula.

Since this formal Latin nomenclature may not be clearly understood by physicians and epidemiologists, who are the most familiar with the names given to the most common serovars, the common serovar names are kept for subspecies I strains, which represent more than 99.5% of the *Salmonella* strains isolated from humans and other warm-blooded animals. The vernacular terminology seems preferred in medical practice, e.g., *Salmonella* ser. Typhimurium (not italicized) or shorter *Salmonella* (or *S.*) Typhimurium.

Antigenic Structure

As with all *Enterobacteriaceae*, the genus *Salmonella* has three kinds of major antigens with diagnostic or identifying applications: somatic, surface, and flagellar.

Somatic (O) or Cell Wall Antigens

Somatic antigens are heat stable and alcohol resistant. Cross-absorption studies individualize a large number of antigenic factors, 67 of which are used for serological identification. O factors labeled with the same number are closely related, although not always antigenically identical.

Surface (Envelope) Antigens

Surface antigens, commonly observed in other genera of enteric bacteria (e.g., *Escherichia coli* and *Klebsiella*), may be found in some *Salmonella* serovars. Surface antigens in *Salmonella* may mask O antigens, and the bacteria will not be agglutinated with O antisera. One specific surface antigen is well known: the Vi antigen. The Vi antigen occurs in only three *Salmonella* serovars (out of about 2,200): Typhi, Paratyphi C, and Dublin. Strains of these three serovars may or may not have the Vi antigen.

Flagellar (H) Antigens

Flagellar antigens are heat-labile proteins. Mixing salmonella cells with flagella-specific antisera gives a characteristic pattern of agglutination (bacteria are loosely attached to each other by their flagella and can be dissociated by shaking). Also, antiflagellar antibodies can immobilize bacteria with corresponding H antigens.

A few *Salmonella enterica* serovars (e.g., Enteritidis, Typhi) produce flagella which always have the same antigenic specificity. Such an H antigen is then called monophasic. Most *Salmonella* serovars, however, can alternatively produce flagella with two different H antigenic specificities. The H antigen is then called diphasic. For example, Typhimurium cells can produce flagella with either antigen i or antigen 1,2. If a clone is derived from a bacterial cell with H antigen i, it will consist of bacteria with i flagellar antigen. However, at a frequency of 10^{-3} - 10^{-5} , bacterial cells with 1,2 flagellar antigen pattern will appear in this clone.

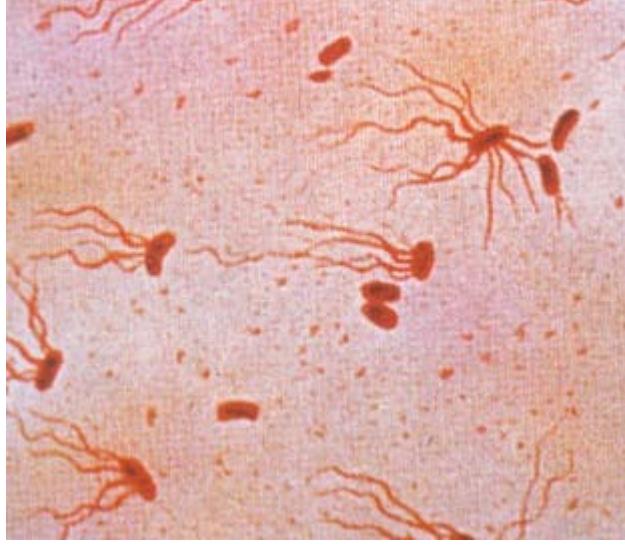


Figure 2. Flagellar stain of a *Salmonella* Typhi. Like *E. coli*, *Salmonella* are motile by means of peritrichous flagella. A close relative that causes enteric infections is the bacterium *Shigella*. *Shigella* is not motile, and therefore it can be differentiated from *Salmonella* on the basis of a motility test or a flagellar stain. (CDC)

Habitats

The principal habitat of the salmonellae is the intestinal tract of humans and animals. *Salmonella* serovars can be found predominantly in one particular host, can be ubiquitous, or can have an unknown habitat. Typhi and Paratyphi A are strictly human serovars that may cause grave diseases often associated with invasion of the bloodstream. Salmonellosis in these cases is transmitted through fecal contamination of water or food. Gallinarum, Abortusovis, and Typhisuis are, respectively, avian, ovine, and porcine *Salmonella* serovars. Such host-adapted serovars cannot grow on minimal medium without growth factors (contrary to the ubiquitous *Salmonella* serovars).

Ubiquitous (non-host-adapted) *Salmonella* serovars (e.g., Typhimurium) cause very diverse clinical symptoms, from asymptomatic infection to serious typhoid-like syndromes in infants or certain highly susceptible animals (mice). In human adults, ubiquitous *Salmonella* organisms are mostly responsible for foodborne toxic infections.

The pathogenic role of a number of *Salmonella* serovars is unknown. This is especially the case with serovars from subspecies II to VI. A number of these serovars have been isolated rarely (some only once) during a systematic

search in cold-blooded animals.

Salmonella in the Natural Environment

Salmonellae are disseminated in the natural environment (water, soil, sometimes plants used as food) through human or animal excretion. Humans and animals (either wild or domesticated) can excrete *Salmonella* either when clinically diseased or after having had salmonellosis, if they remain carriers. *Salmonella* organisms do not seem to multiply significantly in the natural environment (out of digestive tracts), but they can survive several weeks in water and several years in soil if conditions of temperature, humidity, and pH are favorable.

Isolation and Identification of *Salmonella*

A number of plating media have been devised for the isolation of *Salmonella*. Some media are differential and nonselective, i.e., they contain lactose with a pH indicator, but do not contain any inhibitor for non salmonellae (e.g., bromocresol purple lactose agar). Other media are differential and slightly selective, i.e., in addition to lactose and a pH indicator, they contain an inhibitor for nonenterics (e.g., MacConkey agar and eosin-methylene blue agar).

The most commonly used media selective for *Salmonella* are SS agar, bismuth sulfite agar, Hektoen enteric (HE) medium, brilliant green agar and xylose-lysine-deoxycholate (XLD) agar. All these media contain both selective and differential ingredients and they are commercially available.

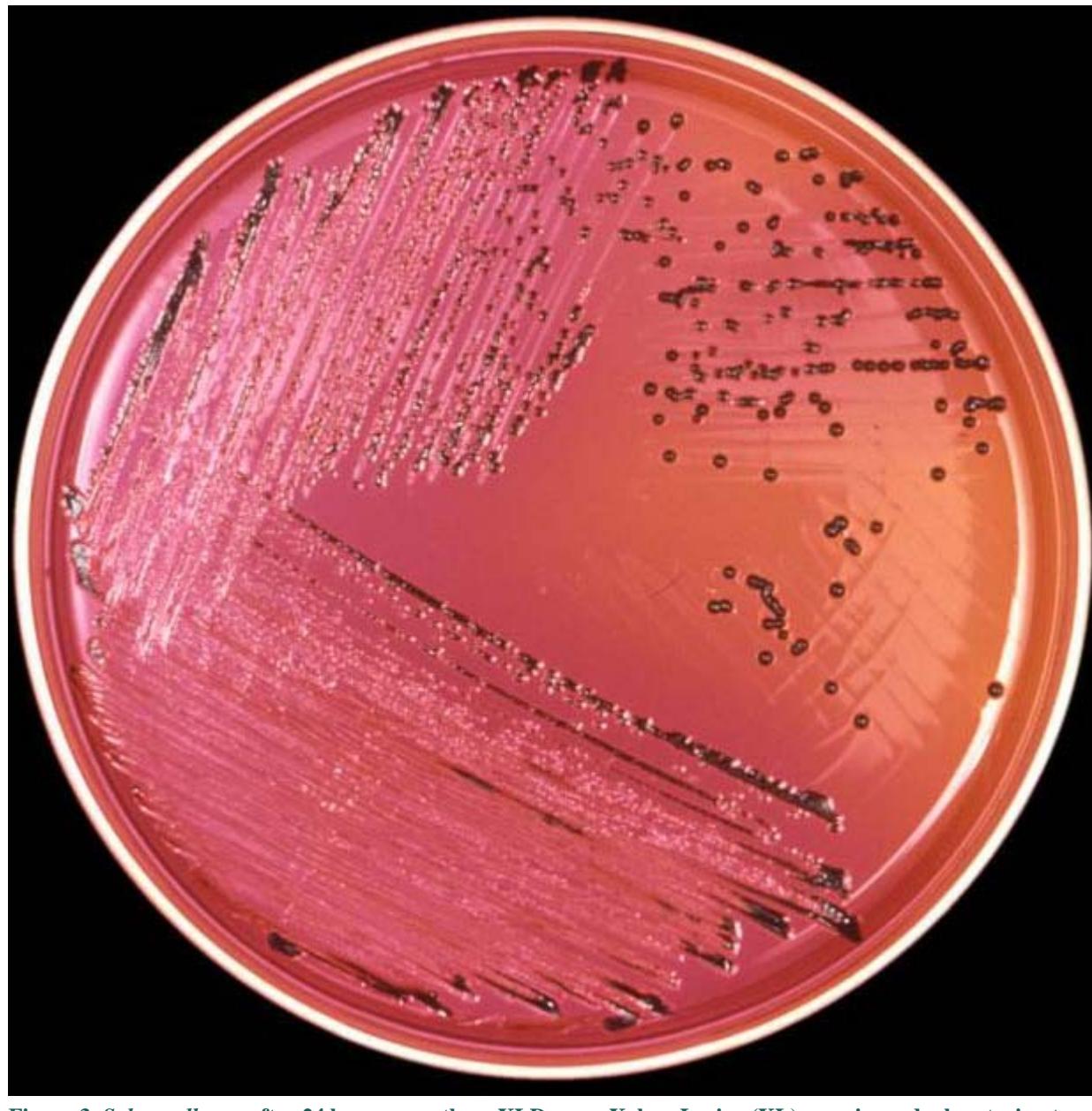


Figure 3. *Salmonella* sp. after 24 hours growth on XLD agar. Xylose Lysine (XL) agar is used when trying to culture and isolate Gram-negative enteric bacilli. When XL agar is supplemented with sodium thiosulfate, ferric ammonium citrate, and sodium deoxycholate, it is then termed XLD agar, and is then an even more selective medium than XL alone. The presence of any black colored area

indicates the deposition of hydrogen sulfide, (H_2S) under alkaline conditions. (CDC)

Media used for *Salmonella* identification are those used for identification of all *Enterobacteriaceae*. Most *Salmonella* strains are motile with peritrichous flagella, however, nonmotile variants may occur occasionally. Most strains grow on nutrient agar as smooth colonies, 2-4 mm in diameter. Most strains are prototrophs, not requiring any growth factors. However, auxotrophic strains do occur, especially in host-adapted serovars such as Typhi and Paratyphi A.

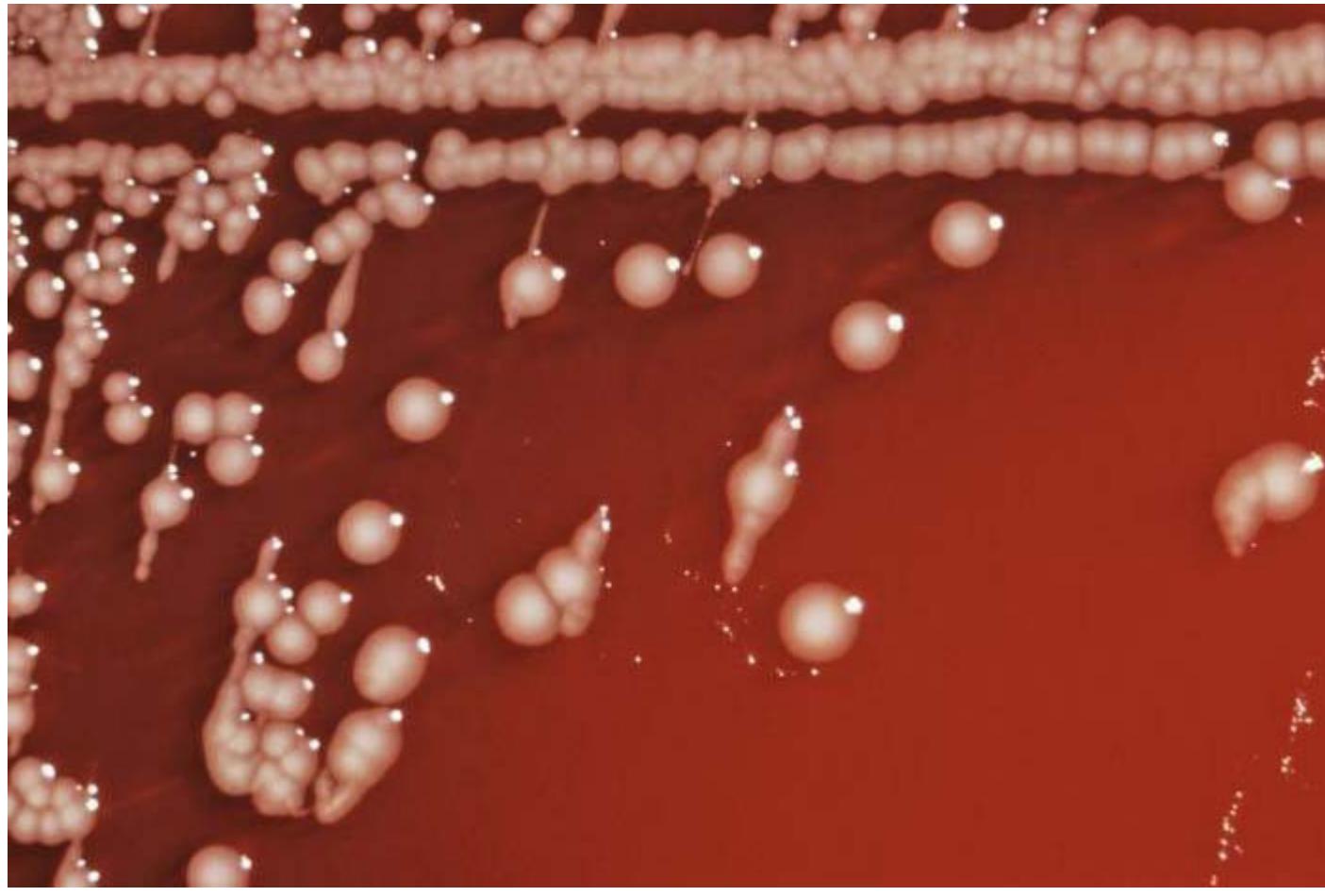


Figure 4. Colonial growth *Salmonella choleraesuis* subsp. *arizonae* bacteria grown on a blood agar culture plate. Also known as *Salmonella Arizonae*, it is a zoonotic bacterium that can infect humans, birds, reptiles, and other animals. (CDC)

Table 1. Characteristics shared by most *Salmonella* strains belonging to subspecies I

Motile, Gram-negative bacteria

Lactose negative; acid and gas from glucose, mannitol, maltose, and sorbitol; no Acid from adonitol, sucrose, salicin, lactose

ONPG test negative (lactose negative)

Indole test negative

Methyl red test positive

Voges-Proskauer test negative

Citrate positive (growth on Simmon's citrate agar)

Lysine decarboxylase positive

Urease negative

Ornithine decarboxylase positive

H_2S produced from thiosulfate

Do not grow with KCN

Phenylalanine and tryptophan deaminase negative

Gelatin hydrolysis negative

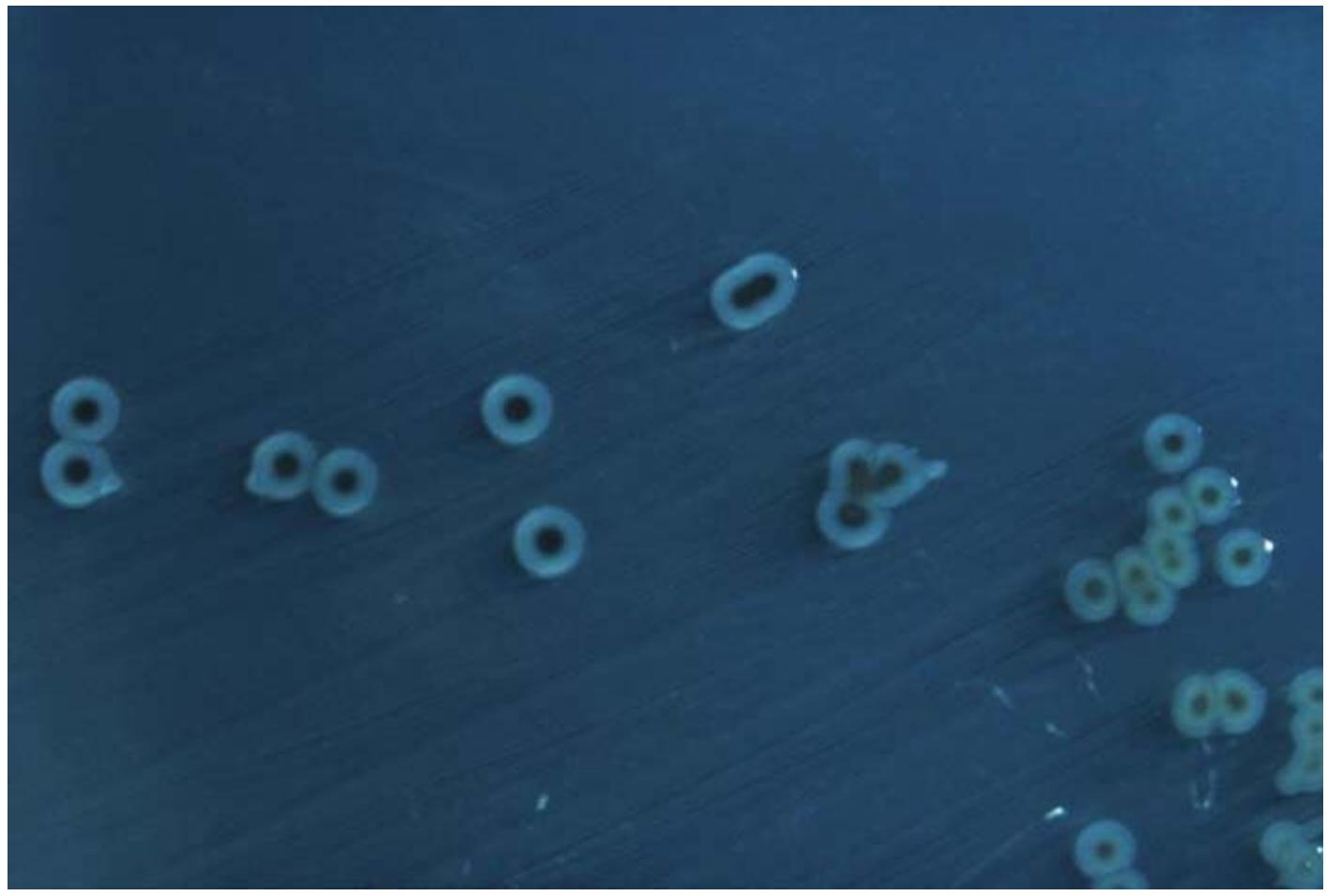


Figure 5. Colonial growth pattern displayed by *Salmonella* Typhimurium cultured on a Hektoen enteric (HE) agar. *S. Typhimurium* colonies grown on HE agar are blue-green in color indicating that the bacterium does not ferment lactose However it does produce hydrogen sulfide, (H_2S), as indicated by black deposits in the centers of the colonies. (CDC)

HE agar is the medium designed for the isolation and recovery of fecal bacteria belonging to the family, Enterbacteriaceae. *S. Typhimurium* causes 25% of the 1.4 million salmonellosis infections a year in the United States. Most persons infected with *Salmonella* sp. develop diarrhea, fever, and abdominal cramps 12 - 72 hours after infection. The illness usually lasts 4 - 7 days, and most people recover without treatment. However, in some cases, the diarrhea may be so severe that the patient needs to be hospitalized.

Genetics of *Salmonella*

The genetic map of the *Salmonella* Typhimurium strain LT2 is not very different from that of *Escherichia coli* K-12. The F plasmid can be transferred to Typhimurium, and an Hfr strain of Typhimurium may subsequently be selected. Conjugative chromosomal transfer may occur from Typhimurium Hfr to *E. coli* or from *E. coli* Hfr to Typhimurium. Chromosomal genes responsible for O, Vi, and H antigens can be transferred from *Salmonella* to *Escherichia*.

Also, *Salmonella* may harbor temperate phages and plasmids. Plasmids in *Salmonella* may code for antibiotic resistance (resistance plasmids are frequent due to the selective pressure of extensive antibiotic therapy), bacteriocins, metabolic characteristics such as lactose or sucrose fermentation, or antigenic changes of O antigen.

Pathogenesis of *Salmonella* Infections in Humans

Salmonella infections in humans vary with the serovar, the strain, the infectious dose, the nature of the contaminated food, and the host status. Certain serovars are highly pathogenic for humans; the virulence of more rare serovars is unknown. Strains of the same serovar are also known to differ in their pathogenicity. An oral dose of at least 10^5 *Salmonella* Typhi cells are needed to cause typhoid in 50% of human volunteers, whereas at least 10^9 *S. Typhimurium* cells (oral dose) are needed to cause symptoms of a toxic infection. Infants, immunosuppressed patients, and those affected with blood disease are more susceptible to *Salmonella* infection than healthy adults.

In the **pathogenesis of typhoid** the bacteria enter the human digestive tract, penetrate the intestinal mucosa (causing no lesion), and are stopped in the mesenteric lymph nodes. There, bacterial multiplication occurs, and part

of the bacterial population lyses. From the mesenteric lymph nodes, viable bacteria and LPS (endotoxin) may be released into the bloodstream resulting in septicemia. Release of endotoxin is responsible for cardiovascular "collapsus and tuphos" (a stuporous state—origin of the name typhoid) due to action on the ventriculus neurovegetative centers.

Salmonella excretion by human patients may continue long after clinical cure. Asymptomatic carriers are potentially dangerous when unnoticed. About 5% of patients clinically cured from typhoid remain carriers for months or even years. Antibiotics are usually ineffective on *Salmonella* carriage (even if salmonellae are susceptible to them) because the site of carriage may not allow penetration by the antibiotic.

Salmonellae survive sewage treatments if suitable germicides are not used in sewage processing. In a typical cycle of typhoid, sewage from a community is directed to a sewage plant. Effluent from the sewage plant passes into a coastal river where edible shellfish (mussels, oysters) live. Shellfish concentrate bacteria as they filter several liters of water per hour. Ingestion by humans of these seafoods (uncooked or superficially cooked) may cause typhoid or other salmonellosis. Salmonellae do not colonize or multiply in contaminated shellfish.

Typhoid is strictly a human disease. The incidence of human disease decreases when the level of development of a country increases (i.e., controlled water sewage systems, pasteurization of milk and dairy products). Where these hygienic conditions are missing, the probability of fecal contamination of water and food remains high and so is the incidence of typhoid.

Foodborne *Salmonella* toxic infections are caused by ubiquitous *Salmonella* serovars (e.g., Typhimurium). About 12-24 hours following ingestion of contaminated food (containing a sufficient number of *Salmonella*), symptoms appear (diarrhea, vomiting, fever) and last 2-5 days. Spontaneous cure usually occurs.

Salmonella may be associated with all kinds of food. Contamination of meat (cattle, pigs, goats, chicken, etc.) may originate from animal salmonellosis, but most often it results from contamination of muscles with the intestinal contents during evisceration of animals, washing, and transportation of carcasses. Surface contamination of meat is usually of little consequence, as proper cooking will sterilize it (although handling of contaminated meat may result in contamination of hands, tables, kitchenware, towels, other foods, etc.). However, when contaminated meat is ground, multiplication of *Salmonella* may occur within the ground meat and if cooking is superficial, ingestion of this highly contaminated food may produce a *Salmonella* infection. Infection may follow ingestion of any food that supports multiplication of *Salmonella* such as eggs, cream, mayonnaise, creamed foods, etc.), as a large number of ingested salmonellae are needed to give symptoms. Prevention of *Salmonella* toxic infection relies on avoiding contamination (improvement of hygiene), preventing multiplication of *Salmonella* in food (constant storage of food at 4°C), and use of pasteurized and sterilized milk and milk products. Vegetables and fruits may carry *Salmonella* when contaminated with fertilizers of fecal origin, or when washed with polluted water.

The incidence of foodborne *Salmonella* infection/toxication remains relatively high in developed countries because of commercially prepared food or ingredients for food. Any contamination of commercially prepared food will result in a large-scale infection. In underdeveloped countries, foodborne *Salmonella* intoxications are less spectacular because of the smaller number of individuals simultaneously infected, but also because the bacteriological diagnosis of *Salmonella* toxic infection may not be available. However, the incidence of *Salmonella* carriage in underdeveloped countries is known to be high.

Salmonella epidemics may occur among infants in pediatric wards. The frequency and gravity of these epidemics are affected by hygienic conditions, malnutrition, and the excessive use of antibiotics that select for multiresistant strains.

***Salmonella Enteritidis* Infection**

Egg-associated salmonellosis is an important public health problem in the United States and several European countries. *Salmonella* Enteritidis, can be inside perfectly normal-appearing eggs, and if the eggs are eaten raw or undercooked, the bacterium can cause illness. During the 1980s, illness related to contaminated eggs occurred mostly frequently in the northeastern United States, but now illness caused by *S. Enteritidis* is increasing in other parts of the country as well.

Unlike eggborne salmonellosis of past decades, the current epidemic is due to intact and disinfected grade A eggs. *Salmonella* Enteritidis silently infects the ovaries of healthy appearing hens and contaminates the eggs before the

shells are formed. Most types of *Salmonella* live in the intestinal tracts of animals and birds and are transmitted to humans by contaminated foods of animal origin. Stringent procedures for cleaning and inspecting eggs were implemented in the 1970s and have made salmonellosis caused by external fecal contamination of egg shells extremely rare. However, unlike eggborne salmonellosis of past decades, the current epidemic is due to intact and disinfected grade A eggs. The reason for this is that *Salmonella Enteritidis* silently infects the ovaries of hens and contaminates the eggs before the shells are formed.

Although most infected hens have been found in the northeastern United States, the infection also occurs in hens in other areas of the country. In the Northeast, approximately one in 10,000 eggs may be internally contaminated. In other parts of the United States, contaminated eggs appear less common. Only a small number of hens seem to be infected at any given time, and an infected hen can lay many normal eggs while only occasionally laying an egg contaminated with *Salmonella Enteritidis*.

A person infected with the *Salmonella Enteritidis* usually has fever, abdominal cramps, and diarrhea beginning 12 to 72 hours after consuming a contaminated food or beverage. The illness usually lasts 4 to 7 days, and most persons recover without antibiotic treatment. However, the diarrhea can be severe, and the person may be ill enough to require hospitalization. The elderly, infants, and those with impaired immune systems (including HIV) may have a more severe illness. In these patients, the infection may spread from the intestines to the bloodstream, and then to other body sites and can cause death unless the person is treated promptly with antibiotics.

Exotoxins

Salmonella strains may produce a thermolabile enterotoxin that bears a limited relatedness to cholera toxin both structurally and antigenically. This enterotoxin causes water secretion in rat ileal loop and is recognized by antibodies against both cholera toxin and the thermolabile enterotoxin (LT) of enterotoxinogenic *E. coli*, but it does not bind in vitro to ganglioside GM1 (the receptor for *E. coli* LT and cholera ctx). Additionally, a cytotoxin that inhibits protein synthesis and is immunologically distinct from Shiga toxin has been demonstrated. Both of these toxins are presumed to play a role in the diarrheal symptoms of salmonellosis.

Antibiotic Susceptibility

During the last decade, antibiotic resistance and multiresistance of *Salmonella* spp. have increased a great deal. The cause appears to be the increased and indiscriminate use of antibiotics in the treatment of humans and animals and the addition of growth-promoting antibiotics to the food of breeding animals. Plasmid-borne antibiotic resistance is very frequent among *Salmonella* strains involved in pediatric epidemics (e.g., Typhimurium, Panama, Wien, Infantis). Resistance to ampicillin, streptomycin, kanamycin, chloramphenicol, tetracycline, and sulfonamides is commonly observed. Colistin resistance has not yet been observed.

Until 1972, Typhi strains had remained susceptible to antibiotics, including chloramphenicol (the antibiotic most commonly used against typhoid) but in 1972, a widespread epidemic in Mexico was caused by a chloramphenicol-resistant strain of *S. Typhi*. Other chloramphenicol-resistant strains have since been isolated in India, Thailand, and Vietnam. Possible importation or appearance of chloramphenicol-resistance strains in the United States is a real threat. *Salmonella* strains should be systematically checked for antibiotic resistance to aid in the choice of an efficient drug when needed and to detect any change in antibiotic susceptibility of strains (either from animal or human source). Indiscriminate distribution and use of antibiotics should be discouraged.

Vaccination Against Typhoid Fever

Three types of typhoid vaccines are currently available for use in the United States: (1) an oral live-attenuated vaccine; (2) a parenteral heat-phenol-inactivated vaccine; (3) a newly licensed capsular polysaccharide vaccine for parenteral use. A fourth vaccine, an acetone-inactivated parenteral vaccine, is currently available only to the armed forces.

1. Live oral vaccines. Although oral killed vaccines are without efficacy, vaccines using living avirulent bacteria have shown promise. A galactose-epimeraseless mutant of Typhi has given very good results in a field trials. Mutants of Typhimurium that have given a good protection in animals include mutants lacking adenylate-cyclase and AMP receptor protein, and mutants auxotrophic for p-aminobenzoate and adenine. Typhi with the same mutations does not cause adverse reactions and is immunogenic in human.

The Live Oral Typhoid Vaccine should not be given to children younger than 6 years of age. It is given in four doses, 2 days apart, as needed for protection. The last dose should be given at least 1 week before travel to allow the vaccine time to work. A booster dose is needed every 5 years for people who remain at risk.

2. The parenteral **heat-phenol-inactivated vaccine** has been widely used for many years. In field trials involving a primary series of two doses of heat-phenol- inactivated typhoid vaccine, efficacy over the 2- to 3-year follow-up periods ranged from 51% to 77%. Efficacy for the acetone- inactivated parenteral vaccine, available only to the armed forces, ranges from 75% to 94%.

Since the inactivated vaccines contain the O antigen (endotoxin), local and general reactions occur. Vi antigen extracted following the methodology used for the meningococcal vaccine seems to avoid reactions to endotoxin.

The inactivated Typhoid Vaccine should not be given to children younger than 2 years of age. One dose provides protection. It should be given at least 2 weeks before travel to allow the vaccine time to work. A booster dose is needed every 2 years for people who remain at risk.

3. The newly licensed parenteral vaccine [**Vi capsular polysaccharide (ViCPS)**] is composed of purified Vi ("virulence") antigen, the capsular polysaccharide elaborated by *S.Typhi* isolated from blood cultures. In recent studies, one 25-ug injection of purified ViCPS produced seroconversion (i.e., at least a fourfold rise in antibody titers) in 93% of healthy U.S. adults. Two field trials in disease-endemic areas have demonstrated the efficacy of ViCPS in preventing typhoid fever. In one trial in Nepal, in which vaccine recipients were observed for 20 months, one dose of ViCPS among persons 5-44 years of age resulted in 74% fewer cases of typhoid fever. ViCPS has not been tested among children less than 1 year of age.

NOTE: No typhoid vaccine is 100% effective and is not a substitute for being careful about what you eat or drink.

Routine typhoid vaccination is not recommended in the United States, but typhoid vaccine is recommended for travellers to parts of the world where typhoid is common, people in close contact with a typhoid carriers, and laboratory workers who work with *Salmonella* Typhi bacteria.

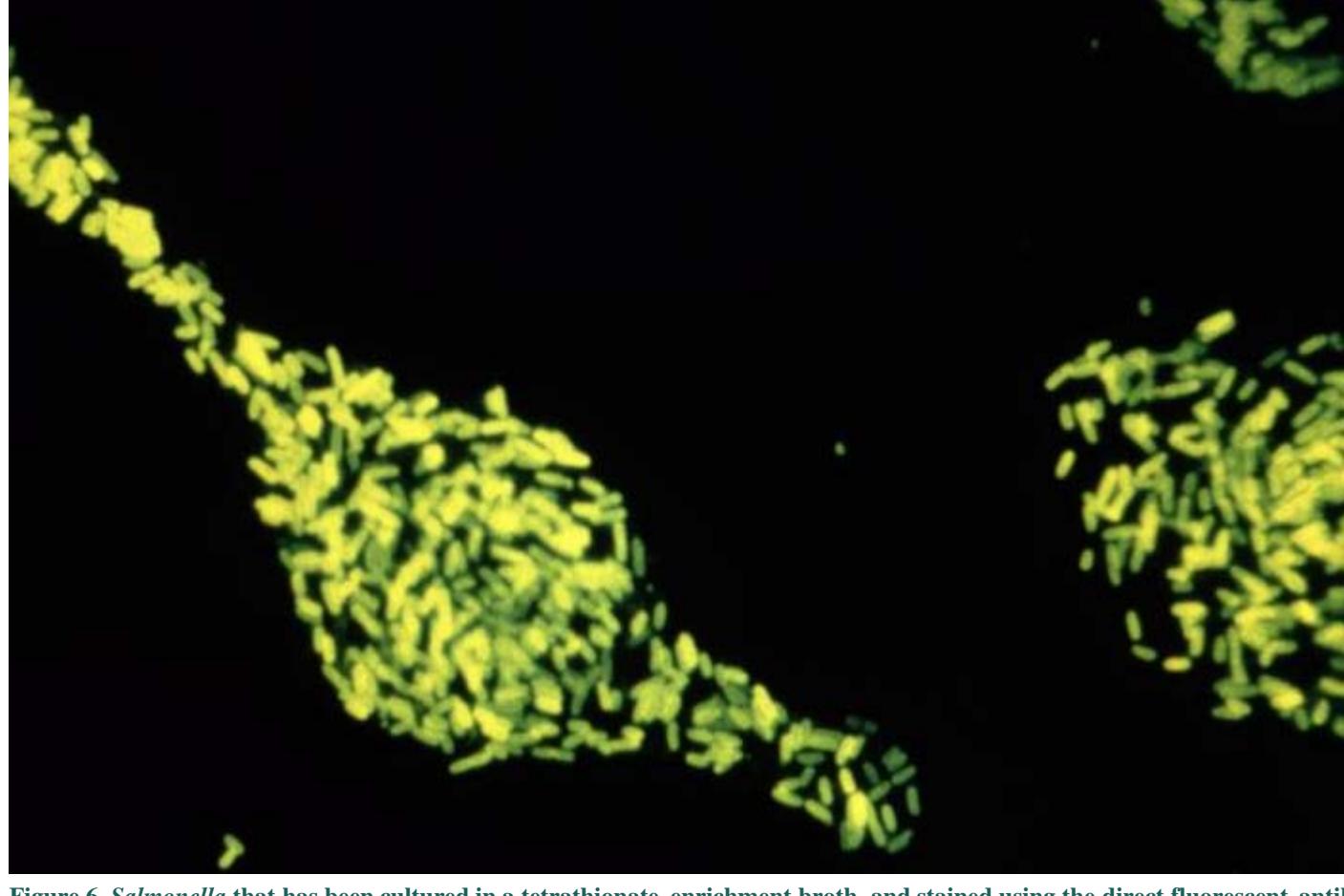


Figure 6. *Salmonella* that has been cultured in a tetrathionate-enrichment broth, and stained using the direct fluorescent-antibody (FA) technique. Tetrathionate-enrichment broth contains bile salts, thereby inhibiting the growth of Gram-positive organisms, while the Gram-negative *Salmonella* sp., being an organism that possess the enzyme tetrathionate reductase, is able to break down

tetrathionate, and grow uninhibited. (CDC)

For more information on salmonella infections please see

[CDC Salmonellosis](#)

[CDC Salmonella enteritidis](#)

[CDC Salmonella Infection \(salmonellosis\) and Animals](#)

[CDC Typhoid General information](#)

[CDC Typhoid traveller's information](#)

[CDC Typhoid vaccine: What You Need to Know](#)

[FDA/CFSAN Bad Bug Book - Salmonella spp](#)

[MedlinePlus Enteric fever](#)

[NOVA | The Most Dangerous Woman in America \(Typhoid Mary\)](#)

[Typhoid and paratyphoid fever \(UK\)](#)

[Typhoid Fever Utah Health Dept](#)

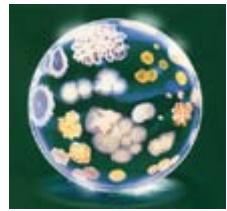
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Shigella and Shigellosis

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Shigella is a genus of the bacterial family *Enterobacteriaceae*. Shigellae are Gram-negative, nonmotile, non-spore forming, rod-shaped bacteria, very closely related to *Escherichia coli*.

Shigellosis is an infectious disease caused by various species of *Shigella*. People infected with *Shigella* develop diarrhea, fever, and stomach cramps starting a day or two after they are exposed to the bacterium. The diarrhea is often bloody. Shigellosis usually resolves in 5 to 7 days, but in some persons, especially young children and the elderly, the diarrhea can be so severe that the patient needs to be hospitalized. A severe infection with high fever may also be associated with seizures in children less than 2 years old. Some persons who are infected may have no symptoms at all, but may still transmit the *Shigella* bacteria to others.

Shigella were discovered over 100 years ago by a Japanese microbiologist named Shiga, for whom the genus are named. There are four species of *Shigella: boydii, dysenteriae, flexneri, and sonnei*. *Shigella sonnei*, also known as "Group D" *Shigella*, accounts for over two-thirds of the shigellosis in the United States. *Shigella flexneri*, or "group B" *Shigella*, accounts for almost all of the rest. Other types of *Shigella* are rare in this country, although they are important causes of disease in the developing world. One type, *Shigella dysenteriae type 1*, causes deadly epidemics in many developing regions and nations.

Diagnosis

Determining that *Shigella* is the cause of the illness depends on laboratory tests that identify the bacteria in the stool of an infected person. Some of the tests may not be performed routinely, so the bacteriology laboratory should be instructed to look for the organism. The laboratory can also do tests to determine which type of *Shigella* is involved, and which antibiotics, if any, would be best for treatment.



Figure 1. Several media have been designed to selectively grow enteric bacteria and allow differentiation of *Salmonella* and *Shigella* from *E. coli*. The primary plating media shown here are eosin methylene blue (EMB) agar, MacConkey agar, ENDO agar, Hektoen enteric (HE) agar and Salmonella-Shigella (SS) agar.

Treatment

Shigellosis can usually be treated with antibiotics. The antibiotics commonly used are ampicillin, trimethoprim/sulfamethoxazole (also known as Bactrim or Septra), nalidixic acid and the fluoroquinolone, ciprofloxacin. Appropriate treatment kills the bacteria present in the gastrointestinal tract and shortens the course of the illness.

Some *Shigella* have become resistant to antibiotics and inappropriate use of antibiotics to treat shigellosis can actually make the organisms more resistant in the future. Persons with mild infections will usually recover quickly without antibiotic treatment. Therefore, when many persons in a community are affected by shigellosis, antibiotics are sometimes used selectively to treat only the more severe cases. Antidiarrheal agents such as loperamide (Imodium) or diphenoxylate with atropine (Lomotil) are likely to make the illness worse and should be avoided.

Reiter's syndrome

Persons with diarrhea usually recover completely, although it may be several months before their bowel habits are entirely normal. About 3% of persons who are infected with *Shigella flexneri* may subsequently develop pains in their joints, irritation of the eyes, and painful urination. This condition is called **Reiter's syndrome**. It can last for months or years, and can lead to chronic arthritis which is difficult to treat. Reiter's syndrome is a late complication of *S. flexneri* infection, especially in persons with a certain genetic predisposition, namely HLA-B27.

Hemolytic Uremic Syndrome (HUS)

Hemolytic-uremic syndrome (HUS) can occur after *S. dysenteriae* type 1 infection. Convulsions may occur in children; the mechanism may be related to a rapid rate of temperature elevation or metabolic alterations, and is associated with the production of the Shiga toxin, which is discussed below.

Transmission

Shigella are transmitted from an infected person to another who become infected. *Shigella* are present in the diarrheal stools of infected persons while they are sick and for a week or two afterwards. Most *Shigella* infections are the result of the bacterium passing from stools or soiled fingers of one person to the mouth of another person. This happens when basic hygiene and handwashing habits are inadequate. It is particularly likely to occur among toddlers who are not fully toilet-trained. Family members and playmates of such children are at high risk of becoming infected. The spread of *Shigella* from an infected person to other persons can be stopped by frequent and careful handwashing with soap, a practice that is important among all age groups.

Part of the reason for the efficiency of transmission is because a very small inoculum (10 to 200 organisms) is sufficient to cause infection. As a result, spread can easily occur by the fecal-oral route and occurs in areas where hygiene is poor. Epidemics may be foodborne or waterborne. *Shigella* can also be transmitted by flies.

Shigella infections may be acquired from eating food that has become contaminated by infected food handlers. Vegetables can become contaminated if they are harvested from a field with contaminated sewage or wherein infected field workers defecate. Flies can breed in infected feces and then contaminate food. *Shigella* infections can also be acquired by drinking or swimming in contaminated water. Water may become contaminated if sewage runs into it, or even if someone with shigellosis swims or bathes or, much less, defecates, in it.

Immunity and Vaccines

Once someone has had shigellosis, they are not likely to get infected with that specific type again for at least several years. However, they can still get infected with other types of *Shigella*. Presumably, this immunity is due to secretory IgA, although CMI may not be ruled out. Circulating antibodies can also be detected in immune individuals.

Currently, there is no vaccine to prevent shigellosis. However, since the virulence of *Shigella* is well-understood, and considering the present art of vaccine development, it seems that vaccination should be feasible.

OFF THE WALL

On the topic of vaccines, the following information is from the Walter Reed Army Institute of Research, [Walter Reed Army Institute of Research, Vaccines: The Best Revenge](#)

"So far, the institute has four vaccines in the works. 'Ideally, the goal would be to have one vaccine that will protect against multiple pathogens that can easily be given to deploying soldiers,' said Maj. David Katz, a senior clinical investigator at WRAIR. 'So soldiers can take it before they deploy to an area, and they'll be protected.'

A vaccine to combat *Shigella flexneri*, called SC602, was developed along with The Institut Pasteur. Since 1992 it has undergone clinical trials in the States and Bangladesh. 'The wonderful thing about the shigella vaccines is... the bacteria (used in them) are alive but weakened to diminish the amount of symptoms,' Katz said. 'The body thinks it's infected and gives an immune response, but you don't get infected like a natural infection because the bacteria don't spread from cell to cell.'

Receiving the oral vaccine before deploying is key, Katz said. 'Most of the soldiers will get hit right when they arrive in a new area, either because they're eating on the economy or they're in a new area and their system has not been primed.'

'Another reason to give the vaccine ahead of time is because of potential side effects,' said Dr. Thomas Hale, Chief of the Department of Enteric Infections at WRAIR. 'The vaccine can cause some short-term fevers and mild diarrhea in 20 percent of the people who

receive it, so soldiers need to take it well before they get on a plane.'

A vaccine for *Shigella sonnei*, which often attacks travelers and stateside daycare centers, is a possible stand alone product, Hale said. 'This one vaccine could make a significant difference in the health of soldiers deployed to the Middle East (where 90 percent of outbreaks occur) and the developing world.'

Drs. Malabi Venkatesan and Antoinette Hartman from WRAIR developed the oral vaccine, called WRSS1, that is currently in clinical trials in conjunction with the University of Maryland Medical School and the National Institute of Allergy and Infectious Diseases.

The Department of Enteric Infections at WRAIR has teamed up with the Israel Defense Force for a vaccine trial evaluating WRSS1 this winter. 'Israel has cities that are very Westernized, but almost everyone has a compulsory military obligation, so they go from cities to field posts and the incidence of diarrheal disease is significant,' Katz said.

To combat the deadly form of diarrhea, dysentery--also called bloody diarrhea--WRAIR researchers are working with the Bloomberg School of Public Health at Johns Hopkins University to test the oral Walter Reed Shigella-Dysentery-1 vaccine, WRSD1.

The other diarrhea-causing bacteria WRAIR and the Navy Medical Research Unit researchers are trying to disable is *E. coli*. Whereas shigella bacteria invades a cell's wall and moves from cell to cell to spread the disease, *E. coli* prefers to stick to the intestine's lining, homestead and crank out toxins that cause diarrhea. To outsmart the unwanted tenant, researchers are trying to make antibodies that will prevent squatters from colonizing because they can't stick to the intestine. The vaccine's been tested in a time-release capsule form as well as a transdermal patch,

'It should be easy for the soldier to use: Just pop the patch on and that's it,' Katz said. Though having one vaccine to combat all major forms of infectious diarrhea is a ways off, the quest to prevent soldiers from needing to run, trot and quick step will be WRAIR researchers' best revenge on Montezuma."

Incidence and Risk of Infection

Meanwhile, back in the USA, there are approximately 14,000 laboratory-confirmed cases of shigellosis and an estimated 448,240 total cases (85% due to *S. sonnei*) that occur each year, according to CDC. In the developing world, *S. flexneri* predominates. Epidemics of *S. dysenteriae* type 1 have occurred in Africa and Central America with case fatality rates of 5-15%.

In the United States, groups at increased risk of shigellosis include children in child-care centers and persons in custodial institutions, where personal hygiene is difficult to maintain; Native Americans; orthodox Jews; international travelers; men who have sex with men; and those in homes with inadequate water for handwashing.

Pathogenesis of *Shigella flexneri*

Shigella flexneri causes bacillary dysentery, the symptoms of which include abdominal pain, diarrhea, fever, vomiting and blood or mucus in the stool. The bacteria are transmitted by the fecal-oral route, and through contaminated food and water. Once ingested, the bacteria survive the gastric environment of the stomach and move on to the large intestine. There, they attach to and penetrate the epithelial cells of the intestinal mucosa. After invasion, they multiply intracellularly and spread to neighboring epithelial cells, resulting in tissue destruction and characteristic pathology of shigellosis.

Entry of *Shigella flexneri* into Epithelial Cells

In order for *S. flexneri* to enter an epithelial cell, the bacterium must first adhere to its target cell. It is then internalized by a process which is similar to the mechanism of phagocytosis. Generally, the bacterium adheres to the membrane of the cell and is internalized via an endosome, which it subsequently lyses to gain access to the cytoplasm where multiplication occurs.

To aid its entry into the epithelial cell, the bacterial DNA encodes a number of plasmid and chromosomal proteins. These proteins are the **invasion plasmid antigens** (*Ipa*), **surface presentation antigens** (*Spa*), **membrane excretion proteins** (*Mxi*), and **virulence proteins** (*Vir*).

When the bacterium grows at 37°C, the virulence protein VirF induces the expression of the VirB protein. The VirB protein then activates the *ipa*, *mxi*, and *spa* promoters leading to expression of the *spa* and *mxi* operons. This results in the synthesis and assembly of a protein complex called the **Mxi-Spa translocon**. When the bacterium makes contact with the epithelial cell membrane, the translocon becomes activated and secretes the pre-synthesized

Ipa proteins. IpaB, IpaC and IpaA associate to form a complex which interacts with the host epithelial cell membrane to induce a cascade of cellular signals which will lead to the internalization of the bacterium via an endosome. The Ipa proteins are also required for escape from the endosome.

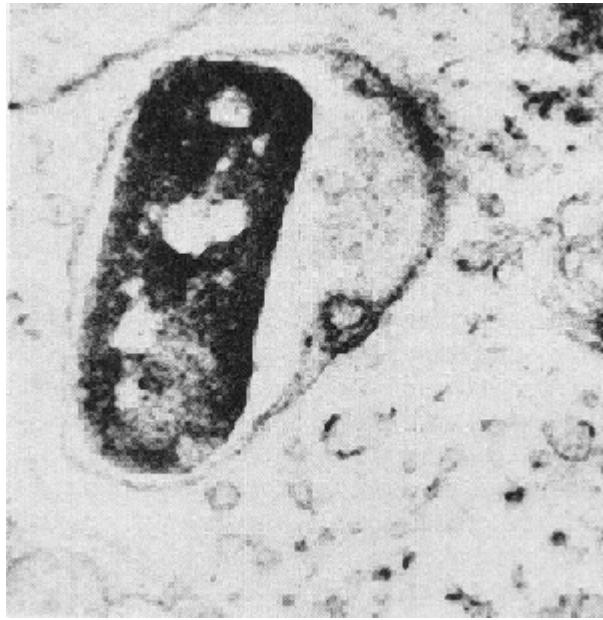


Figure 2 Electron Micrograph of *Shigella* in a membrane-enclosed endosome of an epithelial cell

Intracellular and Intercellular Spread

Extracellular *S. flexneri* cells are nonmotile, but intracellular bacteria move to occupy the entire cytoplasm of the infected cell, and they are able to spread between cells. The genes necessary for intracellular and intercellular spreading are *virG* (*icsA*) and *icsB*.

After entry into the cell, intracellular movement occurs if the bacterium expresses both an "organelle-like movement" (Olm) phenotype, and an alternative Ics phenotype. The expression the Olm phenotype allows the bacteria to "slide" along actin stress cables inside the host cell, while the expression of the Ics phenotype allows the bacteria to "spread" or infect adjacent cells.

Specifically, movement of *S. flexneri* between adjacent cells is mediated via the product of the *virG* (*icsA*) gene. The *icsA* gene elicits actin polymerization at the poles of the bacteria and induces the formation of protrusions. In some instances, these tightly packed actin filaments appear to form a cylinder. The bacteria in the protrusions can move through the host cell and penetrate into an adjacent cell without coming in contact with the extracellular medium where they would be rendered nonmotile.

The *mxiG* gene is required for Ipa protein secretion, and is also essential for entry. This gene and others in the Mxi-Spa translocon are also required for intercellular dissemination.

Pathological Effects

Following host epithelial cell invasion and penetration of the colonic mucosa, *Shigella* infection is characterized by degeneration of the epithelium and inflammation of the lamina propria. This results in desquamation and ulceration of the mucosa, and subsequent leakage of blood, inflammatory elements and mucus into the intestinal lumen. Patients suffering from *Shigella* infection will therefore pass frequent, scanty, dysenteric stool mixed with blood and mucus since under these conditions, the absorption of water by the colon is inhibited. This is in opposition to the diarrheal symptoms seen in patients suffering from extensive *Shigella* colitis, and the pathologic basis for this is unknown. It is possible that prostaglandin interactions induced by the inflammatory response to bacterial invasion contributes to diarrhea in patients with *Shigella* colitis.

The Large Virulence Plasmid of *Shigella flexneri*

All virulent strains of *Shigella flexneri* possess a large 230kb plasmid that mediates its virulence properties. This so-called the **invasion plasmid** has been shown to encode the genes for several aspects of *Shigella* virulence, including

- Ligands that are involved in the adherence of bacteria onto the surface of target epithelial cells
 - The production of invasion plasmid antigens (Ipa) that have a direct role in the *Shigella* invasion process
 - Transport or processing functions that ensure the correct surface expression of the Ipa proteins
 - The induction of endocytic uptake of bacteria and disruption of endocytic vacuoles
 - The intra- and intercellular spreading phenotypes
 - The regulation of plasmid-encoded *vir* genes

The presence of this plasmid was discovered in the 1980s after the observation that essentially the entire chromosome of *S. flexneri* could be transferred to *E. coli* without reconstituting the virulence phenotype of the donor. However, the ability to invade tissue culture cells was transferred to *E. coli* by the conjugal mobilization of this plasmid from *S. flexneri*. The 230kb plasmid has been subjected to SalI endonuclease digestion and 23 fragments labeled A through F have been identified and mapped.

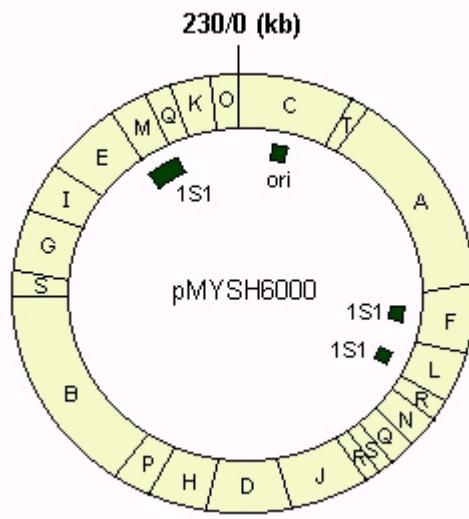


Figure 3.The Circular Sall restriction map of *Shigella flexneri* 2a plasmid pMYSH6000. Adapted from Hale (1991). The large *Shigella* plasmid encodes many of the virulence-associated genes which are summarized in the table below.

Table 1. Virulence-associated Genes and Functions Encoded by the Large *Shigella* Virulence Plasmid

Gene	Protein Product MW	Regulatory or effector function
<i>virF</i>	30 kDa	Positive regulation of <i>virB(invE, ipaR)</i> and <i>virG(icsA)</i>
<i>invA(mxIB)</i>	38 kDa	Necessary for invasion (orients <i>ipa</i> gene products in outer membrane)
<i>mxIA</i>	76 kDa	Same as above
<i>invJ</i>	Unknown	Same as above
<i>invH</i>	Unknown	Necessary for invasion (role unknown)
<i>invF</i>	Unknown	Same as above
<i>invG</i>	24 kDa	Not necessary for invasion (role unknown)
<i>ippI</i>	18 kDa	Same as above
<i>ipaB</i>	62 kDa	Necessary for invasion (may mediate endocytic uptake of shigellae)
<i>ipaC</i>	43 kDa	Same as above
<i>ipaD</i>	38 kDa	Same as above (may mediate adherence of shigellae to host cell membrane)

<i>ipaA</i>	78 kDa	Not necessary for invasion (role unknown)
<i>virB(invE, ipaR)</i>	33 kDa	Positive regulation of <i>ipaABCD</i> and <i>invAKJHFG</i>
<i>virG(icsA)</i>	120 kDa	Associated with intra- and intercellular bacterial spread
<i>ipaH</i>	60 kDa	Unknown (may inhibit coagulation)

Adapted from Hale, T. L. (1991) Genetic Basis of Virulence in *Shigella* Species, *Microbiological Reviews*, 55: 206-224.

The Shiga Toxin

The **Shiga toxin**, also called the **verotoxin**, is produced by the bacteria *Shigella dysenteriae* and enterohemorrhagic *Escherichia coli* (EHEC), of which the strain O157:H7 has become the best known.

The syndromes associated with shiga toxin include dysentery, hemorrhagic colitis, and hemolytic uremic syndrome. The name is dependent upon the causative organism and the symptoms, which may include severe diarrhea, abdominal pain, vomiting, and bloody urine (in the case of hemolytic uremic syndrome).

The onset of symptoms is generally within a few hours, with higher doses leading to more rapid onset. There is no antidote for the toxin. Supportive care requires maintenance of fluid and electrolyte levels, and monitoring and support of kidney function.

Immunoassays are available for rapid diagnosis of the toxin.

Inactivation of the toxin is achieved by steam treatment, oxidizing agents such as bleach, and chemical sterilizing agents such as glutaraldehyde.

The toxicity of Shiga Toxin for the mouse (LD_{50}) is <20 micrograms/kg by intravenous or intraperitoneal administration. There is no published data on the inhalation toxicity of Shiga toxin. However, there are often indirect effects on the lungs when the toxin is taken in as a food contaminant.

Table 2. The toxin has been given several trivial names depending on the bacterium that produces it and the gene that encodes it.

Source Organism	Gene Designation	Toxin Name	Older Names
<i>Shigella dysenteriae</i> , type I	stx	Shiga toxin (Stx)	Shiga toxin
<i>Escherichia coli</i>	stx1	Shiga toxin 1 (Stx1)	Shiga-like toxin I, Verotoxin 1
	stx2	Shiga toxin 2 (Stx2)	Shiga-like toxin II, Verotoxin 2

Structure of the Toxin

The toxin has a molecular weight of 68,000 da. It is a multisubunit protein made up one molecule of an A subunit (32,000 molecular weight) responsible for the toxic action of the protein, and five molecules of the B subunit (7,700 molecular weight) responsible for binding to a specific cell type.

Mechanism of Action

The toxin acts on the lining of the blood vessels, the vascular endothelium. The B subunits of the toxin bind to a component of the cell membrane known as Gb3 and the complex enters the cell. When the protein is inside the cell, the A subunit interacts with the ribosomes to inactivate them. The A subunit of Shiga toxin is an N-glycosidase that modifies the RNA component of the ribosome to inactivate it and so bring a halt to protein synthesis leading to the death of the cell. The vascular endothelium has to continually renew itself, so this killing of cells leads to a breakdown of the lining and to hemorrhage. The first response is commonly a bloody diarrhea. This is because Shiga toxin is usually taken in with contaminated food or water.

The toxin is effective against small blood vessels, such as found in the digestive tract, the kidney, and lungs, but

not against large vessels such as the arteries or major veins. A specific target for the toxin appears to be the vascular endothelium of the glomerulus. This is the filtering structure that is a key to the function of the kidney. Destroying these structures leads to kidney failure and the development of the often deadly and frequently debilitating hemolytic uremic syndrome. Food poisoning with Shiga toxin often also has effects on the lungs and the nervous system.

Shiga Toxin-Producing *Escherichia coli* (STEC)

Shiga toxin-producing *Escherichia coli* is a type of enterohemorrhagic *E. coli* (EHEC) bacteria that can cause illness ranging from mild intestinal disease to severe kidney complications. Enterohemorrhagic *E. coli* include the relatively important serotype *E. coli* O157:H7, but other non-O157 strains, such as O111 and O26, have been associated with shiga toxin production.

The incubation period for STEC ranges from 1 to 8 days, though typically it is 3 to 5 days. Typical symptoms include severe abdominal cramping, sudden onset of watery diarrhea, frequently bloody, and sometimes vomiting and a low-grade fever. Most often the illness is mild and self-limited generally lasting 1-3 days. However, serious complications such as hemorrhagic colitis, Hemolytic Uremic Syndrome (HUS), or postdiarrheal thrombotic thrombocytopenic purpura (TTP) can occur in up to 10% of cases.

Cases and outbreaks of Shiga toxin-producing *Escherichia coli* have been associated with the consumption of undercooked beef (especially ground beef), raw milk, unpasteurized apple juice, contaminated water, red leaf lettuce, alfalfa sprouts, and venison jerky. The bacteria have also been isolated from poultry, pork and lamb. Person-to- person spread, via fecal-oral transmission, may occur in high-risk settings like day care centers and nursing homes.

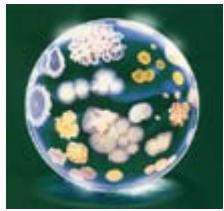
Although anyone can get infected, the highest infection rates are in children under age 5. Elderly patients also account for a large number of cases. Outbreaks have occurred in child-care facilities and nursing homes.

For mild illness, antibiotics have not been shown to shorten the duration of symptoms and may make the illness more severe in some people. Severe complications, such as Hemolytic Uremic Syndrome, require hospitalization.

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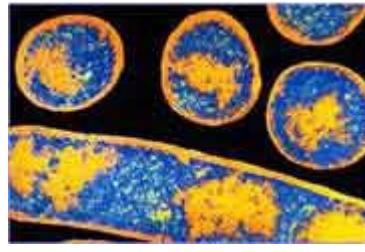




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The Pathogenic Clostridia

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Clostridium botulinum

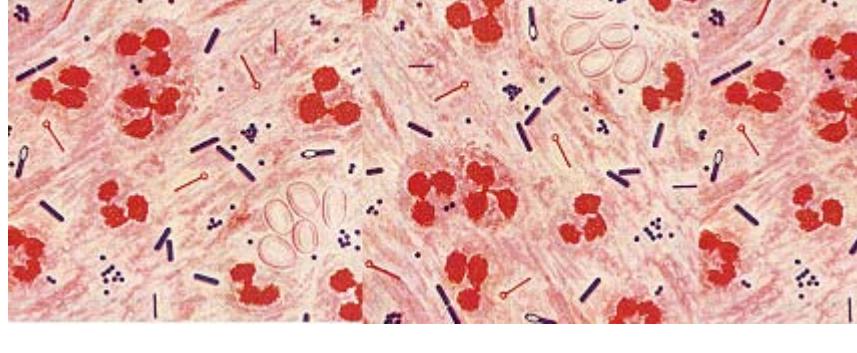
The Genus *Clostridium*

The **clostridia** are relatively large, Gram-positive, rod-shaped bacteria. All species form endospores and have a strictly fermentative mode of metabolism. Most clostridia will not grow under aerobic conditions and vegetative cells are killed by exposure to O₂, but their spores are able to survive long periods of exposure to air.

The clostridia are ancient organisms that live in virtually all of the anaerobic habitats of nature where organic compounds are present, including soils, aquatic sediments and the intestinal tracts of animals.

Clostridia are able to ferment a wide variety of organic compounds. They produce end products such as butyric acid, acetic acid, butanol and acetone, and large amounts of gas (CO₂ and H₂) during fermentation of sugars. A variety of foul smelling compounds are formed during the fermentation of amino acids and fatty acids. The clostridia also produce a wide variety of extracellular enzymes to degrade large biological molecules in the environment into fermentable components. Hence, the clostridia play an important role in nature in biodegradation and the carbon cycle. In anaerobic clostridial infections, these enzymes play a role in invasion and pathology.

Most of the clostridia are saprophytes but a few are pathogenic for humans. Those that are pathogens have primarily a saprophytic existence in nature and, in a sense, are opportunistic pathogens. *Clostridium tetani* and *Clostridium botulinum* produce the most potent biological toxins known to affect humans. As pathogens of tetanus and food-borne botulism, they owe their virulence almost entirely to their toxigenicity. Other clostridia, however, are highly invasive under certain circumstances.



Stained pus from a mixed anaerobic infection. At least three different clostridia are apparent.

Clostridium perfringens



C. perfringens

Clostridium perfringens, which produces a huge array of invasins and exotoxins, causes wound and **surgical infections** that lead to **gas gangrene**, in addition to severe **uterine** infections. Clostridial hemolysins and extracellular enzymes such as proteases, lipases, collagenase and hyaluronidase, contribute to the invasive process. *Clostridium perfringens* also produces an enterotoxin and is an important cause of **food poisoning**. Usually the organism is encountered in improperly sterilized (canned) foods in which endospores have germinated.

OFF THE WALL

Case Report of *C. perfringens* Food Poisoning

Clostridium perfringens is a common cause of outbreaks of foodborne illness in the United States, especially outbreaks in which cooked beef is the implicated source. This is a condensed version of an MMWR report that describes an outbreak of *C. perfringens* gastroenteritis following St.Patrick's Day meals of corned beef. The report typifies outbreaks of *C. perfringens* food poisoning.

Report

On March 18, 1993, the Cleveland City Health Department received telephone calls from 15 persons who became ill after eating corned beef purchased from one delicatessen. After a local newspaper article publicized this problem, 156 persons contacted the health department to report onset of diarrheal illness within 48 hours of eating food from the delicatessen on March 16 or March 17. Symptoms included abdominal cramps (88%) and vomiting (13%); no persons were hospitalized. The median incubation period was 12 hours (range: 2-48 hours). Of the 156 persons reporting illness, 144 (92%) reported having eaten corned beef; 20 (13%), pickles; 12 (8%), potato salad; and 11 (7%), roast beef.

In anticipation of a large demand for corned beef on St. Patrick's Day (March 17), the delicatessen had purchased 1400 pounds of raw, salt-cured product. Beginning March 12, portions of the corned beef were boiled for 3 hours at the delicatessen, allowed to cool at room temperature, and refrigerated. On March 16 and 17, the portions were removed from the refrigerator, held in a warmer at 120 F (48.8 C), and sliced and served. Corned beef sandwiches also were made for catering to several groups on March 17; these sandwiches were held at room temperature from 11 a.m. until they were eaten throughout the afternoon.

Cultures of two of three samples of leftover corned beef obtained from the delicatessen yielded greater than or equal to 10^5 colonies of *C. perfringens* per gram.

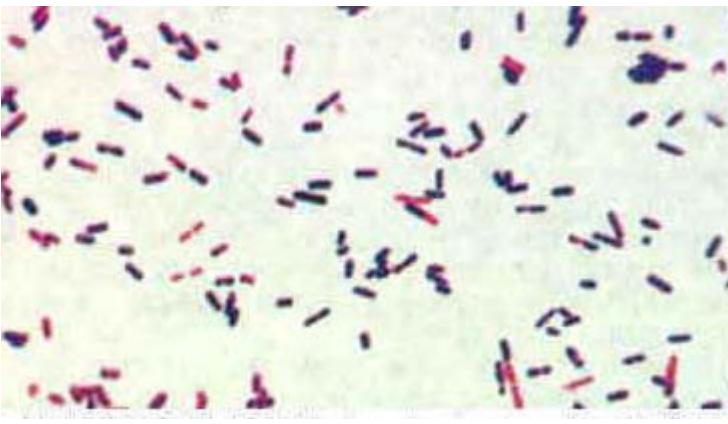
Following the outbreak, public health officials recommended to the delicatessen that meat not served immediately after cooking be divided into small pieces, placed in shallow pans, and chilled rapidly on ice before refrigerating and that cooked meat be reheated immediately before serving to an internal temperature of greater than or equal to 165 F (74 C).

Analysis

C. perfringens is a ubiquitous, anaerobic, Gram-positive, spore-forming bacillus and a frequent contaminant of meat and poultry. *C. perfringens* food poisoning is characterized by onset of abdominal cramps and diarrhea 8-16 hours after eating contaminated meat or poultry. By sporulating, this organism can survive high temperatures during initial cooking; the spores germinate during cooling of the food, and vegetative forms of the organism multiply if the food is subsequently held at temperatures of 60 F-125 F (16 C-52 C). If served without adequate reheating, live vegetative forms of *C. perfringens* may be ingested. The bacteria then elaborate the enterotoxin that causes the characteristic symptoms of diarrhea and abdominal cramping.

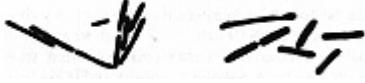
Laboratory confirmation of *C. perfringens* foodborne outbreaks requires quantitative cultures of implicated food or stool from ill persons. This outbreak was confirmed by the recovery of greater than or equal to 10^5 organisms per gram of epidemiologically implicated food. An alternate criterion is that cultures of stool samples from persons affected yield greater than or equal to 10^6 colonies per gram. Stool cultures were not done in this outbreak. Serotyping is not useful for confirming *C. perfringens* outbreaks and, in general, is not available.

Corned beef is a popular ethnic dish that is commonly served to celebrate St. Patrick's Day. The errors in preparation of the corned beef in this outbreak were typical of those associated with previously reported foodborne outbreaks of *C. perfringens*. Improper holding temperatures were a contributing factor in most (97%) *C. perfringens* outbreaks reported to CDC from 1973 through 1987. To avoid illness caused by this organism, food should be eaten while still hot or reheated to an internal temperature of greater than or equal to 165 F (74 C) before serving.



Clostridium perfringens, Gram Stain. Most clostridia are reknowned for staining "Gram-variable".

Clostridium difficile



C. difficile

Clostridium difficile causes **antibiotic associated diarrhea (AAD)** and more serious intestinal conditions such as **colitis** and **pseudomembranous colitis** in humans. These conditions generally result from overgrowth of *Clostridium difficile* in the colon, usually after the normal flora has been disturbed by antimicrobial chemotherapy.

People in good health usually do not get *C. difficile* disease. Individuals who have other conditions that require prolonged use of antibiotics and the elderly are at greater risk of the disease. Also, individuals who have recently undergone gastrointestinal surgery, or have a serious underlying illness, or who are immunocompromised, are at risk.

C. difficile produces two toxins: Toxin A is referred to as an enterotoxin because it causes fluid accumulation in the bowel. Toxin B is an extremely lethal (cytopathic) toxin.

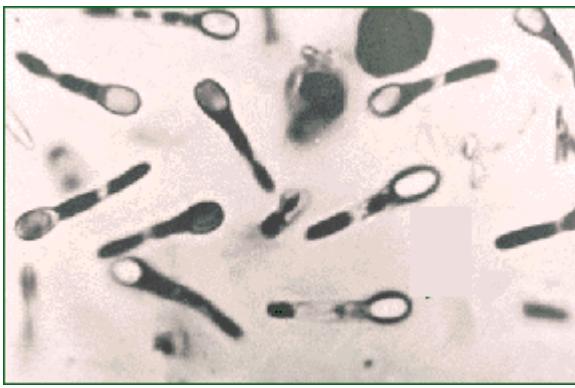
Stool cultures for diagnosis of the bacterium may be complicated by the occurrence and finding of non toxigenic strains of the bacterium, so the most reliable tests involve testing for the presence of the Toxin A and/or Toxin B in the stool. The toxins are very unstable. They degrade at room temperature and may be undetectable within two hours after collection of a stool specimen leading to false negative results of the diagnosis.

In the hospital and nursing home setting, *C. difficile* infections can be minimized by judicious use of antibiotics, use of contact precautions with patients with known or suspected cases of disease, and by implementation of an effective environmental and disinfection strategy.

Clostridium difficile infections can usually be treated successfully with a 10 day course of antibiotics including metronidazole or vancomycin (administered orally).

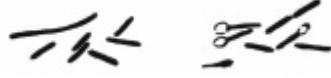


C. difficile colonies on blood agar



C. difficile endospores.

Clostridium tetani



C. tetani

Clostridium tetani is the causative agent of **tetanus**. The organism is found in soil, especially heavily-manured soils, and in the intestinal tracts and feces of various animals. Carrier rates in humans vary from 0 to 25%, and the organism is thought to be a transient member of the flora whose presence depends upon ingestion. The organism produces terminal spores within a swollen sporangium giving it a distinctive drumstick appearance. Although the bacterium has a typical Gram-positive cell wall, it may stain Gram-negative or Gram-variable, especially in older cells.



Clostridium tetani characteristic terminal endospores in a swollen sporangium.

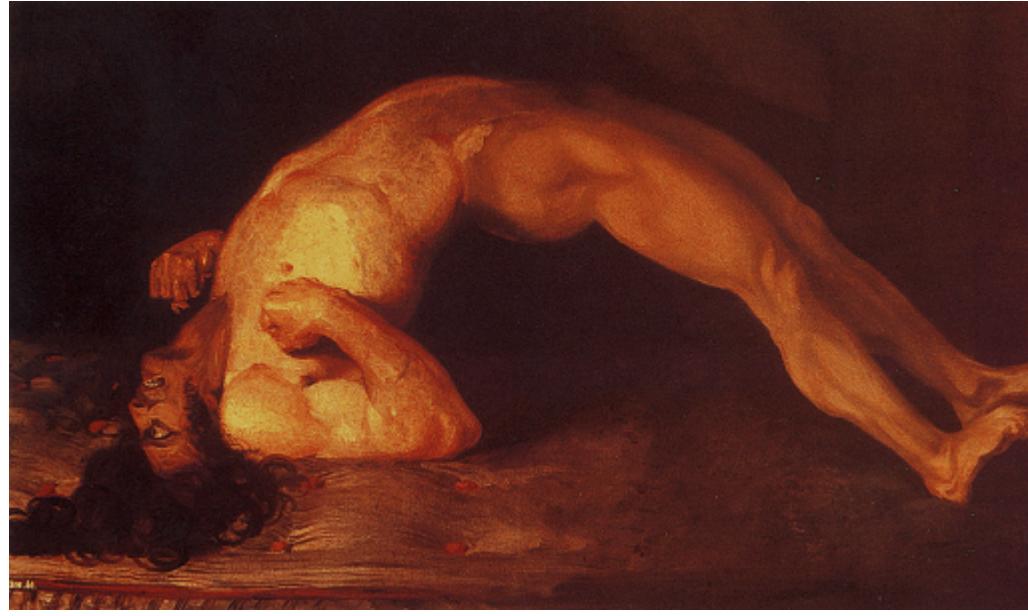
Tetanus is a highly fatal disease of humans. Mortality rates reported vary from 40% to 78%. The disease stems not from invasive infection but from a potent neurotoxin (**tetanus toxin** or **tetanospasmin**) produced when spores germinate and vegetative cells grow after gaining access to wounds. The organism multiplies locally and symptoms appear remote from the infection site.

Because of the widespread use of the **tetanus toxoid** for prophylactic immunization, fewer than 150 cases occur annually in the U.S., but the disease is a significant problem world-wide where there are > more than 300,000 cases annually. Most cases in the U.S. occur in individuals over age 60, which is taken to mean that waning immunity is a significant risk factor.

Pathogenesis

Most cases of tetanus result from small puncture wounds or lacerations which become contaminated with *C. tetani* spores that germinate and produce toxin. The infection remains localized often with only minimal inflammatory damage. The toxin is produced during cell growth, sporulation and lysis. It migrates along neural paths from a local wound to sites of action in the **central nervous system**. The clinical pattern of generalized tetanus consists of severe painful **spasms and rigidity of the voluntary muscles**. The characteristic symptom of "lockjaw" involves

spasms of the masseter muscle. It is an early symptom which is followed by progressive rigidity and violent spasms of the trunk and limb muscles. Spasms of the pharyngeal muscles cause difficulty in swallowing. Death usually results from interference with the mechanics of respiration.



Sir Charles Bell's portrait of a soldier dying of tetanus. The characteristic rigidity of the body is referred to as opisthotonus and risus sardonicus. Original in the Royal College of Surgeons of Edinburgh, Scotland.

Neonatal tetanus accounts for about half of the tetanus deaths in developing countries. In a study of neonatal mortality in Bangladesh, 112 of 330 infant deaths were due to tetanus. Neonatal tetanus follows infection of the umbilical stump in infants born to nonimmune mothers (therefore, the infant has not acquired passive immunity). It usually results from a failure of aseptic technique during the birthing, but certain cultural practices may contribute to infection.

Tetanus Toxin

There have been 11 strains of *C. tetani* distinguished primarily on the basis of flagellar antigens. They differ in their ability to produce tetanus toxin (tetanospasmin), but all strains produce a toxin which is identical in its immunological and pharmacological properties. Tetanospasmin is **encoded on a plasmid** which is present in all toxigenic strains.

Tetanus toxin is one of the three most poisonous substances known, the other two being the toxins of botulism and diphtheria. The toxin is produced by growing cells and released only on cell lysis. Cells lyse naturally during germination the outgrowth of spores, as well as during vegetative growth. After inoculation of a wound with *C. tetani* spores, only a minimal amount of spore germination and vegetative cell growth are required until the toxin is produced.

The bacterium synthesizes the tetanus toxin as a single 150kDa polypeptide chain (called the progenitor toxin), that is cleaved extracellularly by a bacterial protease into a 100 kDa heavy chain (fragment B) and a 50kDa light chain (fragment A) which remain connected by a disulfide bridge. The specific protease that cleaves the progenitor toxin can be found in culture filtrates of *C. tetani*. Cleavage of the progenitor toxin into A and B fragments can also be induced artificially with trypsin.

Tetanus toxin is produced in vitro in amounts up to 5 to 10% of the bacterial weight. Because the toxin has a specific affinity for nervous tissue, it is referred to as a **neurotoxin**. The toxin has no known useful function to *C. tetani*. Why the toxin has a specific action on nervous tissue, to which the organism naturally has no access, may be an anomaly of nature. The toxin is heat labile, being destroyed at 56 degrees C in 5 minutes, and is O₂ labile. The purified toxin rapidly converts to toxoid at 0 degrees C in the presence of formalin.

Toxin Action

Tetanospasmin initially binds to peripheral nerve terminals. It is transported within the axon and across synaptic junctions until it reaches the central nervous system. There it becomes rapidly **fixed to gangliosides at the presynaptic inhibitory motor nerve endings**, and is taken up into the axon by endocytosis. The effect of the toxin is to **block the release of inhibitory neurotransmitters** (glycine and gamma-amino butyric acid) across the synaptic cleft, which is required to check the nervous impulse. If nervous impulses cannot be checked by normal inhibitory mechanisms, it produces the generalized muscular spasms characteristic of tetanus. Tetanospasmin appears to act by selective cleavage of a protein component of synaptic vesicles, **synaptobrevin II**, and this prevents the release of neurotransmitters by the cells.

The receptor to which tetanospasmin binds has been reported as ganglioside GT and/or GD1b, but its exact identity is still in question. Binding appears to depend on the number and position of sialic acid residues on the ganglioside. Isolated B fragments, but not A fragments will bind to the ganglioside. The A fragment has toxic (enzymatic) activity after the B fragment secures its entry. Binding appears to be an irreversible event. Recovery depends on sprouting a new axon terminal.

Immunity

Unlike other diseases, such as diphtheria, recovery from the natural disease usually does not confer immunity, since even a lethal dose of tetanospasmin is insufficient to provoke an immune response.

Prophylactic immunization is accomplished with tetanus toxoid, as part of the DPT (DTP) vaccine or the DT (TD) vaccine. Three injections are given in the first year of life, and a booster is given about a year later, and again on the entrance into elementary school.

Whenever a previously-immunized individual sustains a potentially dangerous wound, a booster of toxoid should be injected. Wherever employed, intensive programs of immunization with toxoid have led to a striking reduction in the incidence of the disease.

Clostridium botulinum



C. botulinum

C. botulinum is a large anaerobic bacillus that forms subterminal endospores. It is widely distributed in soil, sediments of lakes and ponds, and decaying vegetation. Hence, the intestinal tracts of birds, mammals and fish may occasionally contain the organism as a transient. Seven toxigenic types of the organism exist, each producing an immunologically distinct form of botulinum toxin. The toxins are designated A, B, C1, D, E, F, and G). In the U.S. **type A** is the most significant cause of botulism, involved in 62% of the cases. Not all strains of *C. botulinum* produce the botulinum toxin. Lysogenic phages encode toxin serotypes C and D, and non lysogenized bacteria (which exist in nature) do not produce the toxin. Type G toxin is thought to be plasmid encoded.

Pathogenesis of Botulism

Food-borne Botulism

In food-borne botulism the botulinum toxin is ingested with food in which spores have germinated and the organism has grown. The toxin is absorbed by the upper part of the GI tract in the duodenum and jejunum, and passes into the blood stream by which it reaches the peripheral neuromuscular synapses. The toxin binds to the presynaptic stimulatory terminals and blocks the release of the neurotransmitter acetylcholine which is required for a nerve to stimulate the muscle.

Food-borne botulism is not an infection but an **intoxication** since it results from the ingestion of foods that contain the preformed clostridial toxin. In this respect it resembles staphylococcal food poisoning. Botulism results from

eating uncooked foods in which contaminating spores have germinated and produced the toxin. *C. botulinum* spores are relatively heat resistant and may survive the sterilizing process of improper canning procedures. The anaerobic environment produced by the canning process may further encourage the outgrowth of spores. The organisms grow best in neutral or "low acid" vegetables (>pH4.5).

Clinical symptoms of botulism begin 18-36 hours after toxin ingestion with weakness, dizziness and dryness of the mouth. Nausea and vomiting may occur. Neurologic features soon develop: blurred vision, inability to swallow, difficulty in speech, descending weakness of skeletal muscles and respiratory paralysis.

Botulinum toxin may be transported within nerves in a manner analogous to tetanospasmin, and can thereby gain access to the CNS. However, symptomatic CNS involvement is rare.



Clostridium botulinum

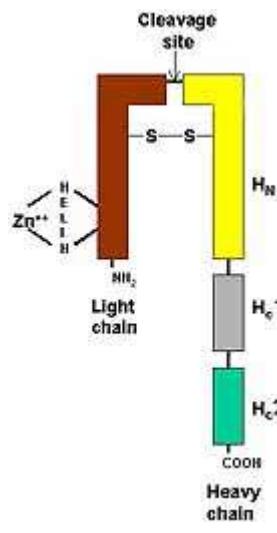
Infant Botulism

Infant botulism is due to **infection** caused by *C. botulinum*. The disease occurs in infants 5 - 20 weeks of age that have been exposed to solid foods, presumably the source of infection (spores). It is characterized by constipation and weak sucking ability and generalized weakness. *C. botulinum* can apparently establish itself in the bowel of infants at a critical age before the establishment of competing intestinal bacteria (normal flora). Production of toxin by bacteria in the GI tract induces symptoms. This "infection-intoxication" is restricted to infants. *C. botulinum* organisms, as well as toxin can be found in the feces of infected infants. Almost all known cases of the disease have recovered. The possible role of infant botulism in "sudden infant death syndrome-SIDS" has been suggested and is under investigation. *C. botulinum*, its toxin, or both have been found in the bowel contents of several infants who have died suddenly and unexpectedly.

The Botulinum Toxins

The botulinum toxins are very similar in structure and function to the tetanus toxin, but differ dramatically in their clinical effects because they target different cells in the nervous system. Botulinum **neurotoxins** predominantly affect the **peripheral nervous system** reflecting a preference of the toxin for **stimulatory motor neurons at a neuromuscular junction**. The primary symptom is weakness or **flaccid paralysis**. Tetanus toxin can affect the same system, but the tetanospasmin shows a tropism for inhibitory motor neurons of the central nervous system, and its effects are primarily rigidity and spastic paralysis.

Botulinum toxin is synthesized as a single polypeptide chain with a molecular weight around 150 kDa. In this form the toxin has a relatively low potency. The toxin is nicked by a bacterial protease (or possibly by gastric proteases) to produce two chains: a light chain (the A fragment) with a molecular weight of 50 kDa; and a heavy chain (the B fragment), with a mw of 100kDa. As with tetanospasmin, the chains remain connected by a disulfide bond. The A fragment of the nicked toxin, on a molecular weight basis, becomes the most potent toxin found in nature.



Structure of the botulinum toxins

Toxin Action

The botulinum toxin is **specific for peripheral nerve endings** at the point where a motor neuron stimulates a muscle. The toxin binds to the neuron and **prevents the release of acetylcholine** across the synaptic cleft.

The heavy chain of the toxin mediates binding to presynaptic receptors. The nature of these receptors is uncertain; different toxin types seem to utilize slightly different receptors. The binding region of the toxin molecule is located near the carboxy terminus of the heavy chain. The amino terminus of the heavy chain is thought to form a channel through the membrane of the neuron allowing the light chain to enter. The toxin (A fragment) enters the cell by receptor mediated endocytosis. Once inside a neuron, the toxin types probably differ in mechanisms by which they inhibit acetylcholine release, but a mechanism similar to or identical to tetanospasmin has been reported (i.e., proteolytic cleavage of synaptobrevin II). The affected cells fail to release a neurotransmitter, thus producing paralysis of the motor system. Once damaged, the synapse is rendered permanently useless. The recovery of function requires sprouting of a new presynaptic axon and the subsequent formation of a new synapse.

As stated above, the mechanism by which acetylcholine release is prevented is not known. However, recent evidence suggests that both botulinum toxin as well as tetanus toxin are **zinc-dependent endopeptidases** that cleave specific proteins that are involved in excretion of neurotransmitters. Both toxins cleave a set of proteins called **synaptobrevins**. Synaptobrevins are a set of proteins found in synaptic vesicle of neurons, the vesicles responsible for release of neurotransmitters. Presumably, proteolytic cleavage of synaptobrevin II would interfere with vesicle function and release of neurotransmitters.

Immunity

On the average there are about 25 cases of botulism annually in the U.S. Prior to the advent of critical care, the case fatality rate exceeded 60%, but currently it is about 20%. The first (or only) patient in an outbreak has a 25% chance of death, whereas subsequent cases which are diagnosed and treated more quickly, carry only a 4% risk.

The toxins that cause botulism are each specifically neutralized by its **antitoxin**. Botulinum toxins can be toxoided and make good antigens for inducing protective antibody. As with tetanus, immunity to botulism does not develop, even with severe disease, because the amount of toxin necessary to induce an immune response is toxic. Repeated occurrence of botulism has been reported.

Once the botulinum toxin has bound to nerve endings, its activity is unaffected by antitoxin. Any circulating ("unfixed") toxin can be neutralized by intravenous injection of antitoxin. Individuals known to have ingested food with botulism should be treated immediately with antiserum.

A multivalent **toxoid** evokes good protective antibody response but its use is unjustified due to the infrequency of the disease. An experimental vaccine exists for laboratory workers.

Prevention

The most important aspect of botulism prevention is proper food handling and preparation. The spores of *C. botulinum* can survive boiling (100 degrees at 1 atm) for more than one hour although they are killed by autoclaving. Because the toxin is heat-labile boiling or intense heating (cooking) of contaminated food will inactivate the toxin. Food containers that bulge may contain gas produced by *C. botulinum* and should not be opened or tasted. Other foods that appear to be spoiled should not be tasted.

OFF THE WALL

Botulinum Toxin in Biowarfare.....of course, it has been thought ofbotulinum toxin is the most potent poison known for humans; 10 grams is a lethal dose for the human population of Los Angeles. Below is an interesting anecdote that appeared in JAMA Vol. 285, No. 21, June 6, 2001

To the Editor:

A historical incident illustrates a number of features of botulinum toxin not discussed in the review of bioweaponry by Dr Arnon and colleagues.

During World War II, the US Office of Strategic Services (OSS) developed a plan for Chinese prostitutes to assassinate high-ranking Japanese officers with whom they sometimes consorted in occupied Chinese cities. Concealing traditional weapons on the women at the appropriate time would obviously be difficult. Therefore, under the direction of Stanley Lovell, the OSS prepared gelatin capsules "less than the size of the head of a common pin" containing a lethal dose of botulinum toxin. Wetted, a capsule could be stuck behind the ear or in scalp hair, later to be detached and slipped into the officer's food or drink. The OSS recognized that the normal background of botulism cases would deflect suspicion from the women.

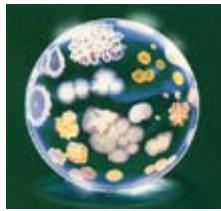
The capsules were shipped to Chunking, China. The Navy detachment there, taking nothing for granted, tested the capsules on stray donkeys. The donkeys lived. Lovell was informed that the capsules were faulty, and the project was abandoned. Much later, Lovell learned of the donkey test with, one imagines, some consternation, since "donkeys are one of the few living creatures immune to botulism."

This incident has been retold in other publications. No source for the donkey-resistance information is ever given. More recent experience shows that botulism can occur in mules and donkeys (R. H. Whitlock, DVM, PhD, written communication, April 27, 2001).

Nevertheless, this incident raises 2 points: (1) botulism need not occur in epidemics when it is being used as a bioweapon, and (2) botulism in animals may be a sign of biowarfare.

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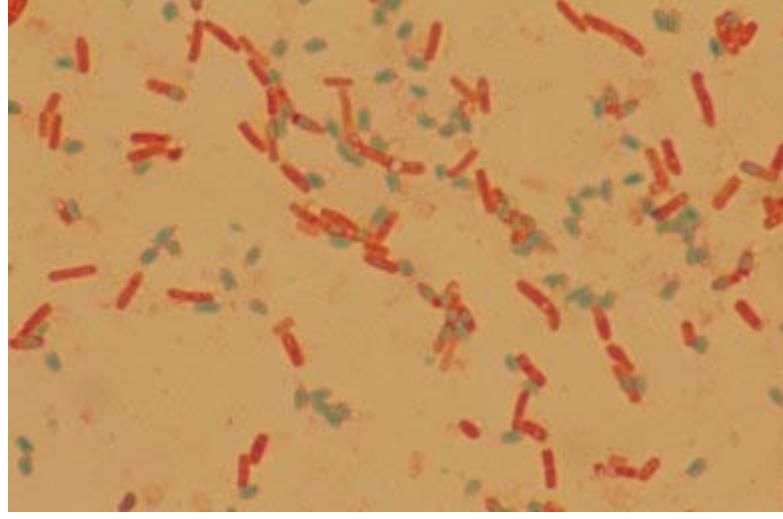


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Bacillus cereus

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Bacillus cereus food poisoning



Bacillus cereus Spore Stain

Bacillus cereus has been recognized as an agent of food poisoning since 1955. Between 1972 and 1986, 52 outbreaks of food-borne disease associated with *B. cereus* were reported to the CDC, but this is thought to represent only 2% of the total cases which have occurred in that time. *B. cereus* causes two types of **food-borne intoxications** (as opposed to infections). One type is characterized by nausea and vomiting and abdominal cramps and has an incubation period of 1 to 6 hours. It resembles *Staphylococcus aureus* food poisoning in its symptoms and incubation period. This is the "short-incubation" or **emetic form** of the disease. The second type is manifested primarily by abdominal cramps and diarrhea with an incubation period of 8 to 16 hours. Diarrhea may be a small volume or profuse and watery. This type is referred to as the "long-incubation" or **diarrheal form** of the disease, and it resembles more food poisoning caused by *Clostridium perfringens*. In either type, the illness usually lasts less than 24 hours after onset. In a few patients symptoms may last longer.

The short-incubation form is caused by a preformed heat-stable enterotoxin of molecular weight less than 5,000 daltons. The mechanism and site of action of this toxin are unknown. The long-incubation form of illness is mediated by a heat-labile enterotoxin (molecular weight of approximately 50,000 daltons) which activates intestinal adenylate cyclase and causes intestinal fluid secretion.

B. cereus food poisoning occurs year-round and is without any particular geographic distribution. The short-incubation form is most often associated with fried rice that has been cooked and then held at warm temperatures for several hours. The disease is often associated with Chinese restaurants. In one reported outbreak, macaroni and cheese made from powdered milk turned out to be the source of the bacterium.

Long-incubation *B. cereus* food poisoning is frequently associated with meat or vegetable-containing foods after cooking. The bacterium has been isolated from 50% of dried beans and cereals and from 25% of dried foods such as spices, seasoning mixes and potatoes. One outbreak of the long-incubation form was traced to a "meals-on-wheels" program in which food was held above room temperature for a prolonged period.

The short-incubation or emetic form of the disease is diagnosed by the isolation of *B. cereus* from the incriminated

food. The long-incubation or diarrheal form is diagnosed by isolation of the organism from stool and food. Isolation from stools alone is not sufficient because 14% of healthy adults have been reported to have transient gastrointestinal colonization with *B. cereus*. Because *B. cereus* gastroenteritis is generally a benign, self-limited illness, antimicrobial agents are of no value in management. Since bacteria grow best at temperatures ranging from 40 to 140°F, infection may be prevented if cold food is refrigerated and if hot food is held at greater than 140°F before serving.

Nonanthrax *Bacillus* species, especially *B. cereus*, are occasionally implicated in local infections especially involving the eye. They can cause conjunctivitis, keratitis, iridocyclitis, dacryocystitis, orbital abscess, and panophthalmitis. The usual setting is that of previous occurrence of penetrating nonsurgical trauma. An intra-ocular foreign body such as a metal projectile is often present, or the injury occurs in a rural or farm location where there is a greater risk of eye contamination with dust or soil. *B. cereus* is one of the most destructive organisms to infect the eye. *Bacillus thuringiensis* has also been known to infect the eye. A case of human infection with *B. thuringiensis* was reported in 1983 in a healthy farmer who splashed the BT insecticide into his eye. The organism was recovered from a corneal ulcer. The clinical course was much less fulminant than with *B. cereus* infection.

OFF THE WALL

Summary and Analysis of a Report of *Bacillus cereus* Food Poisoning Associated with Fried Rice at Two Child Day Care Centers -- Virginia, 1993 (from CDC)

Summary

On July 21, 1993, a regional public health facility received reports of acute gastrointestinal illness that occurred among children and staff at two jointly owned child day care centers following a catered lunch.

The catered lunch was served on July 21 to 82 children aged less than or equal to 6 years, and to nine staff; dietary histories were obtained for 80 persons. 67 ate the catered lunch. A case was defined as vomiting by a person who was present at either day care center on July 21. Fourteen (21%) persons who ate the lunch became ill, compared with none of 13 who did not. Symptoms included nausea (71%), abdominal cramps or pain (36%), and diarrhea (14%). Twelve of the 14 cases occurred among children aged 2.5-5 years, and two occurred among staff. The median incubation period was 2 hours (range: 1.5-3.5 hours). Symptoms resolved a median of 4 hours after onset (range: 1.5-22 hours).

Chicken fried rice prepared at a local restaurant was the only food significantly associated with illness; illness occurred in 14 (29%) of 48 persons who ate chicken fried rice, compared with none of 16 who did not.

The rice had been cooked the night of July 20 and cooled at room temperature before refrigeration. On the morning of the lunch, the rice was pan-fried in oil with pieces of cooked chicken, delivered to the day care centers at approximately 10:30 a.m., held without refrigeration, and served at noon without reheating.

Following the outbreak, health officials recommended to day care staff and restaurant food handlers that the practice of cooling rice or any food at room temperature be discontinued, food be maintained at proper temperatures (i.e., below 41 F {5 C} or above 140 F {60 C}), and a thermometer be used to verify food temperatures.

Analysis

The emetic ("short incubation") form of the disease, which occurred in this outbreak, is mediated by a highly stable toxin that survives high temperatures and exposure to trypsin, pepsin, and pH extremes; the diarrheal syndrome is mediated by a heat- and acid-labile enterotoxin that is sensitive to proteolytic enzymes.

The diagnosis of *B. cereus* food poisoning can be confirmed by the isolation of greater than or equal to 10^5 *B. cereus* organisms per gram from epidemiologically implicated food. Underreporting of such outbreaks is likely because illness associated with *B. cereus* is usually self-limiting and not severe. In addition, findings of a recent survey about culture practices for outbreaks of apparent foodborne illness indicate that 20% of state public health laboratories do not make *B. cereus* testing routinely available.

Fried rice is a leading cause of *B. cereus* emetic-type food poisoning in the United States. *B. cereus* is frequently present in uncooked rice, and heat-resistant spores may survive cooking. If cooked rice is subsequently held at room temperature, vegetative forms multiply, and heat-stable toxin is produced that can survive brief heating, such

as stir frying. In the outbreak described in this report, vegetative forms of the organism probably multiplied at the restaurant and the day care centers while the rice was held at room temperature.

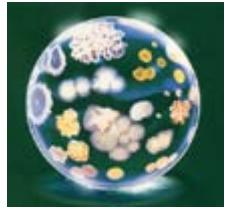
The day care staff and restaurant food handlers in this report were unaware that cooked rice was a potentially hazardous food. This report underscores the ongoing need to educate food handlers about basic practices for safe food handling.



Bacillus cereus Colonies on Blood Agar

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Bacillus anthracis and anthrax

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Introduction

The anthrax bacillus, *Bacillus anthracis*, was the first bacterium shown to be the cause of a disease. In 1877, Robert Koch grew the organism in pure culture, demonstrated its ability to form endospores, and produced experimental anthrax by injecting it into animals.

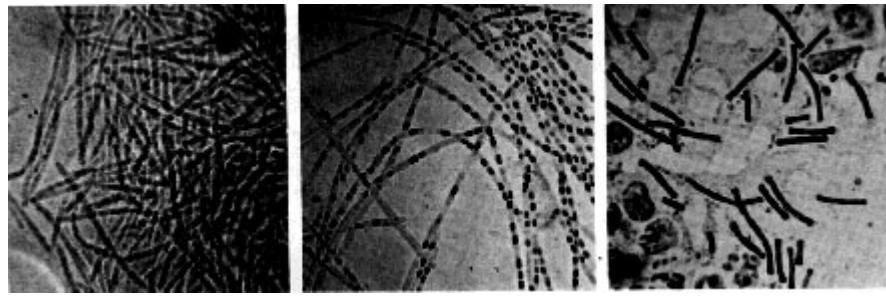


Figure 1. Robert Koch's original photomicrographs of *Bacillus anthracis*, the agent of anthrax. Compare the cell morphology and spore position with the Gram stain below (Figure 2). This is *Bacillus anthracis*. Beware of phony and mislabeled images of *B. anthracis* on the internet, including some that are posted by otherwise credible websites. Look for large cells with square ends and centrally-located ellipsoid spores when identifying *Bacillus anthracis*.

Bacillus anthracis is very large, Gram-positive, sporeforming rod, 1 - 1.2 μm in width x 3 - 5 μm in length. The bacterium can be cultivated in ordinary nutrient medium under aerobic or anaerobic conditions. Genotypically and phenotypically it is very similar to *Bacillus cereus*, which is found in soil habitats around the world, and to *Bacillus thuringiensis*, the pathogen for larvae of *Lepidoptera*. The three species have the same cellular size and morphology and form oval spores located centrally in a nonswollen sporangium.

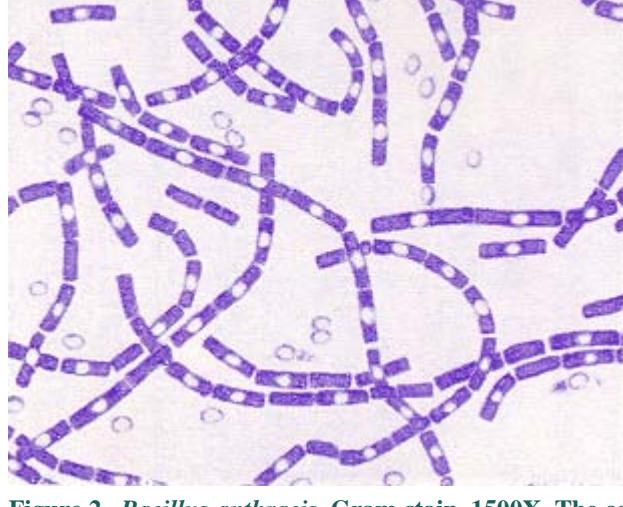


Figure 2. *Bacillus anthracis*. Gram stain. 1500X. The cells have characteristic squared ends. The endospores are ellipsoidal shaped and located centrally in the sporangium. The spores are highly refractile to light and resistant to staining.

Bacillus thuringiensis is distinguished from *B. cereus* or *B. anthracis* by its pathogenicity for Lepidopteran insects (moths and caterpillars) and by production of an intracellular **parasporal crystal** in association with spore formation. The bacteria and protein crystals are sold as "Bt" insecticide, which is used for the biological control of

certain garden and crop pests.

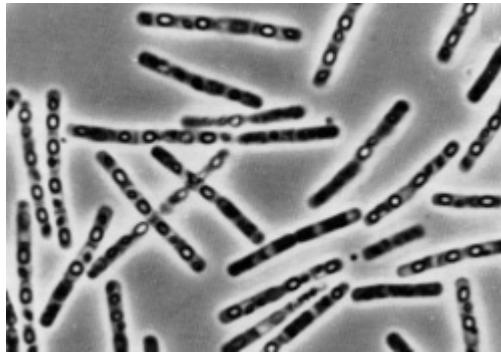


Figure 3. *Bacillus thuringiensis*. Phase Photomicrograph of vegetative cells, intracellular spores (light) and parasporal crystals (dark). 1000X.

Bacillus cereus is a normal inhabitant of the soil, but it can be regularly isolated from foods such as grains and spices. *B. cereus* causes two types of food-borne intoxications (as opposed to infections). One type is characterized by nausea and vomiting and abdominal cramps and has an incubation period of 1 to 6 hours. It resembles *Staphylococcus aureus* food poisoning in its symptoms and incubation period. This is the "short-incubation" or emetic form of the disease. The second type is manifested primarily by abdominal cramps and diarrhea with an incubation period of 8 to 16 hours. Diarrhea may be a small volume or profuse and watery. This type is referred to as the "long-incubation" or diarrheal form of the disease, and it resembles food poisoning caused by *Clostridium perfringens*. In either type, the illness usually lasts less than 24 hours after onset.

The short-incubation form is caused by a preformed heat-stable enterotoxin of molecular weight less than 5,000 daltons. The mechanism and site of action of this toxin are unknown. The long-incubation form of illness is mediated by a heat-labile enterotoxin (molecular weight of approximately 50,000 daltons) which activates intestinal adenylate cyclase and causes intestinal fluid secretion.



Figure 4. *Bacillus cereus*. Gram stain. 450X. Bacilli are large bacteria, so that they are readily observed with the microscope's "high dry objective"but you can't detect anything about their spores. This could be a *Lactobacillus*.

Cultivation

Several nonselective and selective media for the detection and isolation of *Bacillus anthracis* have been described, as well as a rapid screening test for the bacterium based on the morphology of microcolonies. Table 1 provides the differential characteristics that are used to distinguish *Bacillus anthracis* from most strains of *Bacillus cereus* and *Bacillus thuringiensis* but not necessarily from other saprophytic species of *Bacillus*. Otherwise, it is not the intent of this article to provide information on the growth of the bacterium in the laboratory.

Table 1. Differential Characteristics of *B. anthracis*, *B. cereus* and *B. thuringiensis*

Characteristic	<i>B. anthracis</i>	<i>B. cereus</i> and <i>B. thuringiensis</i>
growth requirement for thiamin	+	-
hemolysis on sheep blood agar	-	+
glutamyl-polypeptide capsule	+	-
lysis by gamma phage	+	-

motility	-	+
growth on chloralhydrate agar	-	+
string-of-pearls test	+	-

The following figures (5, 6, and 7) from the CDC are reliable images of *Bacillus anthracis* grown as described in the figure legends.



Figure 5. Colonies of *Bacillus cereus* on the left; colonies of *Bacillus anthracis* on the right. *B. cereus* colonies are larger, more mucoid, and this strain exhibits a slight zone of hemolysis on blood agar.

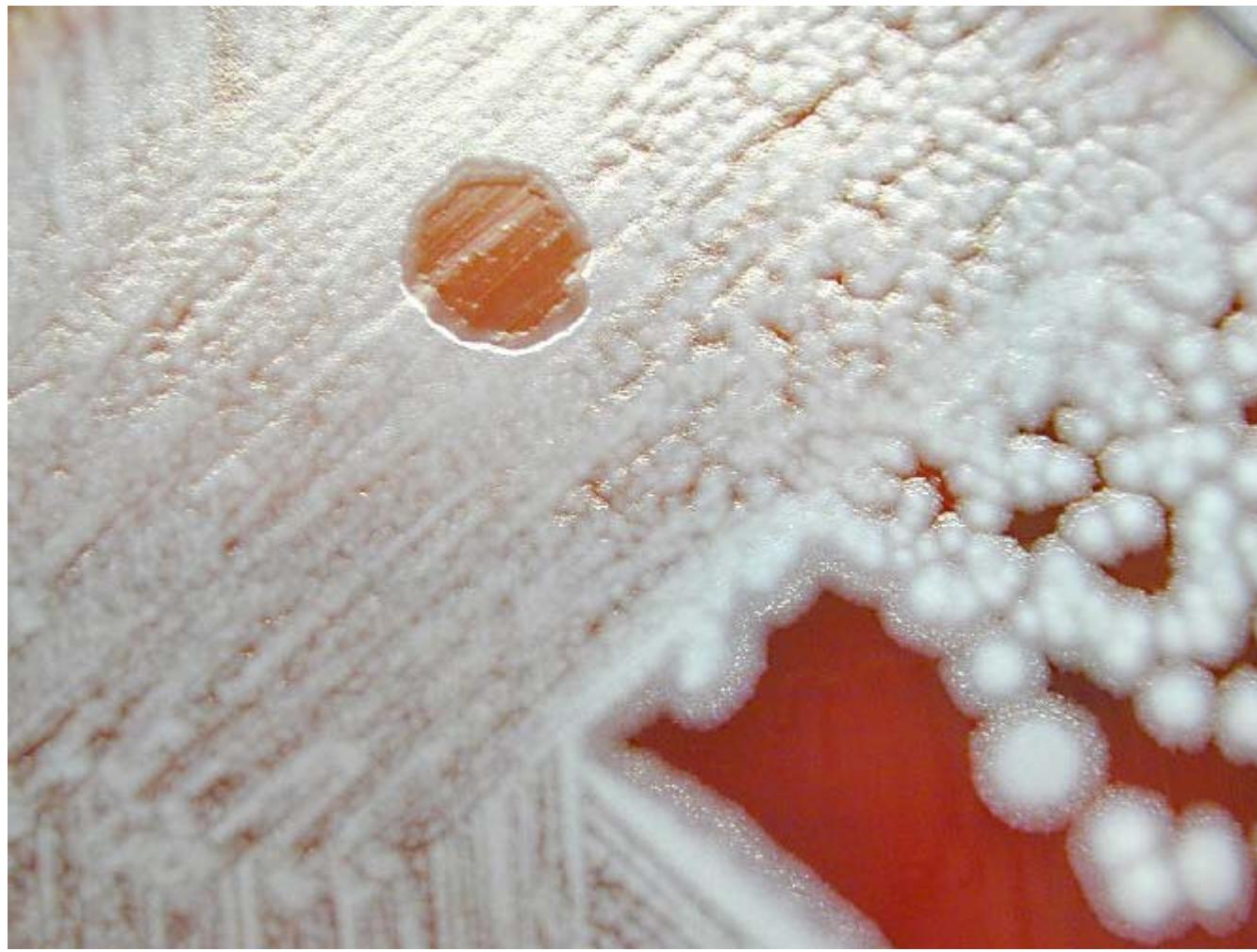


Figure 6. Lysis of *Bacillus anthracis* by the lytic phage gamma. The plaque (clear area) in the region of confluent growth is where the gamma phage was applied. The plaque results from the phage's ability to lyse the bacterial cells. Since the gamma phage is specific for *B. anthracis*, and will not lyse *B. thuringiensis* or *B. cereus*, we know that this is *Bacillus anthracis*. The colony type of is similar to Figure 5.

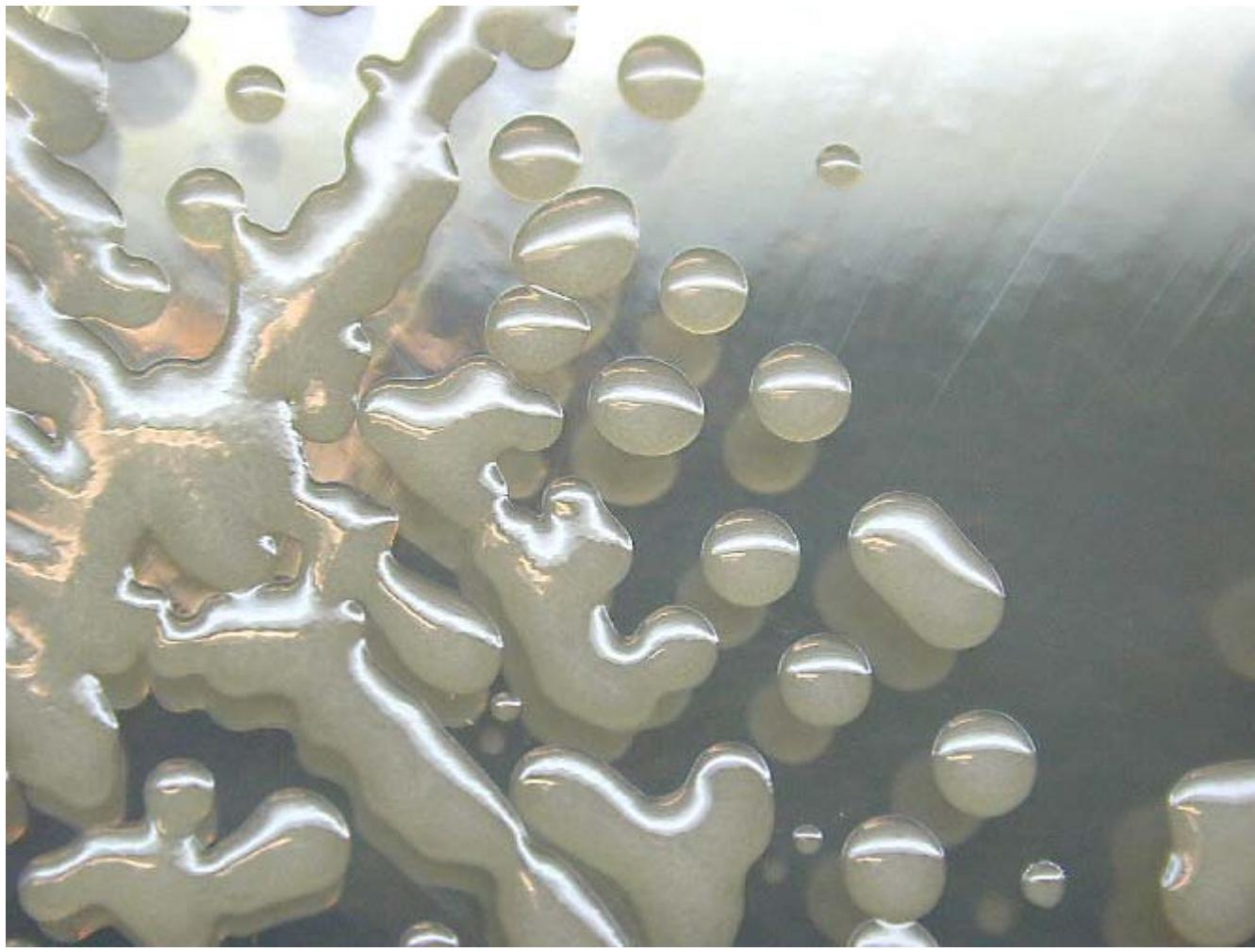


Figure 7. Mucoid colonies of *Bacillus anthracis*. This culture was probably incubated at an increased CO₂ tension (5% CO₂) which greatly enhances production of the poly-D-glutamyl capsule and accounts for the mucoid colony type.

Anthrax

Anthrax is primarily a disease of domesticated and wild animals, particularly herbivorous animals, such as cattle, sheep, horses, mules, and goats. Humans become infected incidentally when brought into contact with diseased animals, which includes their flesh, bones, hides, hair and excrement.

The natural history of *Bacillus anthracis* is obscure. Although the spores have been found naturally in soil samples from around the world, the organisms cannot be regularly cultivated from soils where there is an absence of endemic anthrax. In the United States there are recognized areas of infection in South Dakota, Nebraska, Arkansas, Texas, Louisiana, Mississippi and California; small areas exist in other states. Even in endemic areas, anthrax occurs irregularly, often with many years between occurrences.

In the United States, the incidence of naturally-acquired anthrax is extremely rare (1-2 cases of cutaneous disease per year). Worldwide, the incidence is unknown, although *B. anthracis* is present in most of the world. Unreliable reporting makes it difficult to estimate the true incidence of human anthrax worldwide. However, in fall 2001, 22 cases of anthrax (11 inhalation, 11 cutaneous) were identified in the United States following intentional contamination of the mail.

The most common form of the disease in humans is **cutaneous anthrax**, which is usually acquired via injured skin or mucous membranes. A minor scratch or abrasion, usually on an exposed area of the face or neck or arms, is inoculated by spores from the soil or a contaminated animal or carcass. The spores germinate, vegetative cells multiply, and a characteristic gelatinous edema develops at the site. This develops into papule within 12-36 hours after infection. The papule changes rapidly to a vesicle, then a pustule (malignant pustule), and finally into a necrotic ulcer from which infection may disseminate, giving rise to septicemia. Lymphatic swelling also occurs within seven days. In severe cases, where the blood stream is eventually invaded, the disease is frequently fatal.

Another form of the disease, **inhalation anthrax** (woolsorters' disease), results most commonly from inhalation of

spore-containing dust where animal hair or hides are being handled. The disease begins abruptly with high fever and chest pain. It progresses rapidly to a systemic hemorrhagic pathology and is often fatal if treatment cannot stop the invasive aspect of the infection.

Gastrointestinal anthrax is analogous to cutaneous anthrax but occurs on the intestinal mucosa. As in cutaneous anthrax, the organisms probably invade the mucosa through a preexisting lesion. The bacteria spread from the mucosal lesion to the lymphatic system. Intestinal anthrax results from the ingestion of poorly cooked meat from infected animals. Gastrointestinal anthrax is rare, but may occur as explosive outbreaks associated with ingestion of infected animals. Intestinal anthrax has an extremely high mortality rate.

Meningitis due to *B. anthracis* is a very rare complication that may result from a primary infection elsewhere.

Pathogenicity of *Bacillus anthracis*

Bacillus anthracis clearly owes its pathogenicity to two major determinants of virulence: the formation of a poly-D-glutamyl capsule, which mediates the invasive stage of the infection, and the production of the multicomponent anthrax toxin which mediates the toxigenic stage.

Poly-D-glutamyl capsule

Bacillus anthracis forms a single antigenic type of **capsule** consisting of a poly-D-glutamate polypeptide. All virulent strains of *B. anthracis* form this capsule. Production of capsular material is associated with the formation of a characteristic mucoid or "smooth" colony type. "Smooth" (S) to "rough" (R) colonial variants occur, which is correlated with ability to produce the capsule. R variants are relatively avirulent. Capsule production depends on a 60 megadalton **plasmid, pXO2**; its transfer to nonencapsulated *B. anthracis* via transduction produces the encapsulated phenotype.

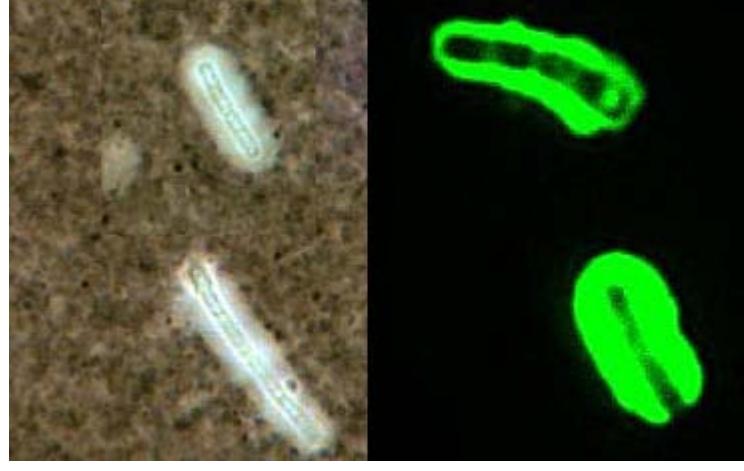


Figure 8. Two microscopic techniques to demonstrate the presence of the poly-D-glutamyl capsule of *Bacillus anthracis*. **Left.** India ink capsule outline 1000X. **Right:** a fluorescent-labeled antibody is reacted specifically with the capsular material which renders the capsule fluorescent - FA stain 1000X.

The poly-D-glutamyl capsule is itself nontoxic, but functions to protect the organism against the bactericidal components of serum and phagocytes, and against phagocytic engulfment. The capsule plays its most important role during the establishment of the infection, and a less significant role in the terminal phases of the disease, which are mediated by the anthrax toxin.

The poly-D-glutamyl capsule is formed in vivo or in the laboratory when the bacterium is grown on serum plates in a 5% CO₂ atmosphere. The capsular material can be detected by the McFadyean reaction which involves staining with polychrome methylene blue. Blue rods in a background of purple/pink-stained capsular material is a positive test (Figure 9). Neither *B. cereus* nor *B. thuringiensis* synthesizes this capsular polymer, so the detection of capsular material can be used to distinguish *B. anthracis* from its closest relatives.

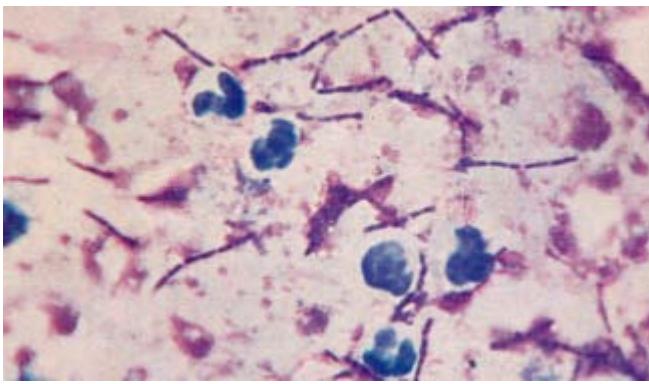


Figure 9. McFadyean's reaction showing short chains of *Bacillus anthracis* cells lying among amorphous, disintegrated capsular material. White blood cells can also be seen.

Anthrax Toxin

The toxigenic properties of *Bacillus anthracis* were not recognized until 1954. Prior to that time, because of the tremendous number of anthrax bacilli observed in the blood of animals dying of the disease (10^9 bacteria/ml), it was assumed that death was due to blockage of the capillaries, popularly known as the "log-jam" theory. But experimentally it was shown that only about 3×10^6 cells/ml are necessary to cause death of the animal. Furthermore, the cell-free plasma of animals dying of anthrax infection contained a toxin which causes symptoms of anthrax when injected into normal guinea pigs. These observations left little doubt that a diffusible exotoxin plays a major role in the pathogenesis of anthrax.

One component of the **anthrax toxin** has a lethal mode of the action that is not understood at this time. Death is apparently due to oxygen depletion, secondary shock, increased vascular permeability, respiratory failure and cardiac failure. Death from anthrax in humans or animals frequently occurs suddenly and unexpectedly. The level of the lethal toxin in the circulation increases rapidly quite late in the disease, and it closely parallels the concentration of organisms in the blood.

Production of the anthrax toxin is mediated by a temperature-sensitive **plasmid, pXO1**, of 110 megadaltons. The toxin consists of three distinct antigenic components. Each component of the toxin is a thermolabile protein with a mw of approximately 80kDa.

Factor I is the **edema factor (EF)** which is necessary for the edema producing activity of the toxin. EF is known to be an **inherent adenylate cyclase**, similar to the *Bordetella pertussis* adenylate cyclase toxin.

Factor II is the **protective antigen (PA)**, because it induces protective antitoxic antibodies in guinea pigs. PA is the **binding (B) domain** of the anthrax toxin which has two active (A) domains, EF (above) and LF (below).

Factor III is known as the **lethal factor (LF)** because it is essential for the **lethal effects** of the anthrax toxin. Apart from their antigenicity, each of the three factors exhibits no significant biological activity in an animal. However, combinations of two or three of the toxin components yield the following results in experimental animals.

PA+LF combine to produce lethal activity

EF+PA produce edema

EF+LF is inactive

PA+LF+EF produces edema and necrosis and is lethal

These experiments suggest that the anthrax toxin has the familiar A-B enzymatic-binding structure of bacterial exotoxins with PA acting as the B fragment and either EF or LF acting as the active A fragment.

EF+PA has been shown to elevate cyclic AMP to extraordinary levels in susceptible cells. Changes in intracellular cAMP are known to affect changes in membrane permeability and may account for edema. In macrophages and neutrophils an additional effect is the depletion of ATP reserves which are needed for the engulfment process. Hence, one effect of the toxin may be to impair the activity of regional phagocytes during the infectious process.

The effects of EF and LF on neutrophils have been studied in some detail. Phagocytosis by opsonized or heat-killed *Bacillus anthracis* cells is not inhibited by either EF or LF, but a combination of EF + LF inhibits engulfment of

the bacteria and the oxidative burst in the PMNs. The two toxin components also increased levels of cAMP in the neutrophils. These studies suggest that the two active components of the toxin, EF + LF, together increase host susceptibility to infection by suppressing neutrophil function and impairing host resistance.

LF+PA have combined lethal activity as stated above. The lethal factor is a Zn⁺⁺ dependent protease that induces cytokine production in macrophages and lymphocytes, and its mechanism of action is slowly becoming understood. The crystal structure of lethal factor, the crucial pathogenic enzyme of anthrax toxin, is known to be a member of the mitogen-activated protein kinase (MAPKK) family of enzymes that disrupts cellular signalling. Furthermore, the identity of the human receptor for anthrax PA, named **anthrax toxin receptor**, has been demonstrated to be a type I membrane protein that binds directly to PA.

In **summary**, the virulence of *Bacillus anthracis* is attributable to three bacterial components:

1. Capsular material composed of poly-D-glutamate polypeptide
2. EF component of exotoxin
3. LF component of exotoxin

Both the capsule and the anthrax toxin may play a role in the early stages of infection, through their direct effects on phagocytes. Virulent anthrax bacilli multiply at the site of the lesion. Phagocytes migrate to the area but the encapsulated organisms can resist phagocytic engulfment, or if engulfed, can resist killing and digestion. A short range effect of the toxin is its further impairment of phagocytic activity and its lethal effect on leukocytes, including phagocytes, at the site. After the organisms and their toxin enter the circulation, the systemic pathology, which may be lethal, will result.

Bacillus anthracis coordinates the expression of its virulence factors in response to a specific environmental signal. Anthrax toxin proteins and the antiphagocytic capsule are produced in response to growth in increased atmospheric CO₂. This CO₂ signal is thought to be of physiological significance for a pathogen which invades mammalian host tissues.

Immunity to Anthrax

Considerable variation in genetic susceptibility to anthrax exists among animal species. Resistant animals fall into two groups: (1) resistant to establishment of anthrax but sensitive to the toxin and (2) resistant to the toxin but susceptible to establishment of disease. This is illustrated in the table below. Neither the source of the inoculum (spores or vegetative cells or a mixture) nor the route of inoculation (subcutaneous, gastrointestinal, or inhalational) is stated. The infectious dose of anthrax is expected to vary widely based on these parameters, as well.

Table 2. The infectious dose of *B. anthracis* and the lethal dose of toxin varies greatly within animal species. The data do not specify the route of infection or whether spores or vegetative cells were used in the inoculum.

Animal model	Infectious dose	Toxic dose causing death	Bacteria per ml blood at time death
Mouse	5 cells	1000 units/kg	10 ⁷
Monkey	3000 cells	2500 unit/kg	10 ⁷
Rat	10 ⁶ cells	15 units/kg	10 ⁵

Animals surviving naturally-acquired anthrax are immune to reinfection. Second attacks are extremely rare. Permanent immunity to anthrax seems to require antibodies to both the toxin and the capsular polypeptide, but the relative importance of the two kinds of antibodies appears to vary widely in different animals.

Vaccines composed of killed bacilli and/or capsular antigens produce no significant immunity. A nonencapsulated toxigenic strain has been used effectively in livestock. The **Sterne Strain** of *Bacillus anthracis* produces sublethal amounts of the toxin that induces formation of protective antibody.

The **anthrax vaccine for humans**, which is used in the U.S., is a preparation of the **protective antigen** recovered from the culture filtrate of an avirulent, nonencapsulated strain of *Bacillus anthracis* that produces PA during active growth. Anthrax immunization consists of three subcutaneous injections given two weeks apart followed by three additional subcutaneous injections given at 6, 12, and 18 months. Annual booster injections of the vaccine are required to maintain a protective level of immunity.

The vaccine is indicated for individuals who come in contact in the workplace with imported animal hides, furs, bone, meat, wool, animal hair (especially goat hair) and bristles; and for individuals engaged in diagnostic or investigational activities which may bring them into contact with anthrax spores. Otherwise, of course, it has been indicated for the military during the current era of biological warfare.

The vaccine should only be administered to healthy individuals from 18 to 65 years of age, since investigations to date have been conducted exclusively in that population. It is not known whether the anthrax vaccine can cause fetal harm, and pregnant women should not be vaccinated.

A new type of **passive vaccine** to anthrax is currently on the horizon. This was recently announced by R.G. Crystal and colleagues from the Medical College of Cornell University, in the February, 2005 issue of the journal, Molecular Therapy. They demonstrated that mice vaccinated with a human adenovirus expressing a single-chain antibody directed against protective antigen (PA) became immune to anthrax within 24 hours of vaccination. This is much quicker than is possible with existing anthrax vaccines, which are a relatively crude preparation of PA.

Currently available anthrax vaccines have limited use in a bioterrorism attack because they are active vaccines in which multiple doses are required over several months to elicit protective immunity against anthrax. Passive vaccines, on the other hand, shuttle fully formed antibodies directly to the body and immunity is achieved much sooner.

In mice receiving the adenovirus-based anti-PA vaccine, PA-specific serum antibodies were detectable within 24 hours. These antibodies had neutralizing activity that protected mice from an intravenous lethal toxin challenge administered 1-14 days post vaccination.

Crystal, et al envision a possible scenario wherein both the passive and active vaccine might be given. Passive vaccines lose their effectiveness fairly rapidly over time, whereas active vaccines do not. The passive vaccine could provide protection that would last a couple of weeks, but that would provide a safety margin for development of more active, long-term immunity stimulated by the active vaccine.

Passive immunotherapy with such adenovirus-based vectors expressing anti-PA antibody, either alone or in combination with antibiotics, may be a rapid, convenient, and highly effective strategy to protect against or treat anthrax in a bioterrorism attack.

Also, in cases of anthrax, coadministration of the passive vaccine with antibiotics may maximize the utility of antibiotic therapy. Coadministration would counter the effects of lethal toxin, and likely prolong the time frame for effective antibiotic treatment and/or reduce the amount of antibiotic therapy required.

Treatment of Anthrax

Antibiotics should be given to unvaccinated individuals exposed to inhalation anthrax. Penicillin, tetracyclines and fluoroquinolones are effective if administered before the onset of lymphatic spread or septicemia, estimated to be about 24 hours. Antibiotic treatment is also known to lessen the severity of disease in individuals who acquire anthrax through the skin. Inhalation anthrax was formerly thought to be nearly 100% fatal despite antibiotic treatment, particularly if treatment is started after symptoms appear. A recent Army study resulted in successful treatment of monkeys with antibiotic therapy after being exposed to anthrax spores. The antibiotic therapy was begun one day after exposure.

Anthrax and Biological Warfare

The inhalation of anthrax spores can lead to infection and disease. The possibility of creating aerosols containing anthrax spores has made *B. anthracis* a chosen weapon of bioterrorism. Several powers may have the ability to load spores of *B. anthracis* into weapons. Domestic terrorists may develop means to distribute spores via mass attacks or small-scale attacks at a local level.

As an agent of biological warfare it is expected that a cloud of anthrax spores would be released at a strategic location to be inhaled by the individuals under attack. Spores of *B. anthracis* can be produced and stored in a dry form and remain viable for decades in storage or after release.

There is no evidence of person-to-person transmission of anthrax. Quarantine of affected individuals is not recommended. Anthrax spores may survive in the soil, water and on surfaces for many years. Spores can only be destroyed by steam sterilization or burning. Chemical disinfection of buildings is problematic. The U.S. Navy Manual on Operational Medicine and Fleet Support, entitled Biological Warfare Defense Information Sheet states "Disinfection of contaminated articles may be accomplished using a 0.05% hypochlorite solution (1 tbsp. bleach per gallon of water). Spore destruction requires steam sterilization."

Anthrax spores are killed by boiling (100C or 212F) for 30 minutes (the actual reported time is considerably less). If boiling as a means of disinfection, the spores must be in liquid suspension (to ensure killing) and in a sealed container (to avoid aerosolization or vaporization of droplet nuclei containing spores).

An infection of local animal populations such as sheep and cattle could follow a biological attack with spores. Infected animals could then transmit the disease to humans through the cutaneous, intestinal or inhalation route by spores from a contaminated animal, carcass or hide.

A segment of the U.S. military population has been vaccinated against anthrax. Anthrax vaccine consists of a series of six doses with yearly boosters. The first vaccine of the series must be given at least four weeks before exposure to the disease. This vaccine protects against anthrax that is acquired through the skin and it is believed that it would also be effective against inhaled spores in a biowarfare situation. Of course, a vaccinated military population would be needed to respond to a terrorist attack with anthrax spores.

OFF THE WALL

One strategy for the development of new vaccines is to expose T-cells to bacterial or viral antigens in order to directly stimulate the mechanisms of cell-mediated immunity (CMI). Such types of vaccines are known as intracellular vaccines, and they theoretically have the potential to stimulate protective CMI, which is rarely accomplished with most present vaccines. Recently, the toxin of *Bacillus anthracis*, specifically its cell-binding domain (PA), has been exploited to transport molecules into T-cells in the search for new vaccines aimed against intracellular parasites. In this case, bacterial or viral antigens were fused to PA creating what is called a model pathogen molecule which is able to recognize and be taken up by T-cells, but which does not produce disease. Though still in early stages of testing, the vaccines show promise, and this work may lead to an entirely new class of human vaccines against most viruses, certain bacteria, and parasites.

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Diphtheria

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Diphtheria

Corynebacteria are Gram-positive, aerobic, nonmotile, rod-shaped bacteria related to the Actinomycetes. They do not form spores or branch as do the actinomycetes, but they have the characteristic of forming irregular shaped, club-shaped or V-shaped arrangements in normal growth. They undergo snapping movements just after cell division which brings them into characteristic arrangements resembling Chinese letters.



Figure 1. Stained *Corynebacterium* cells. The "barred" appearance is due to the presence of polyphosphate inclusions called metachromatic granules. Note also the characteristic "Chinese-letter" arrangement of cells.

The genus *Corynebacterium* consists of a diverse group of bacteria including animal and plant pathogens, as well as saprophytes. Some corynebacteria are part of the normal flora of humans, finding a suitable niche in virtually every anatomic site. The best known and most widely studied species is *Corynebacterium diphtheriae*, the causal agent of the disease diphtheria.

History and Background

No bacterial disease of humans has been as successfully studied as diphtheria. The etiology, mode of transmission, pathogenic mechanism and molecular basis of exotoxin structure, function, and action have been clearly established. Consequently, highly effective methods of treatment and prevention of diphtheria have been developed.

The study of *Corynebacterium diphtheriae* traces closely the development of medical microbiology, immunology and molecular biology. Many contributions to these fields, as well as to our understanding of host-bacterial interactions, have been made studying diphtheria and the diphtheria toxin.

Hippocrates provided the first clinical description of diphtheria in the 4th century B.C. There are also references to the disease in ancient Syria and Egypt.

In the 17th century, murderous epidemics of diphtheria swept Europe; in Spain the disease became known as "El garatillo" (the strangler"), in Italy and Sicily as "the gullet disease".

In the 18th century, the disease reached the American colonies where it reached epidemic proportions about 1735. Often, whole families died of the disease in a few weeks.

The bacterium that caused diphtheria was first described by Klebs in 1883, and was cultivated by Loeffler in 1884, who applied Koch's postulates and properly identified *Corynebacterium diphtheriae* as the agent of the disease.

In 1884, Loeffler concluded that *C. diphtheriae* produced a soluble toxin, and thereby provided the first description of a bacterial exotoxin.

In 1888, Roux and Yersin demonstrated the presence of the toxin in the cell-free culture fluid of *C. diphtheriae* which, when injected into suitable lab animals, caused the systemic manifestation of diphtheria.

Two years later, von Behring and Kitasato succeeded in immunizing guinea pigs with a heat-attenuated form of the toxin and demonstrated that the sera of immunized animals contained an antitoxin capable of protecting other susceptible animals against the disease. This modified toxin was suitable for immunizing animals to obtain antitoxin, but it was found to cause severe local reactions in humans and could not be used as a vaccine.

In 1909, Theobald Smith, in the U.S., demonstrated that diphtheria toxin neutralized by antitoxin (forming a **Toxin-Anti-Toxin complex, TAT**) remained immunogenic and eliminated local reactions seen in the modified toxin. For some years, beginning about 1910, TAT was used for active immunization against diphtheria. TAT had two undesirable characteristics as a vaccine. First, the toxin used was highly toxic, and the quantity injected could result in a fatal toxemia unless the toxin was fully neutralized by antitoxin. Second, the antitoxin mixture was horse serum, the components of which tended to be allergenic and to sensitize individuals to the serum.

In 1913, Schick designed a skin test as a means of determining susceptibility or immunity to diphtheria in humans. Diphtheria toxin will cause an inflammatory reaction when very small amounts are injected intracutaneously. The Schick Test involves injecting a very small dose of the toxin under the skin of the forearm and evaluating the injection site after 48 hours. A positive test (inflammatory reaction) indicates susceptibility (nonimmunity). A negative test (no reaction) indicates immunity (antibody neutralizes toxin).

In 1929, Ramon demonstrated the conversion of diphtheria toxin to its nontoxic, but antigenic, equivalent (**toxoid**) by using formaldehyde. He provided humanity with one of the safest and surest vaccines of all time—the diphtheria toxoid.

In 1951, Freeman made the remarkable discovery that pathogenic (toxigenic) strains of *C. diphtheriae* are lysogenic, (i.e., are infected by a temperate B phage), while non lysogenized strains are avirulent. Subsequently, it was shown that the gene for toxin production is located on the DNA of the B phage.

In the early 1960s, Pappenheimer and his group at Harvard conducted experiments on the mechanism of action of the diphtheria toxin. They studied the effects of the toxin in HeLa cell cultures and in cell-free systems, and concluded that the toxin inhibited protein synthesis by blocking the transfer of amino acids from tRNA to the growing polypeptide chain on the ribosome. They found that this action of the toxin could be neutralized by prior treatment with diphtheria antitoxin.

Subsequently, the exact mechanism of action of the toxin was shown, and the toxin has become a classic model of a bacterial exotoxin.

At the turn of the century, in the United States, diphtheria was common, occurring primarily in children, and was one of the leading causes of death in infants and children. In the 1920's, when data were first gathered, in the United States there were approximately 150,000 cases and 13,000 deaths reported annually. After diphtheria immunization was introduced, the number of cases gradually fell to about 19,000 in 1945. When diphtheria immunization became widespread in the late 1940's, a more rapid decrease in the number of cases and deaths occurred.

From 1970 to 1979, an average of 196 cases per year were reported. Seventeen outbreaks of 15 or more cases occurred in the United States between 1959 and 1980, but there have been none since 1980. From 1980-1989, the number of cases in the United States dropped to 24; two cases were fatal and 18 occurred in persons 20 years of age or older. Most cases have occurred nonimmunized (or inadequately immunized) individuals.

Human Disease

CDC describes diphtheria as "an upper respiratory tract illness characterized by sore throat, low-grade fever, and an adherent membrane of the tonsil(s), pharynx, and/or nose". Diphtheria is a rapidly developing, acute, febrile

infection which involves both local and systemic pathology. A local lesion develops in the upper respiratory tract and involves necrotic injury to epithelial cells. As a result of this injury, blood plasma leaks into the area and a fibrin network forms which is interlaced with rapidly-growing *C. diphtheriae* cells. This membranous network covers over the site of the local lesion and is referred to as the **pseudomembrane**.

The diphtheria bacilli do not tend to invade tissues below or away from the surface epithelial cells at the site of the local lesion. At this site they produce the toxin that is absorbed and disseminated through lymph channels and blood to the susceptible tissues of the body. Degenerative changes in these tissues, which include heart, muscle, peripheral nerves, adrenals, kidneys, liver and spleen, result in the systemic pathology of the disease.

In parts of the world where diphtheria still occurs, it is primarily a disease of children, and most individuals who survive infancy and childhood have acquired immunity to diphtheria. In earlier times, when nonimmune populations (i.e., Native Americans) were exposed to the disease, people of all ages were infected and many were killed.

About one person in 10 who gets diphtheria dies of it. Diphtheria is more severe for those under 5 and over 40 years of age.

Pathogenicity

The pathogenicity of *Corynebacterium diphtheriae* includes **two distinct phenomena**:

1. **Invasion** of the local tissues of the throat, which requires colonization and subsequent bacterial proliferation. Nothing is known about the adherence mechanisms of *C. diphtheriae*
2. **Toxigenesis**: bacterial production of the toxin. The diphtheria toxin causes the death eukaryotic cells and tissues by inhibition protein synthesis in the cells. Although the toxin is responsible for the lethal symptoms of the disease, the virulence of *C. diphtheriae* cannot be attributed to toxigenicity alone, since a distinct invasive phase apparently precedes toxigenesis. However, it has not been ruled out that the diphtheria toxin plays an essential role in the colonization process due to short-range effects at the colonization site.

Three strains of *Corynebacterium diphtheriae* are recognized, **gravis**, **intermedius** and **mitis**. They are listed here by falling order of the severity of the disease that they produce in humans. All strains produce the identical toxin and are capable of colonizing the throat. The differences in virulence between the three strains can be explained by their differing abilities to produce the toxin in rate and quantity, and by their differing growth rates.

The gravis strain has a generation time (in vitro) of 60 minutes; the intermedius strain has a generation time of about 100 minutes; and the mitis stain has a generation time of about 180 minutes. The faster growing strains typically produce a larger colony on most growth media. In the throat (in vivo), a faster growth rate may allow the organism to deplete the local iron supply more rapidly in the invaded tissues, thereby allowing earlier or greater production of the diphtheria toxin. Also, if the kinetics of toxin production follow the kinetics of bacterial growth, the faster growing variety would achieve an effective level of toxin before the slow growing varieties.

Toxigenicity

Two factors have great influence on the ability of *Corynebacterium diphtheriae* to produce the diphtheria toxin: (1) **low extracellular concentrations of iron** and (2) the **presence of a lysogenic prophage** in the bacterial chromosome. The gene for toxin production occurs on the chromosome of the prophage, but a bacterial repressor protein controls the expression of this gene. The repressor is activated by iron, and it is in this way that iron influences toxin production. High yields of toxin are synthesized only by lysogenic bacteria under conditions of iron deficiency.

The role of iron. In artificial culture the most important factor controlling yield of the toxin is the concentration of inorganic iron (Fe++ or Fe+++) present in the culture medium. Toxin is synthesized in high yield only after the exogenous supply of iron has become exhausted (This has practical importance for the industrial production of toxin to make toxoid. Under the appropriate conditions of iron starvation, *C. diphtheriae* will synthesize diphtheria toxin as 5% of its total protein!). Presumably, this phenomenon takes place in vivo as well. The bacterium may not produce maximal amounts of toxin until the iron supply in tissues of the upper respiratory tract has become

depleted. It is the regulation of toxin production in the bacterium that is partially controlled by iron. The tox gene is regulated by a mechanism of negative control wherein a repressor molecule, product of the DtxR gene, is activated by iron. The active repressor binds to the tox gene operator and prevents transcription. When iron is removed from the repressor (under growth conditions of iron limitation), derepression occurs, the repressor is inactivated and transcription of the tox genes can occur. Iron is referred to as a **corepressor** since it is **required for repression of the toxin gene**.

The role of B-phage. Only those strains of *Corynebacterium diphtheriae* that are lysogenized by a specific Beta-phage produce diphtheria toxin. A phage lytic cycle is not necessary for toxin production or release. The **phage contains the structural gene for the toxin molecule.** The original proof rested in the demonstration that lysogeny of *C. diphtheriae* by various mutated Beta phages leads to production of nontoxic but antigenically-related material (called CRM for "cross-reacting material"). CRMs have shorter chain length than the diphtheria toxin molecule but cross react with diphtheria antitoxins due to their antigenic similarities to the toxin. The properties of CRMs established beyond a doubt that the tox genes resided on the phage chromosome rather than the bacterial chromosome.

Even though the tox gene is not part of the bacterial chromosome the regulation of toxin production is under bacterial control since the DtxR (regulatory) gene is on bacterial chromosome and toxin production depends upon bacterial iron metabolism.

It is of some interest to speculate on the role of the diphtheria toxin in the natural history of the bacterium. Of what value should it be to an organism to synthesize up to 5% of its total protein as a toxin that specifically inhibits protein synthesis in eukaryotes (and archaebacteria)? Possibly the toxin assists colonization of the throat (or skin) by killing epithelial cells or neutrophils. There is no evidence to suggest a key role of the toxin in the life cycle of the organism. Since mass immunization against diphtheria has been practiced, the disease has virtually disappeared, and *C. diphtheriae* is no longer a component of the normal flora of the human throat and pharynx. It may be that the toxin played a key role in the colonization of the throat in nonimmune individuals and, as a consequence of exhaustive immunization, toxigenic strains have become virtually extinct.

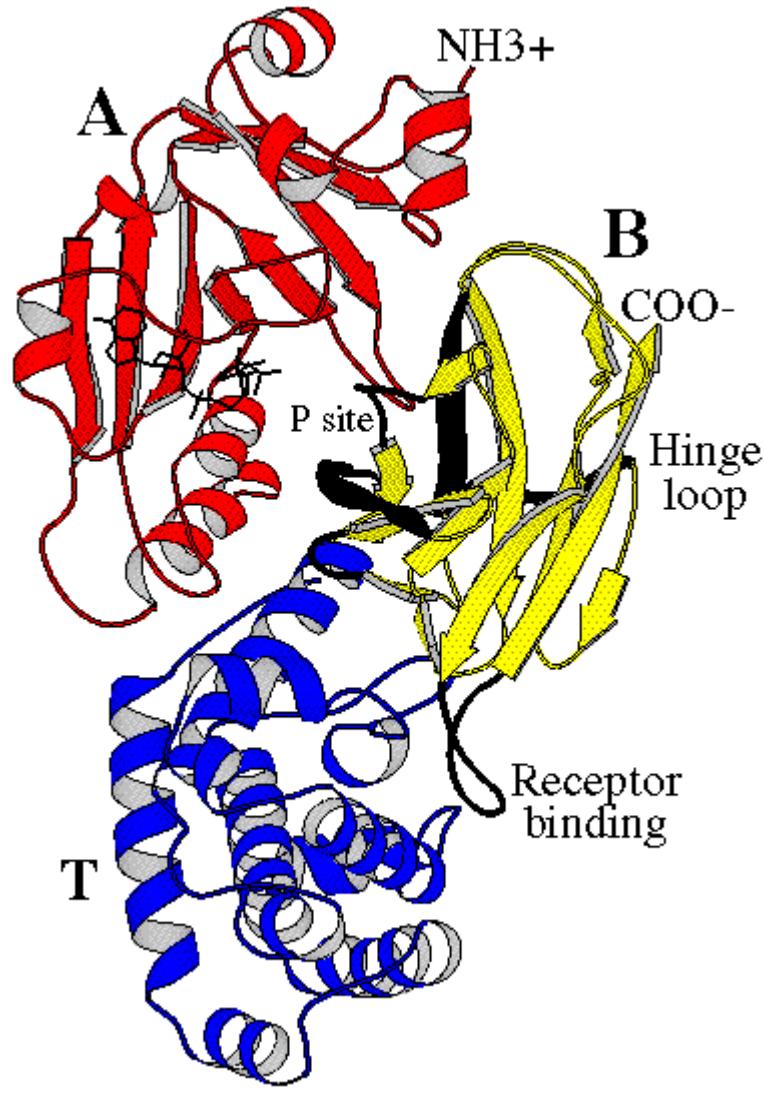


Figure 2a. The Diphtheria Toxin (DT) Monomer

A (red) is the catalytic domain; B (yellow) is the binding domain which displays the receptor for cell attachment; T (blue) is the hydrophobic domain responsible for insertion into the endosome membrane to secure the release of A. The protein is illustrated in its "closed" configuration.



Fig 2b. Mode of Action of the Diphtheria Toxin

The diphtheria toxin is a two component bacterial exotoxin synthesized as a single polypeptide chain containing an A (active) domain and a B (binding) domain. Proteolytic nicking of the secreted form of the toxin separates the A chain from the B chain.

The toxin binds to a specific receptor (now known as the HB-EGF receptor) on susceptible cells and enters by receptor-mediated endocytosis. Acidification of the endosome vesicle results in unfolding of the protein and insertion of a segment into the endosomal membrane. Apparently as a result of activity on the endosome membrane, the A subunit is cleaved and released from the B subunit as it inserts and passes through the membrane.

Once in the cytoplasm, the A fragment regains its conformation and its enzymatic activity. Fragment A catalyzes the transfer of ADP-ribose from NAD to the eukaryotic Elongation Factor 2 which inhibits the function of the latter in protein synthesis. Ultimately, inactivation of all of the host cell EF-2 molecules causes death of the cell. Attachment of the ADP ribosyl group occurs at an unusual derivative of histidine called diphthamide.

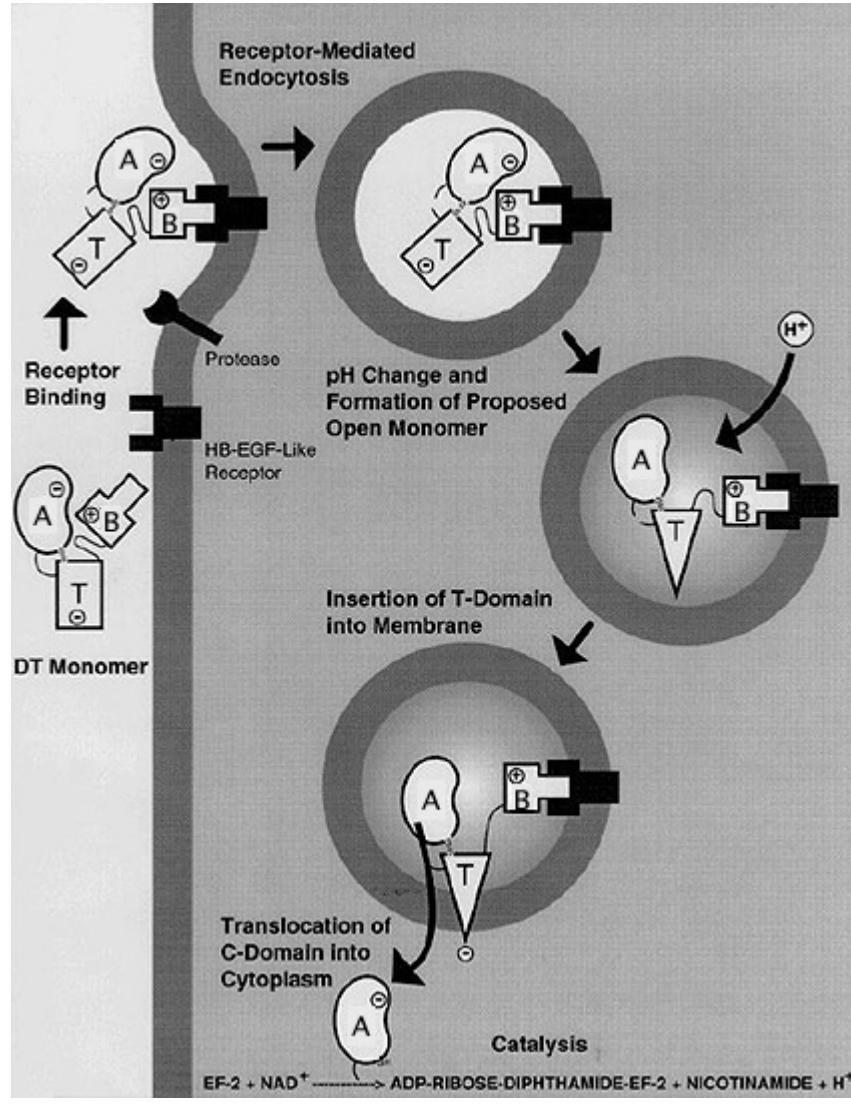


Figure 3. Uptake and activity of the diphtheria toxin in Eukaryotic cells

The figure above was redrawn from the [Diphtheria Toxin Homepage](#) at UCLA. A represents the A/B toxin's A (catalytic) domain; B is the B (receptor) domain; T is the hydrophobic domain that inserts into the cell membrane.

In vitro, the native diphtheria toxin is inactive and can be activated by trypsin in the presence of thiol. The enzymatic activity of fragment A is masked in the intact toxin. Fragment B is required to bind the native toxin to its cognate receptor and to permit the escape of fragment A from the endosome. The C terminal end of Fragment B contains the peptide region that attaches to the HB-EGF receptor on the sensitive cell membrane, and the N-terminal end is a strongly hydrophobic region which will insert into a membrane lipid bilayer.

The specific membrane receptor, heparin-binding epidermal growth factor (HB-EGF) precursor is a protein on the surface of many types of cells. The occurrence and distribution of the HB-EGF receptor on cells determines the susceptibility of an animal species, and certain cells of an animal species, to the diphtheria toxin. Normally, the HB-EGF precursor releases a peptide hormone that influences normal cell growth and differentiation. One hypothesis is that the HB-EGF receptor itself is the protease that nicks the A fragment and reduces the disulfide bridge between it and the B fragment when the A fragment makes its way through the endosomal membrane into the cytoplasm.

Immunity to Diphtheria

Acquired immunity to diphtheria is due primarily to toxin-neutralizing antibody (antitoxin). Passive immunity in utero is acquired transplacentally and can last at most 1 or 2 years after birth. In areas where diphtheria is endemic and mass immunization is not practiced, most young children are highly susceptible to infection. Probably active immunity can be produced by a mild or inapparent infection in infants who retain some maternal immunity, and in adults infected with strains of low virulence (inapparent infections).

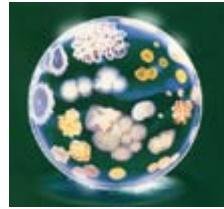
Individuals that have fully recovered from diphtheria may continue to harbor the organisms in the throat or nose for weeks or even months. In the past, it was mainly through such healthy carriers that the disease was spread, and toxigenic bacteria were maintained in the population. Before mass immunization of children, carrier rates of *C. diphtheriae* of 5% or higher were observed.

Because of the high degree of susceptibility of children, artificial immunization at an early age is universally advocated. Toxoid is given in 2 or 3 doses (1 month apart) for primary immunization at an age of 3 - 4 months. A booster injection should be given about a year later, and it is advisable to administer several booster injections during childhood. Usually, infants in the United States are immunized with a trivalent vaccine containing diphtheria toxoid, pertussis vaccine, and tetanus toxoid (DPT or DTP vaccine).

The relative absence of diphtheria in the United States is due primarily to the high level of appropriate immunization in children, and to an apparent reduction in toxin-producing strains of the bacterium. However, the increasing percentage of diphtheria cases in adults suggests that many adults may not be protected against diphtheria, because they have not receive booster immunizations within the past ten years. A similar situation exists with tetanus.

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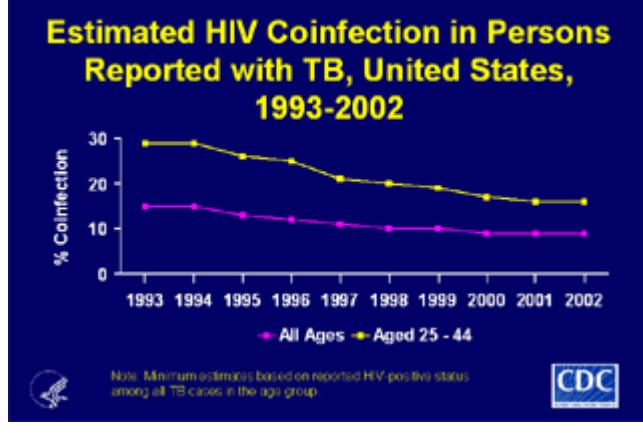
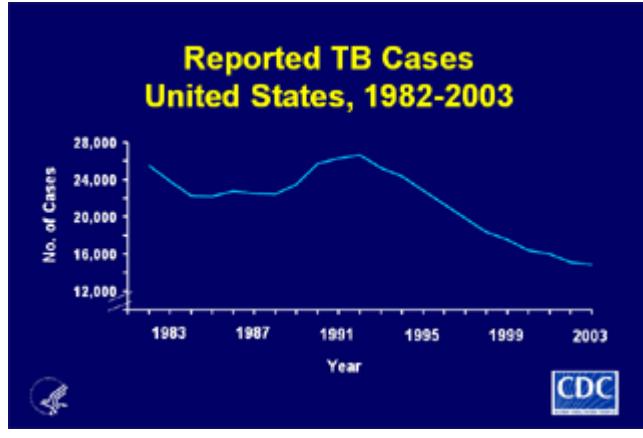
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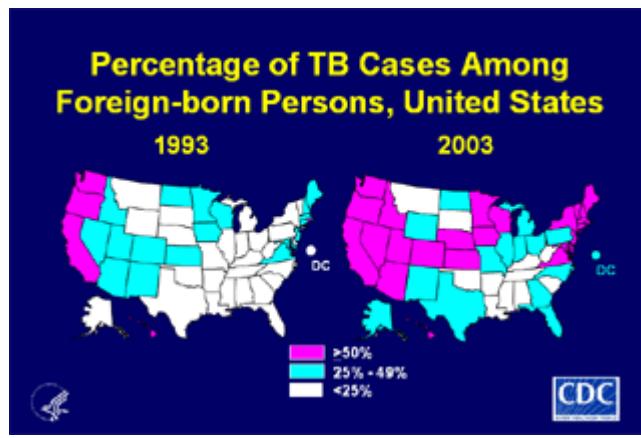
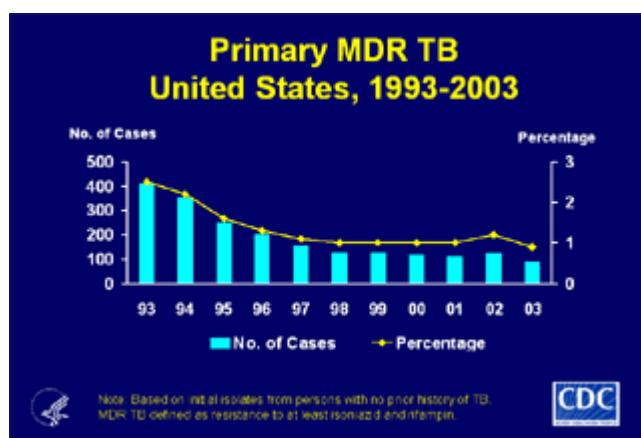
Tuberculosis

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Tuberculosis (TB) is the leading cause of death in the world from a single infectious disease. The disease affects 1.7 billion people/year which is equal to one-third of the entire world population.

In the United States TB is on the decline. There was a resurgence of TB from 1986 to 1992, but since 1993, the numbers of cases have been going down and are now at the lowest they have ever been. Also, since 1993, there has been a gradual decline in the number of TB patients with coinfection with HIV, and the number of cases of multiple drug-resistant (MDR) TB dropped during this period of time. In many states, especially in the West, the upper Midwest, and the Northeast, most new cases of TB now occur in individuals who are foreign born. This is evident in the following statistics provided by the CDC [Division of Tuberculosis Elimination](#).





Mycobacterium tuberculosis

Mycobacterium tuberculosis is the etiologic agent of tuberculosis in humans. Humans are the only reservoir for the bacterium.

Mycobacterium bovis is the etiologic agent of TB in cows and rarely in humans. Both cows and humans can serve as reservoirs. Humans can also be infected by the consumption of unpasteurized milk. This route of transmission can lead to the development of **extrapulmonary TB**, exemplified in history by bone infections that led to hunched backs.

Other human pathogens belonging to the *Mycobacterium* genus include *Mycobacterium avium* which causes a TB-like disease especially prevalent in AIDS patients, and *Mycobacterium leprae*, the causative agent of **leprosy**.

History and Present Day Importance

Mycobacterium tuberculosis (M.TB.) was the cause of the "White Plague" of the 17th and 18th centuries in Europe. During this period nearly 100 percent of the European population was infected with M.TB., and 25 percent of all adult deaths were caused by M.TB. (Note: The White Plague is not to be confused with the "Black Plague", which was caused by *Yersinia pestis* and occurred about 3 centuries earlier).

General Characteristics

Mycobacterium tuberculosis is a fairly large **nonmotile rod-shaped bacterium** distantly related to the Actinomycetes. Many non pathogenic mycobacteria are components of the normal flora of humans, found most often in dry and oily locales. The rods are 2-4 um in length and 0.2-0.5 um in width.

Mycobacterium tuberculosis is an **obligate aerobe**. For this reason, in the classic case of tuberculosis, the M.TB. complexes are always found in the well-aerated upper lobes of the lungs. The bacterium is a **facultative intracellular parasite**, usually of macrophages, and has a **slow generation time**, 15-20 hours, a physiological characteristic that may contribute to its virulence.

Two media are used to grow M.TB. **Middlebrook's medium** which is an agar based medium and **Lowenstein-**

Jensen medium which is an egg based medium. M.TB. colonies are small and buff colored when grown on either medium. Both types of media contain inhibitors to keep contaminants from out-growing M.TB. It takes 4-6 weeks to get visual colonies on either type of media.



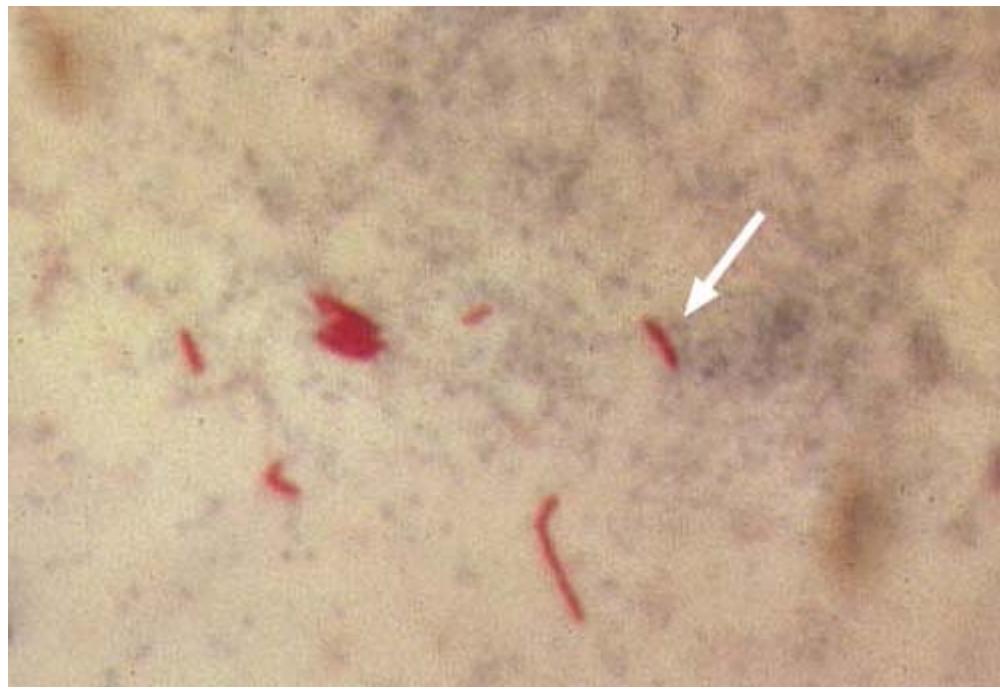
Colonies of *Mycobacterium tuberculosis* on Lowenstein-Jensen medium. CDC.

Chains of cells in smears made from in vitro-grown colonies often form distinctive **serpentine cords**. This observation was first made by Robert Koch who associated **cord factor** with virulent strains of the bacterium.

M.TB. is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although the bacteria do contain peptidoglycan (murein) in their cell wall. If a Gram stain is performed on M.TB., it stains very weakly Gram-positive or not at all (referred to as "ghosts").

Mycobacterium species, along with members of a related genus *Nocardia*, are classified as **acid-fast bacteria** due to their impermeability by certain dyes and stains. Despite this, once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds. One acid-fast staining method for *Mycobacterium tuberculosis* is the **Ziehl-Neelsen stain**. When this method is used, the M.TB. smear is fixed, stained with carbolfuchsin (a pink dye), and decolorized with acid-alcohol. The smear is counterstained with methylene-blue or certain other dyes. Acid-fast bacilli appear pink in a contrasting background.

In order to detect *Mycobacterium tuberculosis* in a sputum sample, in excess of 10,000 organisms per ml of sputum are needed to visualize the bacilli with a 100X microscope objective. One acid-fast bacillus/slide is regarded as "suspicious" of an M.TB. infection.



Mycobacterium tuberculosis. Acid-fast stain. CDC.

Cell Wall Structure

The cell wall structure of *Mycobacterium tuberculosis* deserves special attention because it is unique among prokaryotes and it is a major determinant of virulence for the bacterium. The cell wall complex contains **peptidoglycan**, but otherwise it is composed of complex lipids. Over 60% of the mycobacterial cell wall is lipid. The lipid fraction of M.TB's cell wall consists of three major components.

Mycolic acids are unique alpha-branched lipids found in cell walls of *Mycobacterium* and *Corynebacterium*. They make up 50% of the dry weight of the mycobacterial cell envelope. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. Mycolic Acids are thought to be a significant determinant of virulence in M.TB. Probably, they prevent attack of the mycobacteria by cationic proteins, lysozyme and oxygen radicals in the phagocytic granule. They also protect extracellular mycobacteria from complement deposition in serum.

Cord Factor is responsible for the serpentine cording mentioned above. Cord factor is toxic to mammalian cells and is also an inhibitor of PMN migration. Cord factor is most abundantly produced in virulent strains of M.TB.

Wax-D in the cell envelope is the major component of **Freund's complete adjuvant** (CFA).

In summary, the high concentration of lipids in the cell wall of *Mycobacterium tuberculosis* has been associated with these properties of the bacterium:

- Impermeability to stains and dyes
- Resistance to many antibiotics
- Resistance to killing by acidic and alkaline compounds
- Resistance to osmotic lysis via complement deposition
- Resistance to lethal oxidations and survival inside of macrophages

The Disease Tuberculosis

TB infection means that M.TB. is in the body but the immune system is keeping the bacteria under control. The immune system does this by producing macrophages that surround the tubercle bacilli. The cells form a hard shell that keeps the bacilli contained and under control. Most people with TB infection have a positive reaction to the **tuberculin skin test**. People who have TB infection but not TB disease are NOT infectious, i.e., they cannot spread the infection to other people. These people usually have a normal chest x-ray. TB infection is not considered a case of TB. Major similarities and differences between TB infection and TB disease are shown below.

Tuberculosis: Infection vs Disease

TB Infection	TB disease in lungs
M.TB. present	M.TB. present
Tuberculin skin test positive	Tuberculin skin test positive
Chest X-ray normal	Chest X-ray usually reveals lesion
Sputum smears and cultures negative	Sputum smears and cultures positive
No symptoms	Symptoms such as cough, fever, weight loss
Not infectious	Often infectious before treatment
Not defined as a case of TB	Defined as a case of TB

Predisposing factors for TB infection include:

- Close contact with large populations of people, i.e., schools, nursing homes, dormitories, prisons, etc.
- Poor nutrition
- iv drug use
- Alcoholism
- HIV infection is the #1 predisposing factor for M.TB. infection. 10 percent of all HIV-positive individuals harbor M.TB. This is 400-times the rate associated with the general public

Only 3-4% of infected individuals will develop active disease upon initial infection, 5-10% within one year. These percentages are much higher if the individual is HIV+.

Stages of the Disease

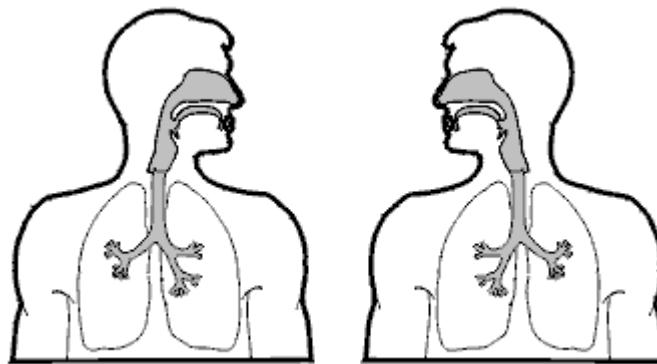
The following stages that will be explained are for a M.TB. sensitive host. It should be realized that, as stated previously, only a small percent of M.TB. infections progress to disease and even a smaller percent progress all the way to stage 5. Usually the host will control the infection at some point.

Disease progression depends on:

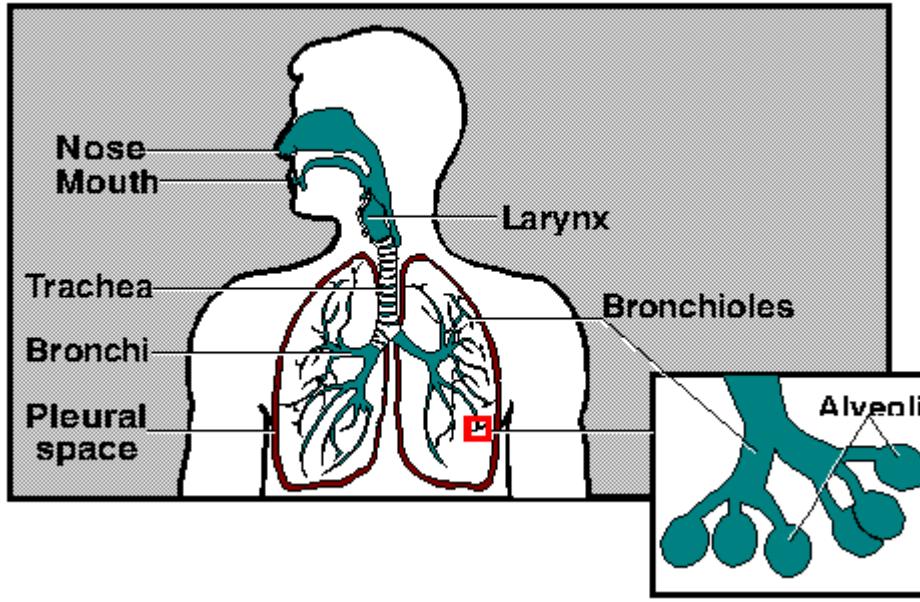
- Strain of M.TB.
- Prior exposure
- Vaccination
- Infectious dose
- Immune status of the host

Stage 1

Droplet nuclei are inhaled. One droplet nuclei contains no more than 3 bacilli. Droplet nuclei are so small that they can remain air-borne for extended periods of time. The most effective (infective) droplet nuclei tend to have a diameter of 5 um. Droplet nuclei are generated by during talking coughing and sneezing. Coughing generates about 3000 droplet nuclei. Talking for 5 minutes generates 3000 droplet nuclei but singing generates 3000 droplet nuclei in one minute. Sneezing generates the most droplet nuclei by far, which can spread to individuals up to 10 feet away.



Spread of droplet nuclei from one individual to another. CDC. After droplet nuclei are inhaled, the bacteria are nonspecifically taken up by alveolar macrophages. However, the macrophages are not activated and are unable to destroy the intracellular organisms.



Tuberculosis begins when droplet nuclei reach the alveoli. When a person inhales air that contains droplets most of the larger droplets become lodged in the upper respiratory tract (the nose and throat), where infection is unlikely to develop. However, the smaller droplet nuclei may reach the small air sacs of the lung (the alveoli), where infection begins.

Stage 2

Begins 7-21 days after initial infection. M.TB. multiplies virtually unrestricted within unactivated macrophages until the macrophages burst. Other macrophages begin to extravasate from peripheral blood. These macrophages also phagocytose M.TB., but they are also unactivated and hence can not destroy M.TB.

Stage 3

At this stage lymphocytes begin to infiltrate. The lymphocytes, specifically T-cells, recognize processed and presented M.TB. antigen in context of MHC molecules. This results in T-cell activation and the liberation of cytokines including gamma interferon (IFN). The liberation of IFN causes in the activation of macrophages. These activated macrophages are now capable of destroying M.TB.

It is at this stage that the individual becomes tuberculin-positive. This positive tuberculin reaction is the result of the host developing a vigorous cell mediated immune (CMI) response. A CMI response must be mounted to control an M.TB. infection. An antibody mediated immune (AMI) will not aid in the control of a M.TB. infection because M.TB. is intracellular and if extracellular, it is resistant to complement killing due to the high lipid concentration in

its cell wall.

Although a CMI response is necessary to control an M.TB. infection, it is also responsible for much of the pathology associated with tuberculosis. Activated macrophages may release lytic enzymes and reactive intermediates that facilitate the development of immune pathology. Activated macrophages and T-cells also secrete cytokines that may also play a role in the development of immune pathology, including Interleukin 1 (IL-1), tumor necrosis factor (TNF), and gamma IFN.

It is also at this stage that **tubercle** formation begins. The center of the tubercle is characterized by "caseation necrosis" meaning semi-solid or "cheesy" consistency. M.TB. cannot multiply within these tubercles because of the low pH and anoxic environment. M.TB. can, however, persist within these tubercles for extended periods.

Stage 4

Although many activated macrophages can be found surrounding the tubercles, many other macrophages present remain unactivated or poorly activated. M.TB. uses these macrophages to replicate and hence the tubercle grows.

The growing tubercle may invade a bronchus. If this happens, M.TB. infection can spread to other parts of the lung. Similarly the tubercle may invade an artery or other blood supply line. The hematogenous spread of M.TB. may result in extrapulmonary tuberculosis otherwise known as **milliary tuberculosis**. The name "milliary" is derived from the fact that metastasizing tubercles are about the same size as a millet seed, a grain commonly grown in Africa.

The **secondary lesions** caused by milliary TB can occur at almost any anatomical location, but usually involve the genitourinary system, bones, joints, lymph nodes, and peritoneum. These lesions are of two types:

1. **Exudative lesions** result from the accumulation of PMN's around M.TB. Here the bacteria replicate with virtually no resistance. This situation gives rise to the formation of a "soft tubercle".
2. **Productive or granulomatous lesions** occur when the host becomes hypersensitive to tuberculoproteins. This situation gives rise to the formation of a "hard tubercle".

Stage 5

For unknown reasons, the caseous centers of the tubercles liquify. This liquid is very conducive to M.TB. growth and hence the organism begins to rapidly multiply extracellularly. After time, the large antigen load causes the walls of nearby bronchi to become necrotic and rupture. This results in cavity formation. This also allows M.TB. to spill into other airways and rapidly spread to other parts of the lung.

As stated previously, only a very small percent of M.TB. infections result in disease, and even a smaller percentage of M.TB. infections progress to an advanced stage. Usually the host will begin to control the infection at some point. When the primary lesion heals, it becomes fibrous and calcifies. When this happens the lesion is referred to as the **Ghon complex**. Depending on the size and severity, the Ghon complex may never subside. Typically the Ghon complex is readily visible upon chest X-ray.

Small metastatic foci containing low numbers of M.TB. may also calcify. However, in many cases these foci will contain viable organisms. These foci are referred to **Simon foci**. The Simon foci are also visible upon chest X-ray and are often the site of disease reactivation.

Virulence Mechanisms and Virulence Factors

Mycobacterium tuberculosis does not possess the classic bacterial virulence factors such as toxins, capsules and fimbriae. However, a number of structural and physiological properties of the bacterium are beginning to be recognized for their contribution to bacterial virulence and the pathology of tuberculosis.

M.TB. has special mechanisms for cell entry. The tubercle bacillus can bind directly to mannose receptors on macrophages via the **cell wall- associated mannosylated glycolipid, LAM**, or indirectly via certain complement receptors or Fc receptors.

M.TB. can grow intracellularly. This is an effective means of evading the immune system. In particular, antibodies and complement are ineffective. Once M.TB. is phagocytosed, it can inhibit phagosome-lysosome fusion. The exact mechanism used by M.TB. to accomplish this is not known but it is thought to be the result of a protein secreted by bacterium that modifies the phagosome membrane. The bacterium may remain in the phagosome or escape from the phagosome, in either case finding a protected environment for growth in the macrophage.

M.TB. interferes with the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis by two mechanisms:

1. Compounds including glycolipids, sulfatides and LAM down regulate the oxidative cytotoxic mechanism.
2. Macrophage uptake via complement receptors may bypass the activation of a respiratory burst.

Antigen 85 complex. This complex is composed of a group of proteins secreted by M.TB. that are known to bind fibronectin. These proteins may aid in walling off the bacteria from the immune system and may facilitate tubercle formation although evidence of this is lacking.

Slow generation time. Because of M.TB's slow generation time, the immune system may not readily recognize the bacteria or may not be triggered sufficiently to eliminate them. Many other chronic disease are caused by bacteria with slow generation times, for example, slow-growing *M. leprae* causes leprosy, *Treponema pallidum* causes syphilis, and *Borrelia burgdorferi* causes Lyme disease.

High lipid concentration in cell wall, as mentioned previously, accounts for impermeability and resistance to antimicrobial agents, resistance to killing by acidic and alkaline compounds in both the intracellular and extracellular environment, and resistance to osmotic lysis via complement deposition and attack by lysozyme.

Cord factor. The cord factor is primarily associated with virulent strains of M.TB. It is known to be toxic to mammalian cells and to be an inhibitor of PMN migration. However, its exact role in M.TB. virulence is unclear.

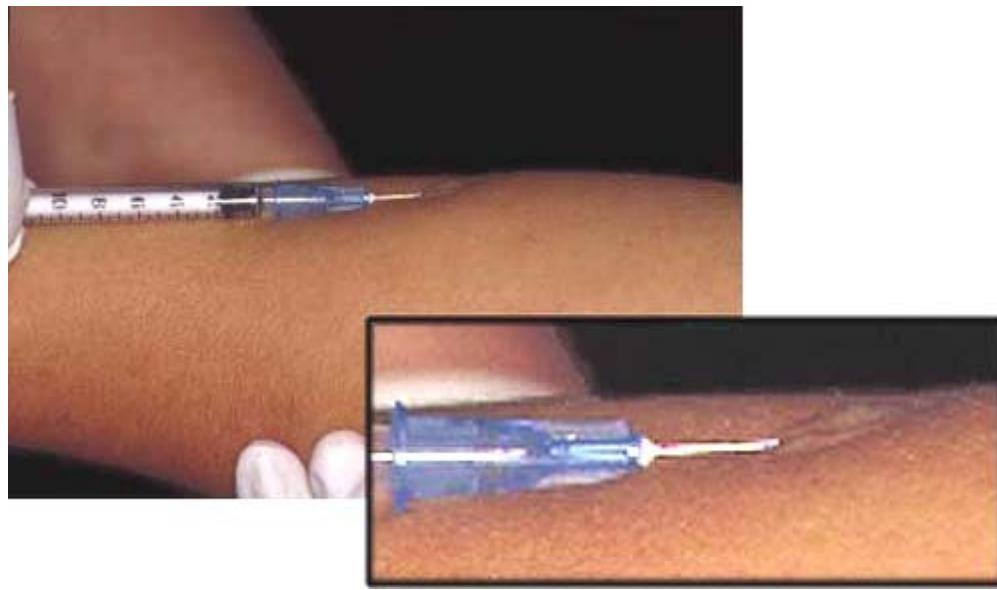
Clinical Identification and Diagnosis of Tuberculosis

The diagnosis of tuberculosis requires **detection of acid-fast bacilli** in sputum via the Ziehl-Neelsen method as previously described.

The organisms must then be **cultured from sputum**. First, the sputum sample is treated with NaOH. This kills other contaminating bacteria but does not kill the M.TB. present because M.TB. is resistant to alkaline compounds by virtue of its lipid layer.

The media used for growth and the resulting colony morphology have been described previously. However, methods of culturing can take 4-6 weeks to yield visible colonies. As a result, another method is commonly used called the **BACTEC System**. The media used in the BACTEC system contains radio-labeled palmitate as the sole carbon source. As M.TB. multiplies, it breaks down the palmitate and liberates radio-labeled CO₂. Using the BACTEC system, M.TB. growth can be detected in 9-16 days vs 4-6 weeks using conventional media.

Skin Testing is performed as the **tuberulin** or **Mantoux test**. PPD (purified protein derivative) is employed as the test antigen in the **Mantoux test**. PPD is generated by boiling a culture of M.TB., specifically Old Tuberculin (OT). 5 TU (tuberculin units), which equals 0.0001mg of PPD, in a 0.1 ml volume is intracutaneously injected in the forearm. The test is read within 48-72 hours.



Administering the Mantoux test. CDC.

The test is considered positive if the diameter of the resulting lesion is 10 mm or greater. The lesion is characterized by erythema (redness) and swelling and induration (raised and hard). 90% of people that have a lesion of 10 mm or greater are currently infected with M.TB. or have been previously exposed to M.TB. 100% of people that have a lesion of 15 mm or greater are currently infected with M.TB. or have been previously exposed to M.TB.

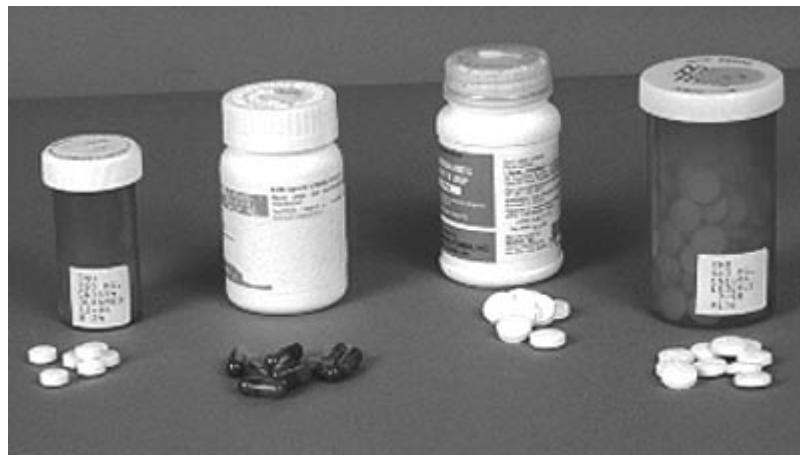
False positive tests usually manifest themselves as lesser reactions. These lesser reactions could indicate prior exposure or infection with other Mycobacteria or vaccination with BCG. However, in places where the vaccine is not used, lesser reactions should be regarded as highly suspicious.

False negatives are more rare than false positives but are especially common in AIDS patients as they have an impaired CMI response. Other conditions such as malnutrition, steroids, etc., can rarely result in a false negative reaction.

Tuberculosis Treatment

Because administration of a single drug often leads to the development of a bacterial population resistant to that drug, **effective regimens for the treatment of TB must contain multiple drugs to which the organisms are susceptible**. When two or more drugs are used simultaneously, each helps prevent the emergence of tubercle bacilli resistant to the others. However, when the in vitro susceptibility of a patient's isolate is not known, which is generally the case at the beginning of therapy, selecting two agents to which the patient's isolate is likely to be susceptible can be difficult, and improper selection of drugs may subsequently result in the development of additional drug-resistant organisms.

Hence, tuberculosis is usually treated with four different antimicrobial agents. The course of drug therapy usually lasts from 6-9 months. The most commonly used drugs are rifampin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB) or streptomycin (SM). When adherence with the regimen is assured, this four-drug regimen is highly effective. Based on the prevalence and characteristics of drug-resistant organisms, at least 95% of patients will receive an adequate regimen (at least two drugs to which their organisms are susceptible) if this four-drug regimen is used at the beginning of therapy (CDC, unpublished data). Furthermore, a patient who is treated with the four-drug regimen, but who defaults therapy, is more likely to be cured and not relapse when compared with a patient treated for the same length of time with a three-drug regimen.



Drugs used to treat TB disease. From left to right isoniazid, rifampin, pyrazinamide, and ethambutol. Streptomycin (not shown) is given by injection. CDC.

Prevention

A vaccine against M.TB. is available. It is called **BCG** (Bacillus of Calmette and Guerin, after the two Frenchmen that developed it). BCG consists of a live attenuated strain derived from *Mycobacterium bovis*. This strain of *Mycobacterium* has remained avirulent for over 60 years.

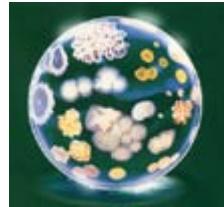
The vaccine is not 100% effective. Studies suggest a 60-80% effective rate in children.

The vaccine is not administered in the U.S. for several reasons:

- The vaccine cannot circumvent disease reactivation in previously exposed individuals.
- The vaccine does not prevent infection, only disease. Therefore, the entire population would have to be vaccinated if the vaccine was to be considered efficacious.
- Vaccination may complicate the way the tuberculin skin test is read in this country. In places that do not vaccinate, the skin test may be used to monitor the effectiveness of antibiotic therapy.

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Lyme Disease

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Borrelia burgdorferi the spirochete that causes Lyme Disease. FA stain (CDC)

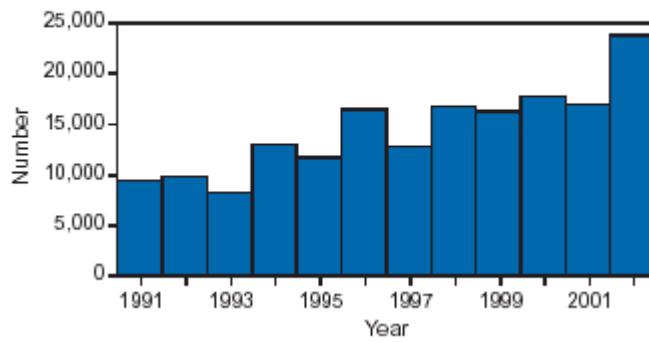
Introduction

Lyme disease was first recognized in the United States in 1975 by Dr. Allen Steere, following a mysterious outbreak of juvenile rheumatoid arthritis near the community of Lyme, Connecticut. The rural location of the Lyme outbreak and the onset of illness during summer and early fall suggested that the transmission of the disease was by an arthropod vector.

In 1982, the etiologic agent of Lyme disease was discovered by Willy Burgdorfer who isolated spirochetes belonging to the genus *Borrelia* from the mid-guts of *Ixodes* ticks. He showed that these spirochetes reacted with immune serum from patients that had been diagnosed with Lyme disease. Subsequently, the etiologic agent was given the name ***Borrelia burgdorferi***.

Since then, reports of Lyme disease have increased dramatically to the point that the disease has become an important public health problem in some areas of the United States. Today Lyme disease is the most prevalent tick-borne illness in the US. Lyme disease has been reported in 49 states and on four different continents. In 2002, there were 23,763 new cases reported in the U.S. Between 1996 and 2001 the number was level at about 17,000 new cases per year, but increased by nearly 7,000 cases in 2002. See Figure 1 below from the CDC.

**FIGURE 1. Number of reported cases of Lyme disease, by year
— United States, 1991–2002**



Biology of *Borrelia burgdorferi*

Borrelia burgdorferi, like the human pathogen *Treponema pallidum*, is a **spirochete**. Spirochetes are a group of phylogenetically-distinct bacteria that have a unique mode of motility by means of axial filaments (endoflagella). Spirochetes are widespread in viscous environments and they are found in the intestinal tracts of animals and the oral cavity of humans. The spirochetes have a unique cell surface which accompanies their unique type of motility. The endoflagella are contained within the periplasmic space between a semi rigid peptidoglycan helix and a multi-layer, flexible outer membrane sheath. When the filaments rotate within this space, the spirochetes move in corkscrew fashion. This type of movement may be an adaptation to viscous environments such as aquatic sediments, biofilms, mucosal tissues and the intestinal tracts of animals. For pathogens, this allows the spirochetes to hide their flagella, which are normally antigenic, from the host immune defenses.



Schematic representation of a spirochete

Spirochetes are usually much longer than they are wide, and often their width is below the resolving power of the light microscope. For example, *Borrelia* may have a length of 20-30μm but a width of only .2-.3μm. Hence, most spirochetes cannot be viewed using conventional light microscopy. **Dark-field microscopy** must be used to view spirochetes. Dark field microscopy utilizes a special condenser which directs light toward an object at an angle, rather than from the bottom. As a result, particles or cells are seen as light objects against a dark background.



B. burgdorferi dark field illumination (CDC)

The spirochetes are not classified as either Gram-positive or Gram-negative. When *Borrelia burgdorferi* is Gram-stained, the cells stain a weak Gram-negative by default, as safranin is the last dye used. *Borrelia*, like most spirochetes, does have an outer membrane that contains an LPS-like substance, an inner membrane, and a periplasmic space which contains a layer of peptidoglycan. Therefore, it has a Gram-negative bacterial type cell wall, despite its staining characteristics.

Cultivation

Unlike *Treponema pallidum*, *Borrelia burgdorferi* can be cultivated in vitro. However, the bacterium is fastidious and requires a very complex growth medium. The medium used to grow *Borrelia burgdorferi* is called Barbour-Stoenner-Kelly (BSK) medium. It contains over thirteen ingredients in a rabbit serum base. *Borrelia burgdorferi* has an optimal temperature for growth of 32 C, in a microaerobic environment. Even under optimal conditions, the generation time is slow, about 12-24 hours.

Borrelia from ticks and from the blood, skin, and cerebrospinalfluid of Lyme disease patients have been

successfully cultivated in BSK medium. BSK solidified with 1.3% agarose allows the production of colonies from single organisms.

Strains of *Borrelia*

Recently, the *Borrelia* causing Lyme disease were divided into several "genospecies", three of which have been firmly established and are well accepted:

I. *Borrelia burgdorferi sensu stricto*

II. *Borrelia garinii*

III. *Borrelia afzelii*

The term used to collectively describe all three genospecies is ***Borrelia burgdorferi sensu lato***. The differences in genospecies are revealed by restriction fragment length polymorphism, (RFLP), multi-locus enzyme electrophoresis (MLEE) and ssuRNA sequences. All US isolates fall into genospecies I. Examples of all three genospecies have been found in Europe and Asia, although II and III predominate there.

Outer Surface Proteins

The outer membrane of *Borrelia burgdorferi* is composed of various unique outer surface proteins (Osp) that have been characterized (Osp A through OspF). They are presumed to play a role in virulence. Osp A and Osp B are by far the most abundant outer surface proteins. The genes encoding these proteins are transcribed from a common promoter, and are located on a 49 kb linear plasmid. The chromosome of *Borrelia burgdorferi* is also linear and is almost 1100 kb in size.

Pathogenicity

Borrelia burgdorferi invades the blood and tissues of various infected mammals and birds. The natural reservoir for *Borrelia burgdorferi* is thought to be the white-footed mouse. Ticks transfer the spirochetes to the white-tailed deer, humans, and other warm-blooded animals after a blood meal on an infected animal. In humans, dogs, and many other animals, infection with *Borrelia burgdorferi* results in the pathology of Lyme Disease.

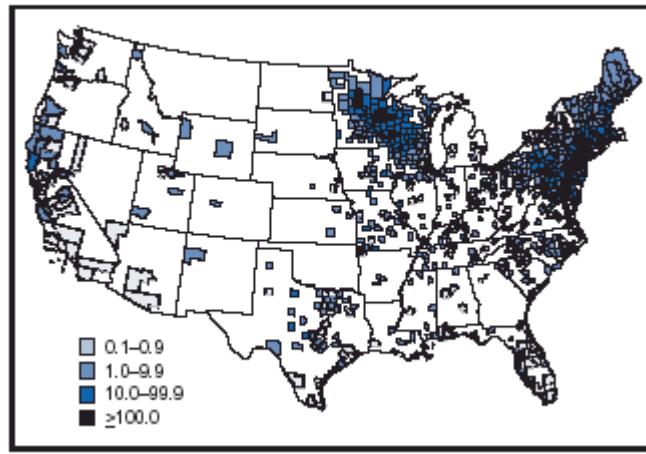
Incidence and Distribution of Lyme Disease in the United States

Lyme disease has a wide distribution in northern temperate regions of the world. In the United States, the highest incidence occurs in the Northeast, from Massachusetts to Maryland and the North-central states, especially Wisconsin and Minnesota.

In 2002, more than 23,000 cases of Lyme disease were reported in the U.S., the highest number ever reported. This increase could be caused by an increase in human contact with infected ticks and enhanced reporting of cases.

Twelve states reported an incidence of Lyme Disease that was higher than the national average in both 2001 and 2002: Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, and Wisconsin. These 12 states account for 95% of cases reported nationally. See Figure 2 below from CDC.

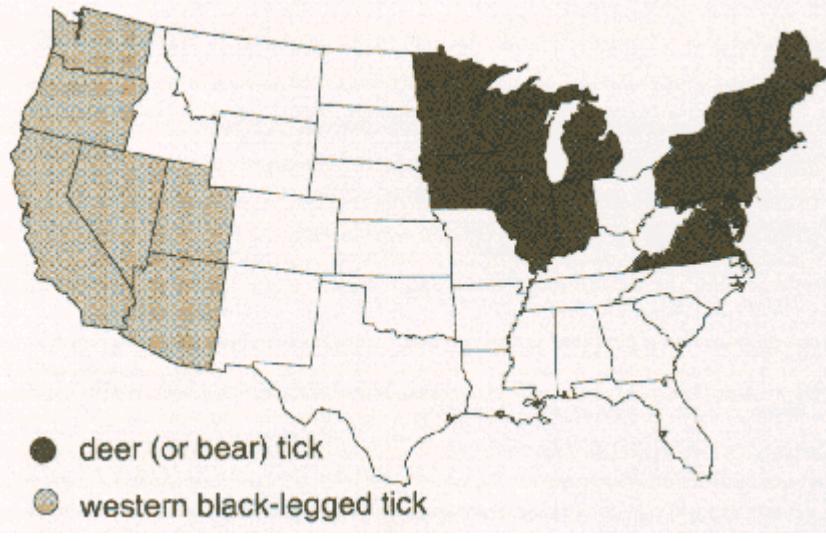
**FIGURE 2. Incidence* of Lyme disease, by county of residence
— United States, 2002**



* Per 100,000 population.

Transmission of Lyme Disease

Lyme disease is spread by the bite of ticks of the genus *Ixodes* that are infected with *Borrelia burgdorferi*. *Ixodes*, commonly known as the **deer tick** (or bear tick), normally feeds on the white-footed mouse, the white-tailed deer, and certain other mammals. It is responsible for transmitting the spirochetes to humans in the northeastern and north-central United States. On the Pacific Coast, the bacteria are transmitted to humans by the **western black-legged tick**, and in the southeastern states by the related black-legged tick.



Distribution of *Ixodes* ticks that transmit Lyme disease in the U.S. (CDC)

Ixodes ticks are much smaller than common dog and cattle ticks. In their larval and nymphal stages, they are no bigger than a pinhead. Adult ticks are slightly larger. The tick nymphs, which are most likely to feed on a person and are rarely noticed because of their small size (less than 2 mm), are usually involved in the transmission of the disease.

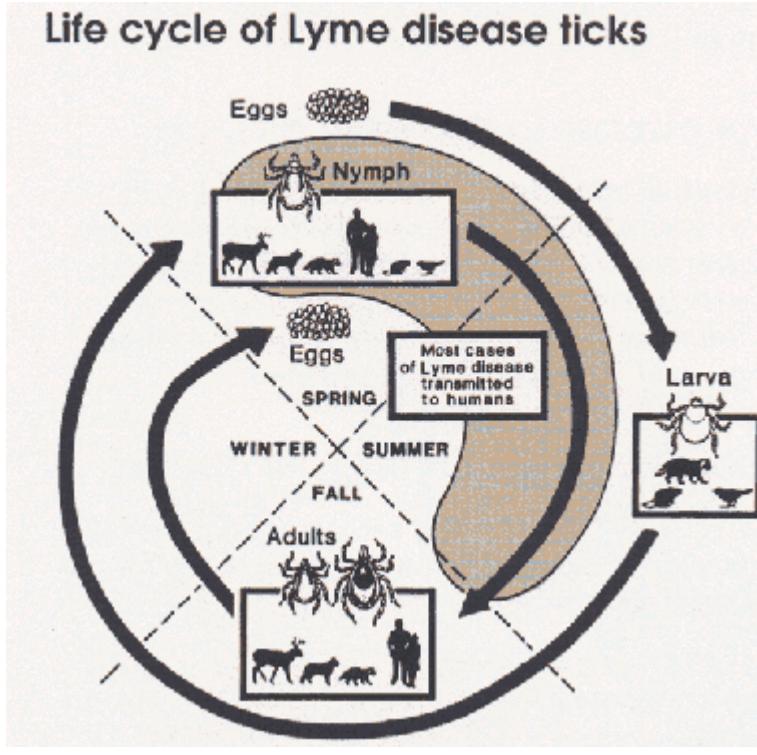


Ixodes ticks (CDC)

Spirochete prevalence in adult *Ixodes* ticks has been shown to be approximately 35% (for example, in the Baraboo Hills, northwest of Madison, Wisconsin), but varies greatly among geographic locations (e.g. California = 2%,

New York =50%).

For Lyme disease to exist in an area, at least three closely interrelated elements must be present in nature: the Lyme disease bacteria, ticks that can transmit them, and mammals (such as mice and deer) to provide food for the ticks in their various life stages. The tick life cycle consists of three distinctive stages: larvae, nymphs, and adults. A blood meal is required for ticks to molt from the larvae stage to the nymph stage and from the nymph stage to the adult stage. The tick larvae and nymphs typically become infected with Lyme disease bacteria when they feed on infected small animals, particularly the white-footed mouse. The bacteria remain in the tick as it changes from larva to nymph or from nymph to adult. Infected nymphs and adult ticks then bite and transmit Lyme disease bacteria to other small rodents, other animals, and humans, all in the course of their normal feeding behavior. Adult ticks preferentially feed on the white-tailed deer, which thereby becomes an important reservoir in regions of infestation. The tick life cycle takes two years to complete (See diagram below).



Lyme disease occurs in domestic animals, as well. In dogs, the disease usually presents as arthritis. Domestic animals can carry infected ticks into areas where humans live, but whether pet owners are more likely than others to get Lyme disease is not known.

Symptoms of Lyme disease

The symptoms of Lyme disease in humans occur in three stages.

Stage one (early infection). The early stage of Lyme disease is often characterized by a distinctive, expanding red rash that usually develops at the site of the tick bite. This rash, known as **erythema migrans**, is seen in 60-80% of infected individuals (it is important to remember that the converse is true: no rash is ever observed in 20-40 % of the cases!). Spirochetes can be isolated from the leading edge of the rash. Erythema migrans is a red circular patch that appears usually 3 days to 1 month following the bite of the tick. The patch then expands, often to a large size and develops a characteristic "bull's eye" appearance. However, not all rashes that occur at the site of a tick bite are due to Lyme disease. An allergic reaction to tick saliva often occurs at the site of a tick bite. This rash can be confused with the rash of Lyme disease. Allergic reactions to tick saliva usually occur within hours to a few days after the tick bite, usually do not expand, and disappear within a few days. Erythema migrans persists longer, but usually subsides within 3-4 weeks.



The presentation of erythema migrans in Stage 1

Stage two (dissemination stage): occurs days to weeks following infection. At this stage the spirochetes spread hematogenously to additional body tissues. One or more of the following symptoms and signs may be noted:

- fatigue
- chills and fever
- headache
- muscle and joint pain
- swollen lymph nodes
- secondary annular skin lesions

Stage three (persistent infection). Some symptoms and signs of Lyme disease may not appear until weeks, months, or years after a tick bite. Stage three typically involves intermittent episodes of joint pain. Common clinical manifestations at this stage may include meningitis, Bell's palsy, cardiac involvement, and migratory pain to joints, tendons, muscle and bone:

Arthritis is most likely to appear as brief bouts of pain and swelling, usually in one or more large joints, especially the knees.

Nervous system abnormalities can include numbness, pain, Bell's palsy (paralysis of the facial muscles, usually on one side), and meningitis (fever, stiff neck, and severe headache).

Less frequently, irregularities of the heart rhythm occur.

In a minority of individuals (11%) the development of chronic Lyme arthritis may lead to erosion of cartilage and/or bone. Other clinical manifestations associated with stage three Lyme disease include neurologic complications such as disturbances in memory, mood, or sleep patterns, and sensations of numbness and tingling in the hands or feet.

Lyme disease mimics other diseases and pathologies and is highly variable in its presentation. In some persons the rash never forms; in some, the first and only sign of Lyme disease is arthritis, and in others, nervous system problems are the only evidence of Lyme disease. There is an increasing and alarming number of reports of neuropsychiatric effects associated with Lyme Disease.

Diagnosis of Lyme disease

Lyme disease is often difficult to diagnose because its symptoms and signs mimic those of so many other diseases. The fever, muscle aches, and fatigue of Lyme disease can easily be mistaken for viral infections, such as influenza or infectious mononucleosis. Joint pain can be mistaken for other types of arthritis, such as rheumatoid arthritis, and neurologic signs can mimic those caused by other conditions, such as multiple sclerosis. At the same time, other types of arthritis or neurologic diseases can be misdiagnosed as Lyme disease.

The clinical diagnosis of Lyme disease is usually based on history of possible exposure to ticks, especially in areas where Lyme disease is known to occur and a combination of symptoms and signs of infection. Serodiagnosis to detect anti-borrelia antibodies is not useful until in later stages of illness. Serologic testing may, however, provide valuable supportive diagnostic information in patients with endemic exposure and/or clinical findings that suggest

late stage or disseminated Lyme disease.

When serologic testing is indicated, CDC recommends testing first with an enzyme-linked immunosorbent assay (ELISA) or an indirect fluorescent antibody (IFA) test, followed by a more specific Western immunoblot (WB) test to corroborate equivocal or positive results obtained with the first test. None of these tests is useful in the diagnosis of early stages of Lyme disease since a primary serum immune response is just beginning. Furthermore, these tests are associated with a high degree of cross-reactivity, since sera from patients with Rocky Mountain spotted fever, relapsing fever, mononucleosis, syphilis, and rheumatoid arthritis often test positive for Lyme disease.

Patients with early disseminated or late-stage disease usually have strong serological reactivity. Antibodies may persist for months or years following successfully treated or untreated infection. Thus, seroreactivity alone cannot be used as a marker of active disease.

Neither a positive serologic activity nor a history of previous Lyme disease assures that an individual has protective immunity. Repeated infection with *B. burgdorferi* has been documented.

B. burgdorferi can be cultured from 80% or more of biopsy specimens taken from early erythema migrans lesions. However, the diagnostic value of this procedure is limited because of the need for special bacteriologic media (BSK medium) and protracted observation of cultures.

Polymerase chain reaction (PCR) has been used to amplify genomic DNA of *B. burgdorferi* in skin, blood, cerebro-spinal fluid, and synovial fluid, but PCR has not been standardized for routine diagnosis of Lyme disease.

Treatment of Lyme disease

Since the diagnosis of Lyme disease is based primarily on clinical findings, it is often appropriate to treat patients with early disease solely on the basis of objective signs and a known exposure.

Several antibiotics are effective in the treatment of Lyme disease. The present drug of choice is doxycycline, a semisynthetic derivative of tetracycline. Even patients who are treated in later stages of the disease respond well to antibiotics. In a few patients who are treated for Lyme disease, symptoms of persisting infection may continue or recur, making additional antibiotic treatment necessary. Varying degrees of permanent damage to joints or the nervous system can develop in patients with late chronic Lyme disease. Typically these are patients in whom Lyme disease was unrecognized in the early stages or for whom the initial treatment was unsuccessful.

Prevention

Removing leaves and clearing brush and tall grass around houses and at the edges of gardens may reduce the numbers of ticks that transmit Lyme disease. A relationship has been observed between the abundance of deer and the abundance of deer ticks in some parts United States. Reducing and managing deer populations in geographic areas where Lyme disease occurs may reduce tick abundance.

CDC recommends the following for personal protection from tick bites and Lyme disease:

Avoid tick-infested areas, especially in May, June, and July.

Wear light-colored clothing so that ticks can be spotted more easily. Tuck pant legs into socks or boots and shirt into pants or ape the area where pants and socks meet so that ticks cannot crawl under clothing.

Spray insect repellent containing DEET on clothes and on exposed skin other than the face, or treat clothes (especially pants, socks, and shoes) with permethrin, which kills ticks on contact.

Wear a hat and a long-sleeved shirt for added protection.

Walk in the center of trails to avoid overhanging grass and brush.

After being outdoors, remove clothing and wash and dry it at a high temperature; inspect body carefully and

remove attached ticks with tweezers, grasping the tick as close to the skin surface as possible and pulling straight back with a slow steady force; avoid crushing the tick's body. In some areas, ticks (saved in a sealed container) can be submitted to the local health department for identification.

Preventive antibiotic treatment with erythromycin or doxycycline to prevent Lyme disease after a known tick bite may be warranted.

Personal protective measures, such as repellent use and routine tick checks, are key components of primary prevention. Removing infected ticks within 48 hours of attachment can reduce the likelihood of transmission, and prompt antimicrobial prophylaxis of tick bites, although controversial, might be beneficial under certain circumstances. Exposure to ticks in yards and recreational areas can be reduced 50-90% through simple landscaping practices, such as removing brush and leaf litter or creating a buffer zone of wood chips or gravel between forest and lawn or recreational areas. Correctly timed applications of pesticides to yards once or twice a year can decrease the number of nymphal ticks 68%--100%.

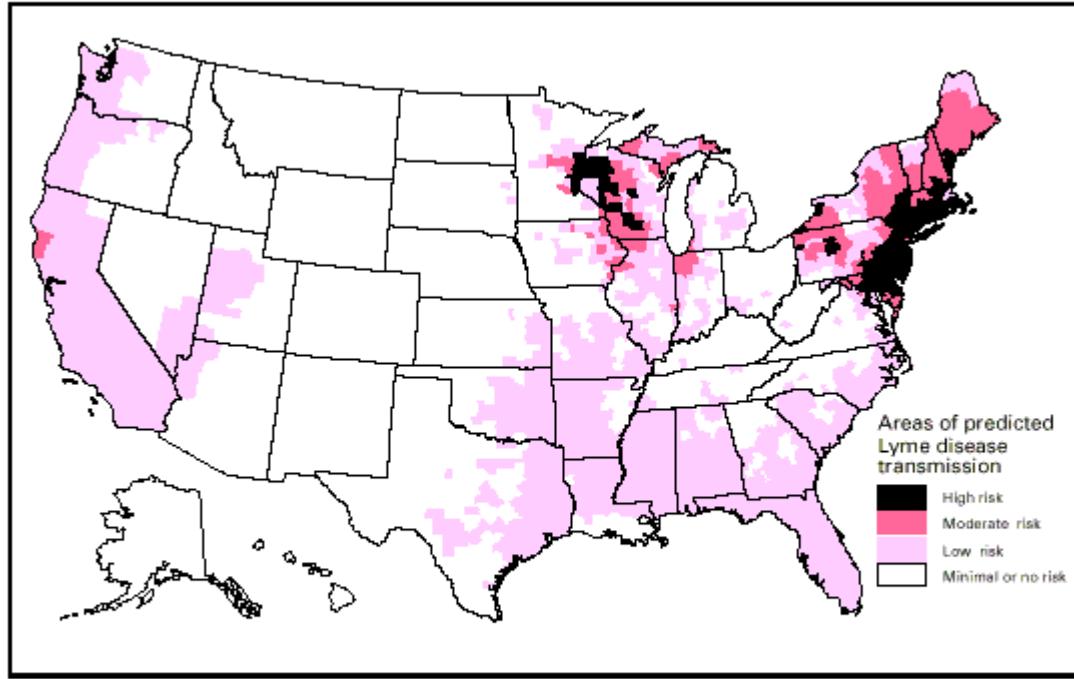
In addition to these interventions, several novel approaches to Lyme disease prevention are under investigation or will soon be available. These include bait boxes and "four-poster" devices that deliver acaricides to rodents and deer without harming them, and the use of biologic agents, such as fungi that kill *Ixodes* ticks.

Vaccines for Lyme disease

In 1998, the Food and Drug Administration licensed the LYMErixTM vaccine against Lyme disease for human use. LYMErixTM contains lipidated recombinant outer surface protein A (OspA) of *Borrelia burgdorferi sensu stricto*, the causative agent of Lyme disease in North America, adsorbed onto aluminum adjuvant. It was indicated for use in persons aged 15-70 years. Three doses of the vaccine are administered by intramuscular injection. The initial dose is followed by a second dose 1 month later and a third dose 12 months after the first. Vaccine administration should be timed so the second dose and the third dose are given several weeks before the beginning of the *B. burgdorferi* transmission season which usually begins in April.

The vaccine is targeted at persons at risk for exposure to infected vector ticks. This risk should be assessed by considering the regional distribution of the disease and the extent to which a person's activities place them in contact with ticks. A Lyme disease risk map (below) is available from CDC. Vaccination of persons with frequent or prolonged exposure to ticks in areas endemic for Lyme disease is expected to be an important preventive strategy. Recommendations for use of the LYMErixTM vaccine have been developed by the Advisory Committee for Immunization Practices of the CDC and are available at [CDC Lyme Disease Vaccine Recommendations](#).

In February, 2002, the manufacturer of the FDA-approved LYMErixTM Lyme disease vaccine withdrew it from the market, reportedly because of poor sales. However, several other effective preventive measures remain available to persons living in areas where the disease is endemic.

National Lyme disease risk map with four categories of risk

Note: This map demonstrates an approximate distribution of predicted Lyme disease risk in the United States. The true relative risk in any given county compared with other counties might differ from that shown here and might change from year to year. Risk categories are defined in the accompanying text. Information on risk distribution within states and counties is best obtained from state and local public health authorities.

Lyme Disease Websites

[CDC Lyme Disease Home Page](#)

[Lyme Disease: Questions and Answers - CDC](#)

[Lyme Disease: Diagnosis - CDC](#)

[Lyme Disease: Prevention and Control - CDC](#)

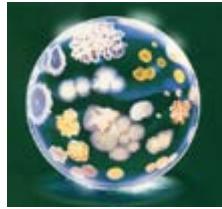
[PBS Documentary on Lyme Disease - CDC](#)

[Lyme Disease: Vaccine Recommendations - CDC](#)

[Lyme Disease, NIH Division of Microbiology and Infectious Diseases](#)

[Lyme Disease: Scientific Literature](#)

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The Rickettsiae

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Introduction to the Rickettsiae

The **rickettsiae** are small (0.3-0.5 x 0.8-2.0 um), Gram-negative, aerobic, coccobacilli that are obligate intracellular parasites of eukaryotic cells. They may reside in the cytoplasm or within the nucleus of the cell that they invade. They divide by binary fission and they metabolize host-derived glutamate via aerobic respiration and the citric acid (TCA) cycle. They have typical Gram-negative cell walls, and they lack flagella. The rickettsiae frequently have a close relationship with arthropod vectors that may transmit the organism to mammalian hosts. The rickettsiae have very small genomes of about 1.0-1.5 million bases.

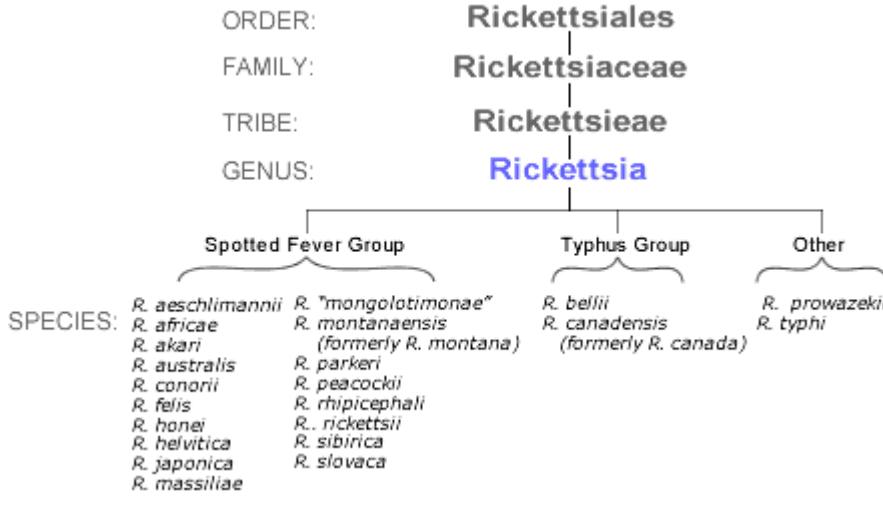
Rickettsiae must be grown in the laboratory by co-cultivation with eukaryotic cells, and they have not been grown by in axenic culture. The basis of their obligate relationship with eukaryotic cells has been explained by rickettsial possession of "leaky membranes" that require the osmolarity and nutritional environment supplied by an intracellular habitat.

The rickettsiae, in spite of their small size and obligate intracellular habitat, are a group of **alphaproteobacteria**, which include many well-known organisms such as *Acetobacter*, *Rhodobacter*, *Rhizobium* and *Agrobacterium*. Very few of the alphaproteobacteria are pathogens of humans. *Brucella*, *Bartonella*, *Rickettsia*, and a related intracellular parasite, *Ehrlichia*, are the main exceptions.

Taxonomy

The genus *Rickettsia* is included in the bacterial tribe *Rickettsiaeae*, family *Rickettsiaceae*, and order *Rickettsiales*. This genus includes many other species of bacteria associated with human disease, including those in the spotted fever group and the typhus group (figure 1).

Figure 1. Taxonomic classification of the order *Rickettsiales*



The rickettsiae can be subdivided into two or three major groups, depending on your taxonomic point of view (1. spotted fever; 2. typhus; and 3. scrub typhus groups) based on clinical characteristics of disease and phylogenetic relationships.

Spotted Fever Group (SFG)

Rickettsia rickettsii is the cause of **Rocky Mountain spotted fever (RMSF)** and is the prototype bacterium in the spotted fever group of rickettsiae. *Rickettsia rickettsii* is found in the Americas and is transmitted to humans through the bite of infected ticks. The bacterium infects human vascular endothelial cells, producing an inflammatory response. The pathogenesis of RMSF is discussed in some detail below.

Other spotted fever group rickettsiae that produce human rickettsioses include *R. conorii*, *R. mongolotimonae* and *R. slovaca* (boutonneuse fever and similar illnesses), *R. akari* (rickettsial pox), *R. japonica* (Japanese spotted fever), *R. sibirica* (North Asian tick typhus), *R. africae* (African tick bite fever), *R. helvetica* (perimyocarditis), *R. australis* (Queensland tick typhus) and *R. honei* (Flinders Island spotted fever). The spotted fever rickettsiae have been found on every continent except Antarctica.

Typhus Group (TG)

Rickettsia prowazekii is the cause of epidemic or louse-borne typhus and is the prototypical bacterium from the typhus group of rickettsiae. *R. prowazekii* infects human vascular endothelial cells, producing widespread vasculitis. In contrast to RMSF, louse-borne typhus tends to occur in the winter. Infection usually is transmitted from person to person by the body louse and, therefore, tends to manifest under conditions of crowding and poor hygiene. The southern flying squirrel is apparently the reservoir in the United States, but the vector involved in transmission from the flying squirrel to humans is unknown. The disease has a worldwide distribution.

Other rickettsiae in the typhus group include *R. typhi* and *R. felis*. Murine typhus is caused by transmission of *R. typhi* from rats, cats and opossums to humans via a flea vector. Murine typhus is found worldwide and is endemic to areas of Texas and southern California in the United States. Although *R. felis* is phylogenetically more closely related to the spotted fever group of rickettsiae than the typhus group, it shares antigens with *R. typhi* and produces a murine typhus-like illness. *Rickettsia felis* has been detected in cat fleas and opossums.

Scrub Typhus Group (STG)

Orientia (Rickettsia) tsutsugamushi is the cause of scrub typhus. Originally called *Rickettsia tsutsugamushi*, this organism was given its own genus designation because it is phylogenetically distinct from the other rickettsiae, though closely related. *Orientia tsutsugamushi* is transmitted to humans by the bite of trombiculid mites (chiggers), which are the vector and host. Scrub typhus occurs throughout much of Asia and Australia.

Virulence of Rickettsiae

Adherence to the Host Cell

Rickettsiae are inoculated into the dermis of the skin by a tick bite or through damaged skin from the feces of lice or fleas. The bacteria spread through the bloodstream and infect the endothelium. Adherence to the host cell is the first step of rickettsial pathogenesis. The adhesins are presumed to be outer membrane proteins. The outer membrane protein OmpA has been implicated in adherence of *R. rickettsii* because antibodies to OmpA have been shown to block adherence.

The host cell receptor for any *Rickettsia* has yet to be identified. Although the main target cells of *Rickettsia* in vivo are endothelial cells, rickettsiae can infect virtually every cell line in vitro. Thus, either the receptor for *Rickettsia* is ubiquitous among cells, or rickettsiae can bind to different receptors.

Invasion of Host Cells

Upon attaching to the host cell membrane, rickettsiae are phagocytosed by the host cell. The rickettsiae are believed to induce host cell phagocytosis because they can enter cells that normally do not phagocytose particles. Once phagocytosed by the host cell, rickettsiae are observed to quickly escape from the phagosome membrane and enter the cytoplasm. The mechanism of escape from the phagosome membrane is not well understood, but it is thought to be mediated by a rickettsial enzyme, phospholipase A2.

Movement within and Release from the Host Cell

Observations in cell culture systems suggest that the mechanisms of intracellular movement and destruction of the host cells differ among the spotted fever group and typhus group rickettsiae.

Typhus group rickettsiae are released from host cells by lysis of the cells. After infection with *R. prowazekii* or *R. typhi*, the rickettsiae continue to multiply until the cell is packed with organisms and then bursts. Phospholipase A2

may be involved in cell lysis. Typhus group rickettsia-infected host cells have a normal ultrastructural appearance.

Spotted fever group rickettsiae seldom accumulate in large numbers and do not lyse the host cells. They escape from the cell by stimulating polymerization of host cell-derived actin tails, which propel them through the cytoplasm and into tips of membranous extrusions, from which they emerge. Infected cells exhibit signs of membrane damage associated with an influx of water, but the means by which rickettsiae damage host cell membranes is uncertain. There is evidence to suggest a role for free radicals of oxygen, phospholipase, and a protease. The protein responsible for the actin-based movement in spotted fever group rickettsiae has yet to be identified, but it is apparently different than the proteins responsible for actin polymerization by *Listeria monocytogenes* and *Shigella flexneri*.

Diseases

Rickettsial diseases vary in clinical severity according to the virulence of the *Rickettsia* and host factors, such as age, male gender, alcoholism, and other underlying diseases. The most virulent rickettsiae are *R. rickettsii* and *R. prowazekii*, which kill a significant portion of infected persons, unless the diseases are treated sufficiently early in the course of infection with an effective antimicrobial agent, usually doxycycline.

All rickettsial infections begin with introduction of the organisms into the skin, either through a tick bite or cutaneous abrasions contaminated by flea or louse feces. Rickettsiae enter dermal cells including endothelium and proliferate locally intracellularly with endothelial cell-to-cell spread for most SFG rickettsioses resulting in an eschar or tache noire, a zone of dermal and epidermal necrosis approximately 1 cm in diameter with a surrounding zone of erythema. Eschars do not occur in epidemic and murine typhus and are rarely observed in Rocky Mountain spotted fever.

SFG rickettsioses often manifest regional lymphadenopathy in the drainage of the eschar, suggesting that rickettsiae may spread via lymphatic vessels from the tick bite inoculation site early in the infection. Rickettsiae spread throughout the body and infect mainly endothelial cells, establishing many foci of contiguous infected blood vessel-lining cells. Injury in these local sites causes vascular damage manifesting as rash, interstitial pneumonia, encephalitis, interstitial nephritis, and interstitial myocarditis, as well as lesions in the liver, gastrointestinal wall, pancreas, and potentially any vascularized tissue of the body.

The most important pathophysiologic effect is increased vascular permeability with consequent edema, loss of blood volume, hypoalbuminemia, decreased osmotic pressure, and hypotension. These effects can be life threatening resulting in pulmonary edema and adult respiratory distress syndrome, shock, or acute tubular necrosis.

Rocky Mountain Spotted Fever

Rocky Mountain spotted fever is the most severe and most frequently reported rickettsial disease in the United States. In the pre-antibiotic era, 20-25% of previously healthy, infected persons died of the illness. Today, even with antimicrobial agents that are highly effective, 3-5% of persons die mainly because of late or mis-diagnosed infection and delayed or ineffective antimicrobial treatment.

The disease is caused by *Rickettsia rickettsii*, a species of bacteria that are spread to humans by *Ixodes* ticks. The onset of disease follows an infective bite by a week (range 2-14 days), beginning with fever, severe headache, and muscle pain, followed by development of rash. The disease can be difficult to diagnose in the early stages, and without prompt and appropriate treatment it can be fatal.

The reasons are that up to 40% of patients are unaware of a tick bite, which is painless and may go unnoticed or be forgotten, and that the rash does not usually appear until 3-5 days after onset of illness. To further confound the diagnosis, symptoms such as nausea, vomiting, diarrhea, abdominal pain, and cough may suggest other diagnoses such as enterocolitis, acute surgical abdomen, or pneumonia. The rash typically appears on the ankles and wrists as faint pink 1-5 mm macules that represent a focus of vascular infection and surrounding vasodilation. These lesions may progress to become maculopapular, owing to the leakage of edema fluid from the affected blood vessels, with the development of a hemorrhage (petechia) in the center of the lesions.



Figure 2. Characteristic spotted rash of late-stage Rocky Mountain spotted fever on legs of a patient. (CDC)

Rocky Mountain spotted fever was first recognized in 1896 in the Snake River Valley of Idaho and was originally called "black measles" because of the characteristic rash. It was a dreaded and frequently fatal disease that affected hundreds of people in this area. By the early 1900s, the geographic distribution of the disease in United States was recognized as far north as Washington and Montana and as far south as California, Arizona, and New Mexico.

Howard Ricketts was the first to establish the identity of the infectious organism that causes Rocky Mountain spotted fever in . He and others characterized the basic epidemiologic features of the disease, including the role of tick vectors. Their studies found that Rocky Mountain spotted fever is caused by *Rickettsia rickettsii*, and involves a complex cycle between ticks and mammals. Humans are accidental hosts, but are not involved in the natural transmission cycle of the pathogen.

The name Rocky Mountain spotted fever is somewhat of a misnomer. Beginning in the 1930s, it became clear that this disease occurred in many areas of the United States other than the Rocky Mountain region. It is now recognized that this disease is broadly distributed throughout the continental United States, as well as southern Canada, Central America, Mexico, and parts of South America. Between 1981 and 1996, this disease was reported from every U.S. state except Hawaii, Vermont, Maine, and Alaska.

Rocky Mountain spotted fever remains a serious and potentially life-threatening infectious disease today. Despite the availability of effective treatment and advances in medical care, approximately 3- 5% of individuals who become ill with Rocky Mountain spotted fever die from the infection. However, effective antibiotic therapy has dramatically reduced the number of deaths caused by Rocky Mountain spotted fever. Before the discovery of tetracycline and chloramphenicol in the late 1940s, as many as 30% of individuals infected with *R. rickettsii* died.

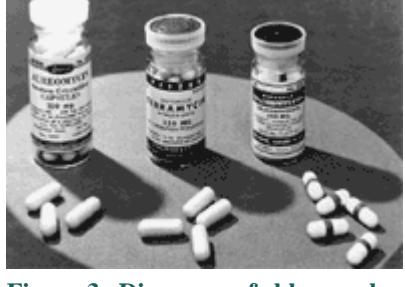


Figure 3. Discovery of chloramphenicol and tetracycline antibiotics in the 1940s led to a sharp decline in RMSF-related mortality. Today, doxycycline is the drug of choice for treatment of RMSF. (CDC)

Rickettsia rickettsii, is a very small bacterium that must live inside the cells of its hosts. Consequently, they are difficult to see in tissues by using routine histologic stains and generally require the use of special staining methods (Figure 4).

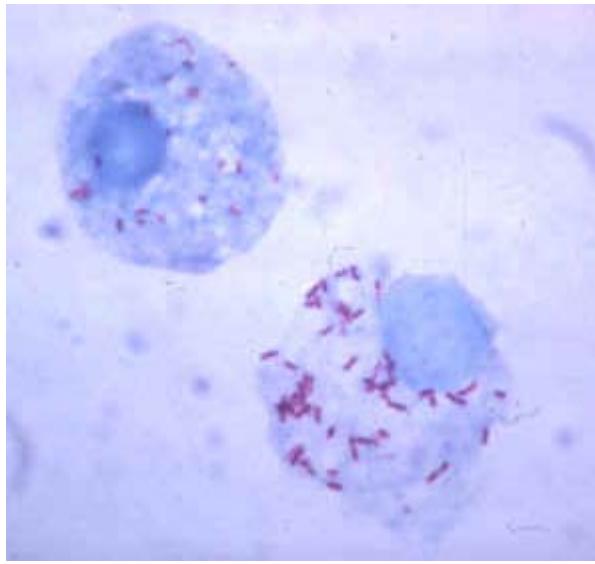


Figure 4. Gimenez stain of tick hemolymph cells infected with *R. rickettsii*. (CDC)

In humans, *Rickettsia rickettsii* live and multiply primarily within cells that line small- to medium-sized blood vessels. Spotted fever group rickettsiae can grow in the nucleus or in the cytoplasm of the host cell. Once inside the host the rickettsiae multiply, resulting in damage and death of these cells. This causes blood to leak through tiny holes in vessel walls into adjacent tissues. This process causes the rash that is traditionally associated with Rocky Mountain spotted fever and causes damage to organs and tissues.

Natural History

Rocky Mountain spotted fever, like all rickettsial infections, is classified as a zoonosis. **Zoonoses** are diseases of animals that can be transmitted to humans. Many zoonotic diseases require a vector (e.g., a mosquito, tick, or mite) in order to be transmitted from the animal host to the human host. In the case of Rocky Mountain spotted fever, ticks are the natural hosts, serving as both reservoirs and vectors of *R. rickettsii*. Ticks transmit the organism to vertebrates primarily by their bite. Less commonly, infections may occur following exposure to crushed tick tissues, fluids, or feces.

Only members of the tick family *Ixodidae* (hard ticks) are naturally infected with *Rickettsia rickettsii*. These ticks have four stages in their life cycle: egg, larva, nymph, and adult. After the eggs hatch, each stage must feed once to develop into the next stage. Both male and female ticks will bite.

A female tick can transmit *R. rickettsii* to her eggs in a process called transovarial transmission. Ticks can also become infected with *R. rickettsii* while feeding on blood from the host in either the larval or nymphal stage. After the tick develops into the next stage, *R. rickettsii* may be transmitted to the second host during the feeding process. Furthermore, male ticks may transfer *R. rickettsii* to female ticks through body fluids or spermatozoa during mating.. In this manner generations of each life stage of infected ticks are maintained. Once infected, the tick can carry the rickettsiae for life.

Rickettsiae are transmitted to a vertebrate host through saliva while a tick is feeding. It usually takes several hours of attachment and feeding before the rickettsiae are transmitted to the host. The risk of exposure to a tick carrying *R. rickettsii* is low. Generally, about 1 -3% of the tick population carries *R. rickettsii*, even in areas where the majority of human cases are reported.

Major Tick Vectors in the United States

There are two major vectors of *R. rickettsii* in the United States, the American dog tick and the Rocky Mountain wood tick.

The American dog tick (*Dermacentor variabilis*) is widely distributed east of the Rocky Mountains and also occurs in limited areas along the Pacific Coast. Dogs and medium-sized mammals are the preferred hosts of adult *D. variabilis*, although it feeds on other large mammals, including humans. This tick is the most commonly identified species responsible for transmitting *R. rickettsii* to humans.



Figure 5. American dog tick (*Dermacentor variabilis*). (CDC)

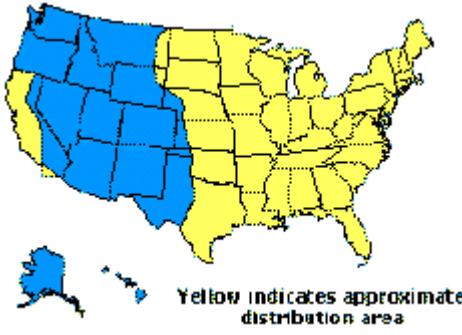


Figure 6. Approximate distribution of the American dog tick. (CDC)

The Rocky Mountain wood tick (*Dermacentor andersoni*) is found in the Rocky Mountain states and in southwestern Canada. The life cycle of this tick may require up to 2 to 3 years for completion. Adult ticks feed primarily on large mammals. Larvae and nymphs feed on small rodents.



Figure 7. Rocky Mountain wood tick (*Dermacentor andersoni*). (CDC)



Figure 8. Approximate distribution of the Rocky Mountain wood tick. (CDC)

Other tick species have been shown to be naturally infected with *R. rickettsii* but these species are likely to play only a minor role in the ecology of *R. rickettsii*.

Epidemiology

Rocky Mountain spotted fever has been a reportable disease in the United States since the 1920s. In the last 50 years, approximately 250-1200 cases of Rocky Mountain spotted fever have been reported annually, although it is likely that many more cases go unreported.

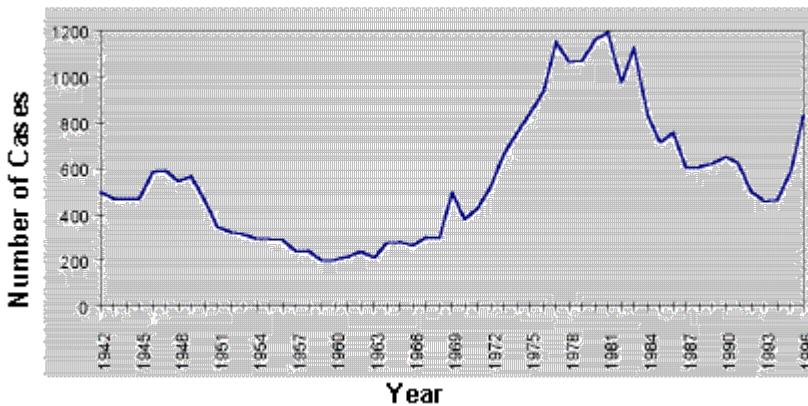


Figure 9. Reported cases of Rocky Mountain spotted fever in the United States, 1942-1996. CDC compiles the number of cases reported by the state health departments. (CDC)

Over 90% of patients with Rocky Mountain spotted fever are infected during April through September. This period is the season for increased numbers of adult and nymphal *Dermacentor* ticks. A history of tick bite or exposure to tick-infested habitats is reported in approximately 60% of all cases of Rocky Mountain spotted fever.

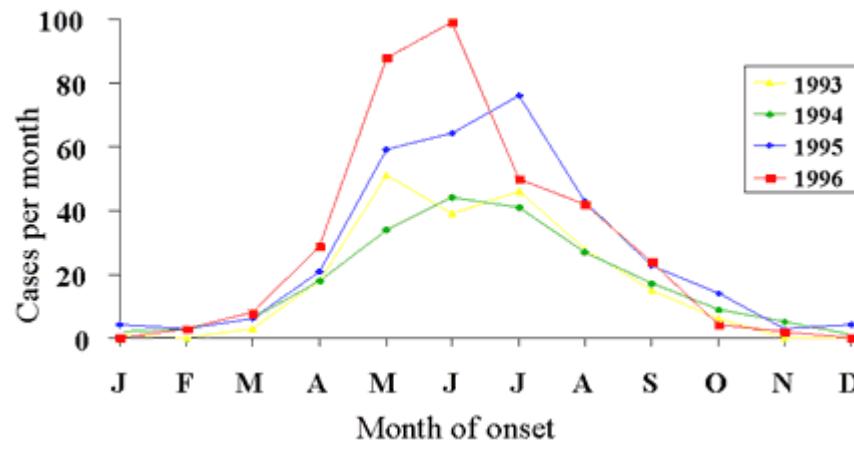


Figure 10. Seasonal distribution of reported cases of Rocky Mountain spotted fever, 1993-1996. (CDC)

Over half of Rocky Mountain spotted fever infections are reported from the south-Atlantic region of the United States (Delaware, Maryland, Washington D.C., Virginia, West Virginia, North Carolina, South Carolina, Georgia, and Florida). Infection also occurs in other parts of the United States, namely the Pacific region (Washington, Oregon, and California) and west south-central (Arkansas, Louisiana, Oklahoma, and Texas) region.

The states with the highest incidences of Rocky Mountain spotted fever are North Carolina and Oklahoma. These two states combined accounted for 35% of the total number of U.S. cases reported to CDC during 1993 through 1996. Although Rocky Mountain spotted fever was first identified in the Rocky Mountain states, actually less than 3% of the U.S. cases were reported from that area during the same interval (1993-1996).



Figure 11. Number of reported cases of Rocky Mountain spotted fever by state and region, 1994-1998. (CDC)

Certain individuals are at higher risk of disease. The frequency of reported cases of Rocky Mountain spotted fever is highest among males, Caucasians, and children. Two-thirds of the Rocky Mountain spotted fever cases occur in children under the age of 15 years, with the peak age being 5 to 9 years old (see Figure 12). Individuals with frequent exposure to dogs and who reside near wooded areas or areas with high grass may also be at increased risk of infection.

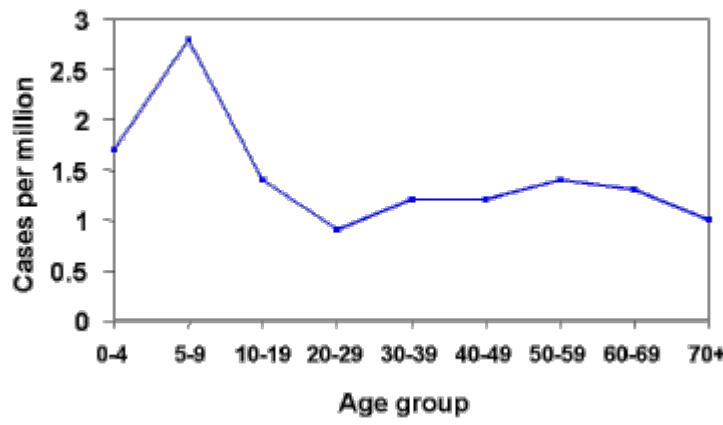


Figure 12. Average annual incidence of Rocky Mountain spotted fever by age group, 1993-1996. (CDC)

Signs and Symptoms

Rocky Mountain spotted fever can be very difficult to diagnose in its early stages, even among experienced physicians who are familiar with the disease. Patients infected with *R. rickettsii* generally visit a physician in the first week of their illness, following an incubation period of about 5-10 days after a tick bite. The early clinical presentation of Rocky Mountain spotted fever is nonspecific and may resemble a variety of other infectious and non-infectious diseases.

Initial symptoms may include fever, nausea, vomiting, severe headache, muscle pain, and lack of appetite. Later signs and symptoms include rash, abdominal pain, joint pain and diarrhea.

The classic triad of findings for this disease are fever, rash, and history of tick bite. However, this combination is often not identified when the patient initially presents for care. The rash first appears 2-5 days after the onset of fever and is often not present or may be very subtle when the patient is initially seen by a physician. Younger patients usually develop the rash earlier than older patients. Most often it begins as small, flat, pink, non-itchy spots (macules) on the wrists, forearms, and ankles (Figure 13). These spots turn pale when pressure is applied and eventually become raised on the skin. The characteristic red, spotted (petechial) rash of Rocky Mountain spotted fever is usually not seen until the sixth day or later after onset of symptoms, and this type of rash occurs in only 35% to 60% of patients with Rocky Mountain spotted fever (Figure 14). The rash involves the palms or soles in as many as 50% to 80% of patients; however, this distribution may not occur until later in the course of the disease.

As many as 10% to 15% of patients may never develop a rash.



Figure 13. Early (macular) rash on sole of foot. (CDC)



Figure 14. Late (petechial) rash on palm and forearm. (CDC)

Rocky Mountain spotted fever can be a very severe illness and patients often require hospitalization. Because *R. rickettsii* infects the cells lining blood vessels throughout the body, severe manifestations of this disease may involve the respiratory system, central nervous system, gastrointestinal system, or renal system. Host factors associated with severe or fatal Rocky Mountain spotted fever include advanced age, male sex, African-American race, chronic alcohol abuse, and glucose-6-phosphate dehydrogenase (G6PD) deficiency. Deficiency of G6PD is a sex-linked genetic condition affecting approximately 12% of the U.S. African-American male population; deficiency of this enzyme is associated with a high proportion of severe cases of Rocky Mountain spotted fever. This is a rare clinical course that is often fatal within 5 days of onset of illness.

Long-term health problems following acute Rocky Mountain spotted fever infection include partial paralysis of the lower extremities, gangrene requiring amputation of fingers, toes, or arms or legs, hearing loss, loss of bowel or bladder control, movement disorders, and language disorders. These complications are most frequent in persons recovering from severe, life-threatening disease, often following lengthy hospitalizations.

Laboratory Diagnosis

There is no widely available laboratory assay that provides rapid confirmation of early Rocky Mountain spotted fever. Treatment decisions must be based on epidemiologic and clinical clues, and should never be delayed while waiting for confirmation by laboratory results.

Serologic assays are the most widely available and frequently used methods for confirming cases of Rocky Mountain spotted fever. The indirect immunofluorescence assay (IFA) is generally considered the reference

standard in Rocky Mountain spotted fever serology and is the test currently used by CDC and most state public health laboratories (Figure 15).

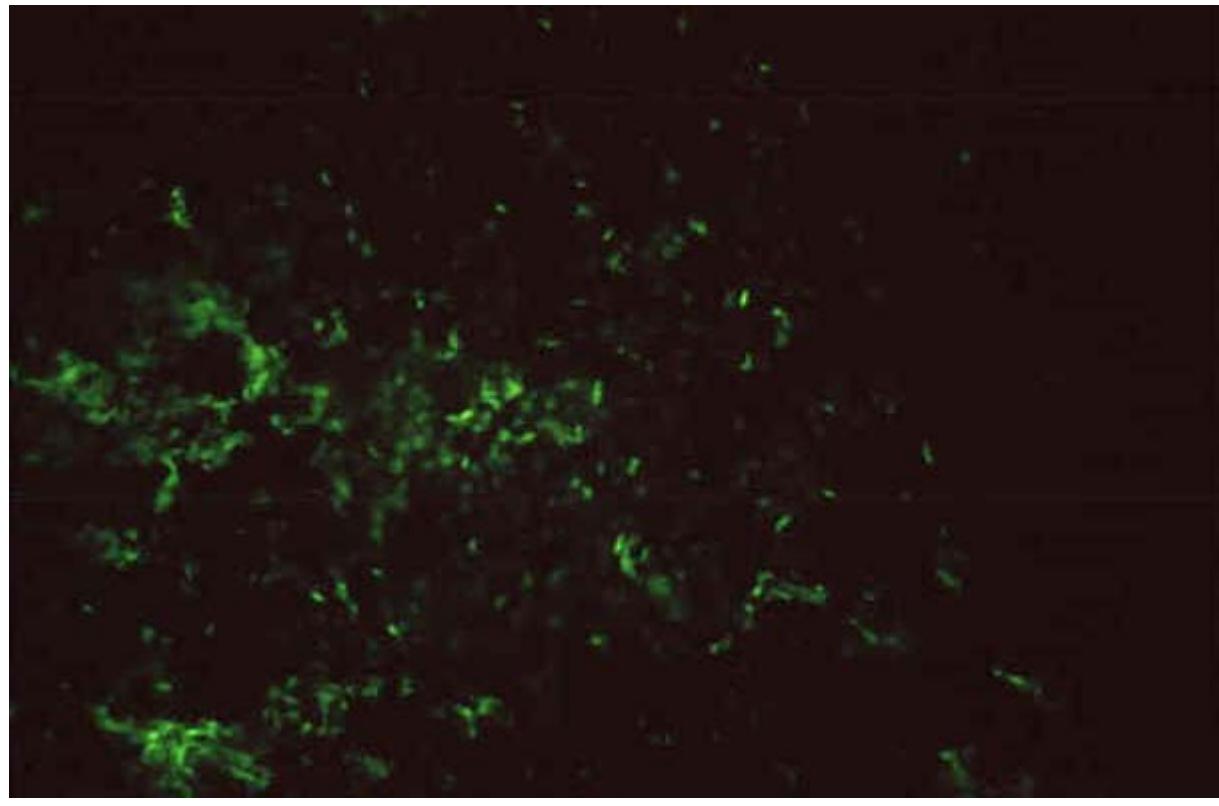


Figure 15. IFA reaction of a positive human serum on *Rickettsia rickettsii* grown in chicken yolk sacs, 400X. (CDC)

IFA can be used to detect either IgG or IgM antibodies. Blood samples taken early (acute) and late (convalescent) in the disease are the preferred specimens for evaluation. Most patients demonstrate increased IgM titers by the end of the first week of illness. Diagnostic levels of IgG antibody generally do not appear until 7-10 days after the onset of illness. It is important to consider the amount of time it takes for antibodies to appear when ordering laboratory tests, especially because most patients visit their physician relatively early in the course of the illness, before diagnostic antibody levels may be present. The value of testing two sequential serum or plasma samples together to show a rising antibody level is considerably more important in confirming acute infection with rickettsial agents because antibody titers may persist in some patients for years after the original exposure.

Another approach to Rocky Mountain spotted fever diagnostics is immunostaining. This method is used by taking a skin biopsy of the rash from an infected patient prior to therapy or within the first 48 hours after antibiotic therapy has been started. Because rickettsiae are focally distributed in lesions of Rocky Mountain spotted fever, this test may not always detect the agent. Even in laboratories with expertise in performing this test, the sensitivity is only about 70% on biopsied tissues. This assay may also be used to test tissues obtained at autopsy and has been used to confirm Rocky Mountain spotted fever in otherwise unexplained deaths (Figure 16). Immunostaining for spotted fever group rickettsiae is offered by the CDC, a few state health departments, and some university-based hospitals and commercial laboratories in the United States.

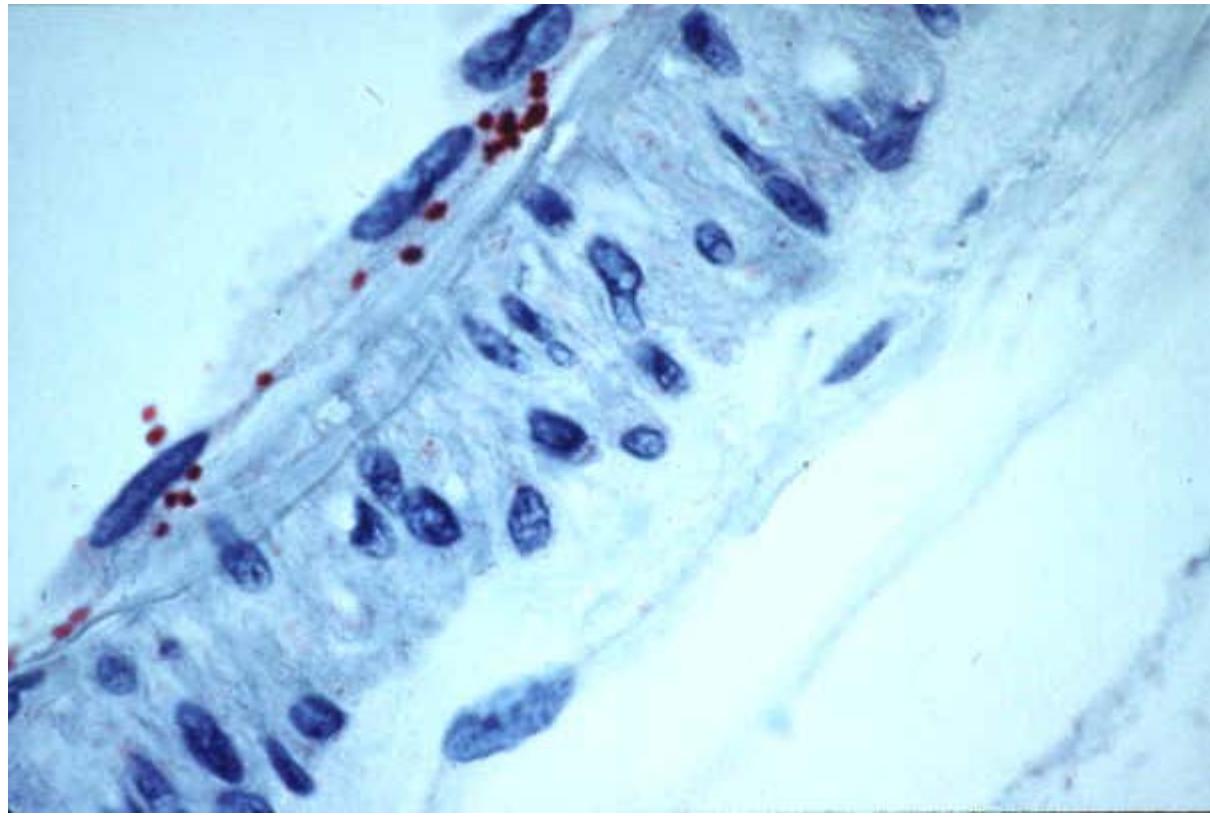


Figure 16. Red structures indicate immunohistological staining of *Rickettsia rickettsii* in endothelial cells of a blood vessel from a patient with fatal RMSF. (CDC)

Treatment

Appropriate antibiotic treatment should be initiated immediately when there is a suspicion of Rocky Mountain spotted fever on the basis of clinical and epidemiologic findings. Treatment should not be delayed until laboratory confirmation is obtained.

If the patient is treated within the first 4-5 days of the disease, fever generally subsides within 24-72 hours after treatment with an appropriate antibiotic (usually a tetracycline). In fact, failure to respond to a tetracycline antibiotic argues against a diagnosis of RMSF. Severely ill patients may require longer periods before their fever resolves, especially if they have experienced damage to multiple organ systems. Prophylactic therapy in non-ill patients who have had recent tick bites is not recommended and may, in fact, only delay the onset of disease.

Doxycycline (100 mg every 12 hours for adults or 4 mg/kg body weight per day in two divided doses for children under 45 kg [100 lbs]) is the drug of choice for patients with Rocky Mountain spotted fever. Therapy is continued for at least 3 days after fever subsides and until there is unequivocal evidence of clinical improvement, generally for a minimum total course of 5 to 10 days. Severe or complicated disease may require longer treatment courses. Doxycycline is also the preferred drug for patients with ehrlichiosis, another tick-transmitted infection with signs and symptoms that may resemble Rocky Mountain spotted fever.

Tetracyclines are usually not the preferred drug for use in pregnant women because of risks associated with malformation of teeth and bones in unborn children. Chloramphenicol is an alternative drug that can be used to treat Rocky Mountain spotted fever; however, this drug may be associated with a wide range of side effects including aplastic anemia, and may require careful monitoring of blood levels.

Prevention and Control

Limiting exposure to ticks is the most effective way to reduce the likelihood of Rocky Mountain spotted fever infection. In persons exposed to tick-infested habitats, prompt careful inspection and removal of crawling or attached ticks is an important method of preventing disease. It may take several hours of attachment before organisms are transmitted from the tick to the host.

Currently, no licensed vaccine is available for Rocky Mountain spotted fever.

It is unreasonable to assume that a person can completely eliminate activities that may result in tick exposure.

Therefore, prevention measures should be aimed at personal protection. CDC recommends the following prevention measures:

- Wear light-colored clothing to allow you to see ticks that are crawling on your clothing.
- Tuck your pants legs into your socks so that ticks cannot crawl up the inside of your pants legs.
- Apply repellants to discourage tick attachment. Repellents containing permethrin can be sprayed on boots and clothing, and will last for several days. Repellents containing DEET(diethyltoluamide) can be applied to the skin, but will last only a few hours before reapplication is necessary. Use DEET with caution on children. Application of large amounts of DEET on children has been associated with adverse reactions.
- Conduct a body check upon return from potentially tick-infested areas by searching your entire body for ticks. Use a hand-held or full-length mirror to view all parts of your body.
- Remove any tick you find on your body. Parents should check their children for ticks, especially in the hair, when returning from potentially tick-infested areas. Additionally, ticks may be carried into the household on clothing and pets. Both should be examined carefully.

Tick Control

Strategies to reduce populations of vector ticks through area-wide application of acaricides (chemicals that will kill ticks and mites) and control of tick habitats (e.g., leaf litter and brush) have been effective in small-scale trials. New methods being developed include applying acaricides to rodents by using baited tubes, boxes, and feeding stations in areas where these pathogens are endemic. Biological control with fungi, parasitic nematodes, and parasitic wasps may play alternate roles in integrated tick control efforts. Community-based, integrated, tick-management strategies may prove to be an effective public health response to reduce the incidence of tick-borne infections. However, limiting exposure to ticks is currently the most effective method of prevention.

Boutonneuse Fever and African Tick-bite Fever

Boutonneuse fever and its agent were first described in North Africa in 1910, and variants of *R. conorii* have been identified in South Africa, Kenya, Somalia, Israel, Morocco, Ethiopia, Russia, India and Pakistan. In parts of Africa, tick-transmitted diseases caused by *R. conorii* and *R. africae* overlap geographically. Although their clinical manifestations also overlap, there are differences sufficient to distinguish two different disease agents. Generally milder than boutonneuse fever, African tick bite fever has a lower incidence of rash, which is more often vesicular and sparse, a higher incidence of eschars that are frequently multiple, and more prominent regional lymphadenopathy. Each of these diseases has been diagnosed in the United States after patients return from vacation abroad, particularly from African safaris.

Rickettsialpox

R. akari has been recognized mainly in the urban United States as an agent maintained in a mite-mouse cycle with humans as an accidental host. The organism may, however, have a broader host range and geographic distribution.

A papule appears at the site of mite feeding in the skin during the incubation period, and over 2-7 days, evolves into an eschar. Later fever, chills, malaise, headache, and myalgia develop, followed after 2-6 days by a macular rash that becomes maculopapular and then vesicular before crusting and healing. Fatalities have not been reported.

Cat Flea Typhus

Despite the widespread geographic distribution and prevalence of *R. felis* in cat fleas, there have been only a handful of clinical investigations of undertaken to diagnose cat flea typhus.

Among eight reported cases of human infection with *R. felis* (five diagnosed by polymerase chain reaction [PCR] and three by differential antibody titers), all had fever and constitutional symptoms. The majority manifested rash, headache, and central nervous system (CNS) involvement, and variable proportions suffered nausea, vomiting, diarrhea, abdominal pain, myalgia and conjunctivitis. The actual spectrum of illness of this infection requires further clinical studies.

Typhus Fever

Rickettsia prowazekii infections occur in three situations: louse-transmitted epidemics, reactivation of a longstanding latent infection, and zoonotic infection transmitted from flying squirrels by their ectoparasites. Onset of disease is characterized by fever, chills, headache, and myalgia. Macules of 2-6 mm usually appear first on the trunk on day 5 and later spread to the extremities. Rales, conjunctival injection, and delirium are frequent manifestations. Reactivated typhus is a milder version with the same signs and symptoms. Flying squirrel-associated typhus has also been described as less severe; whether this is due to antimicrobial treatment or less virulent strains of rickettsiae is unclear.

Murine Typhus

Flea-borne *R. typhi* infections cause extreme discomfort but are seldom fatal healthy young individuals. The difficulty in detecting a rash in darkly pigmented skin was evident in a study finding only 20% of experimentally infected African-American volunteers had rashes, compared to 80% of Caucasian volunteers. The infection can follow a mild course in children with as many as half suffering only fever at night, but necessitates intensive care unit support in 10% of hospitalized adult patients. Pneumonitis or meningoencephalitis can be the major manifestation in some patients.

Treatment of Rickettsioses

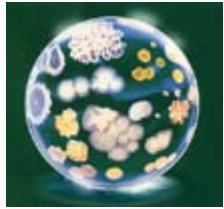
Doxycycline is the drug of choice for the treatment of infections caused by *Rickettsia* except in cases of pregnancy and tetracycline hypersensitivity. Some studies have shown that doxycycline is superior to chloramphenicol for the treatment of Rocky Mountain spotted fever as it is associated with a lower case fatality rate and a lower hospitalization rate. Several fluoroquinolones, azithromycin, and clarithromycin, have been used successfully to treat boutonneuse fever but are not recommended for more pathogenic rickettsioses. It should be emphasized that rickettsiae are highly resistant to most antibiotics. Most fatal cases of Rocky Mountain spotted fever have received substantial courses of antimicrobial treatment, including beta lactams, aminoglycosides, and erythromycin. Sulfonamide antimicrobials actually appear to exacerbate the severity of rickettsial infections.

Immunity

Rickettsial infection stimulates an early innate immune response with activation of natural killer cells and production of gamma interferon (gammaIFN), which act in concert to dampen rickettsial growth. Acquired immunity develops with clonal expansion of CD4 and CD8 T lymphocytes as well as antibody-producing B cells. Clearance of intraendothelial rickettsiae is achieved by rickettsicidal effects due to cytokine activation of the infected endothelial cells themselves. Cell mediated immunity (CMI) plays an important role as expected in infection by an intracellular parasite, but antibodies (including those directed at epitopes of OmpA and OmpB) also play a role in protective immunity.

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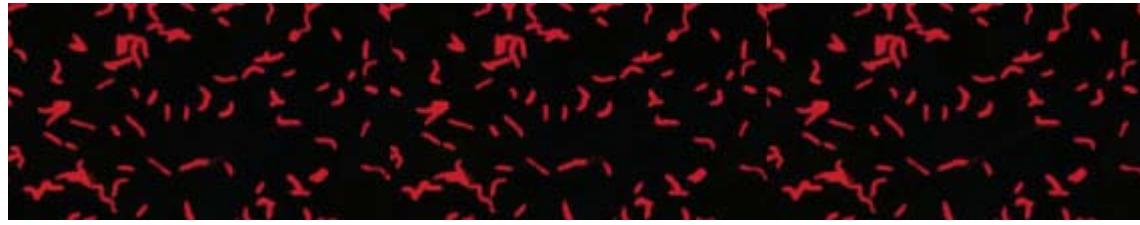
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Vibrio vulnificus

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Vibrio vulnificus is scarcely recognized by many microbiologists, much less by the public. Yet, in this country, the bacterium causes a disease with over a 50 percent mortality rate, and it causes 95 percent of all seafood-related deaths.

Vibrio vulnificus is a Gram-negative, motile curved bacterium found in marine and estuarine environments. It has been isolated from seawater, sediments, plankton and shellfish (oysters, clams and crabs) located in the Gulf of Mexico, the Atlantic Coast as far north as Cape Cod, and the entire U.S. West Coast. The bacterium thrives in warm seawater and is part of a group of vibrios that are "moderate halophiles", meaning they require salt for growth. The vibrios are frequently isolated from oysters and other shellfish in warm coastal waters during the summer months. This correlates with the peak incidence of disease caused by the bacterium.

Vibrio vulnificus is in the Bacterial family *Vibrionaceae*, the same as *Vibrio cholerae*, the agent of epidemic cholera in humans. Vibrios are one of the most common organisms in surface waters of the world. They occur in both marine and freshwater habitats and in associations with aquatic animals. Some species are bioluminescent and live in mutualistic associations with fish and other marine life. Other species are pathogenic for fish, eels, and frogs, as well as other vertebrates and invertebrates.

V. cholerae and *V. parahaemolyticus* are pathogens of humans. Both produce diarrhea, but in ways that are entirely different. *V. parahaemolyticus* is an invasive organism affecting primarily the colon; *V. cholerae* is noninvasive, affecting the small intestine through secretion of an enterotoxin.

Vibrio vulnificus is an emerging pathogen of humans. It causes wound infections, gastroenteritis, or a syndrome known as primary septicemia. It was first recognized as an agent of disease in 1976. The first documented case of disease caused by the bacterium was in 1979.

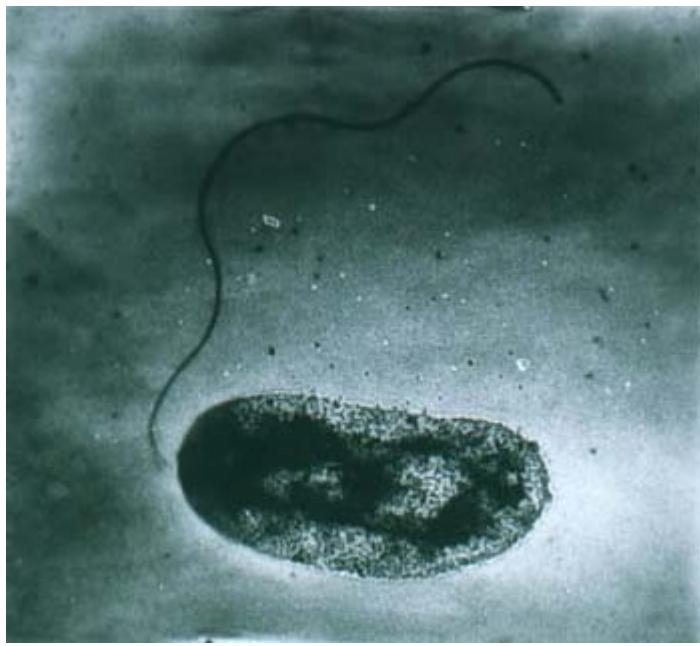


Figure 1. *Vibrio vulnificus* is a typical marine vibrio - a slightly curved bacterium, motile by means of a single polar flagellum.

Disease

V. vulnificus causes disease in individuals who eat contaminated seafood (usually raw or undercooked oysters) or have an open wound that is exposed to seawater. Among healthy people, ingestion of *V. vulnificus* can cause vomiting, diarrhea, and abdominal pain. Most *V. vulnificus* infections are acute and have no long-term consequences.

In immunocompromised persons, particularly those with chronic liver disease, *V. vulnificus* can invade the bloodstream from either a wound or from the GI tract, causing a severe and life-threatening illness called primary septicemia, characterized by fever, chills, septic shock and death. Blistering skin lesions accompany the disease in about 70% of the cases. *V. vulnificus* bloodstream infections are fatal about 50% of the time.

Although *V. vulnificus* is a rare cause of disease, it is likely that it is unrecognized and underreported (one estimate of the total number of cases annually in the U.S. is as high as 45,000). Between 1988 and 1995, CDC received reports of over 300 *V. vulnificus* infections from the Gulf Coast states, where the majority of cases occur.

Persons who are immunocompromised, especially those with chronic liver disease, are at risk for *V. vulnificus* when they eat raw seafood, particularly oysters. These individuals are 80-200 times more likely to develop *V. vulnificus* primary septicemia than are healthy people. For this particular risk group, the infection carries one of the highest mortality rates of all bacterial infections.

Health conditions that place a person at risk for serious illness or death from *V. vulnificus* infection include liver disease, hemochromatosis, diabetes, stomach problems, kidney disease, cancer, immune disorders (including HIV) and long-term steroid use. In these individuals, the bacterium enters the blood stream, resulting in septic shock, rapidly followed by death in many cases. These individuals are strongly advised not to consume raw or inadequately cooked seafood. Many of these health conditions may exist but be unrecognized in a person, so that they do not realize they are at risk of *V. vulnificus* disease.

Pathogenesis

Wound infections result from contaminating an existing open wound with seawater harboring the organism, or by cutting part of the body on coral, fish, fishhooks, etc., followed by contamination with the organism.

Also, people who consume foods contaminated with this organism are susceptible to gastroenteritis, which usually develops within 16 hours of eating the contaminated food. They experience vomiting, diarrhea, and abdominal pain. Many patients develop distinctive bullous skin lesions.

The bacterium invades directly from the GI tract or broken skin to produce bacteraemia and septicemia. Invasion is characterized by the occurrence of blister-like skin lesions or bullae, and rapidly-spreading necrosis resembling

necrotizing fasciitis.

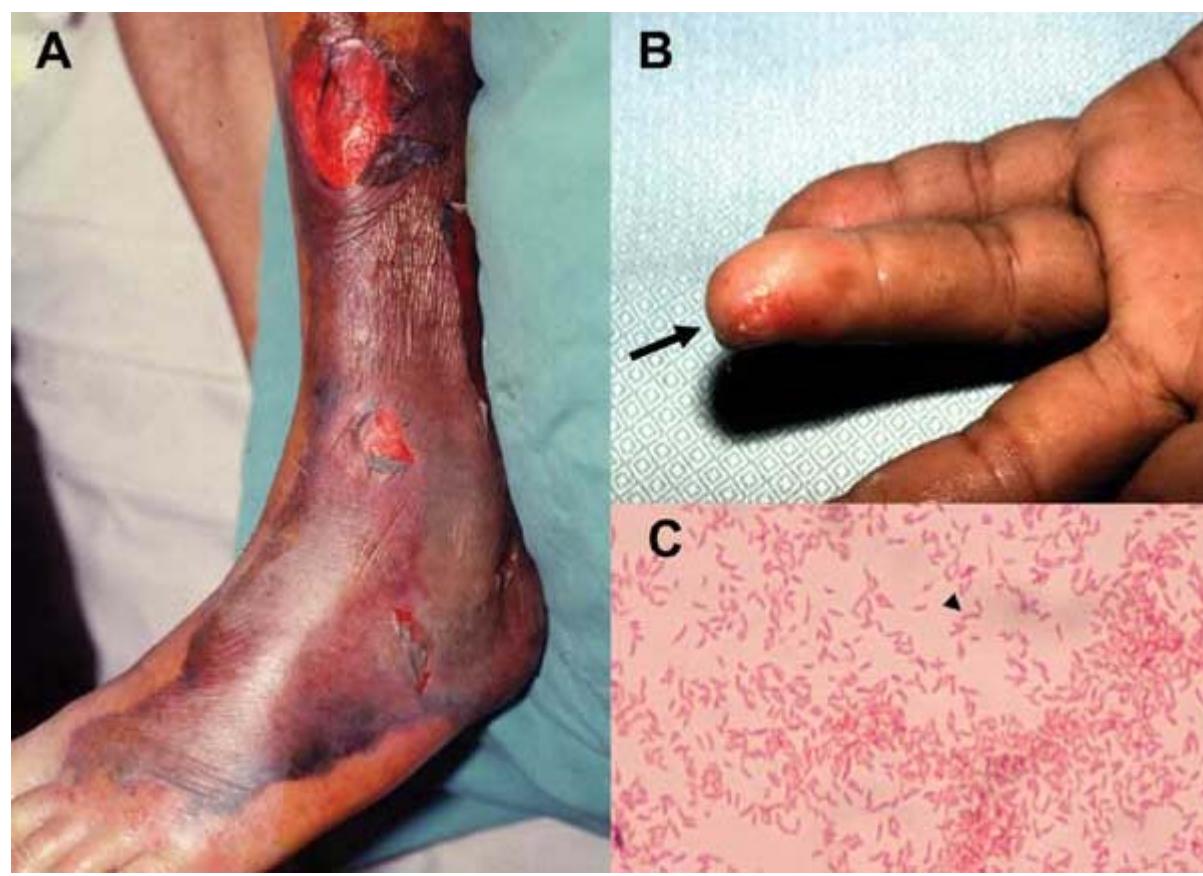


Figure 2. A. Characteristic skin lesions associated with *Vibrio vulnificus* infection on the leg in a 75-year-old patient with liver cirrhosis in whom septic shock and bacteremia developed. B. *V. vulnificus* bacteremia developed one day after a fish bone injury on the fourth finger of the left hand (arrow) in a 45-year-old patient with uremia. C. Gram-negative curved bacilli isolated from a blood sample of the 45-year-old patient with uremia. (Photos from Hsueh, et al. *Vibrio vulnificus* in Taiwan..CDC Emerging Infectious Diseases Volume 10, Number 8, August 2004)

Determinants of Virulence

Attempts to associate phenotypic or genotypic characteristics of *Vibrio vulnificus* with strain virulence have been largely unsuccessful. *V. vulnificus* exhibits considerable strain-to-strain variation in virulence. More than 100 strains of the bacterium have been identified, and it is possible that many thousands more exist. The bacterium also exhibits a large number of potential determinants of virulence, on the order of *V. cholerae* and *Pseudomonas aeruginosa* combined, but their role in disease has not been elucidated.

There are at least three ways that *Vibrio vulnificus* strains have been divided into two "biotypes", one of which is pathogenic for humans, and the other of which is found in shellfish or fish, or is free-living. One way is based on the difference in a 17-bp nucleotide sequence of the 16S rRNA gene. By this criterion two major groups of *V. vulnificus* have been identified, designated types A and B. The majority of nonclinical isolates are type A, and there is a positive correlation between the type B genotype and the cause of human disease. Similarly, a homogeneous LPS type is found in vibrios that live in associations with eels (biotype 2), and distinct heterogeneous LPS types are observed in clinical isolates (Biotype 1). And while the presence of a capsule occurs in virulent strains, noncapsulated strains are nonvirulent. The significance of these observations is not known and they do not explain how the bacteria are able to switch from free-swimming and colonizing oysters to colonizing human tissues.

Generalized Stress Response

Many of the heat shock proteins produced by *V. vulnificus*, such as the chaperonins DnaK and GroEL, and the proteases, Clp and Lon, are induced by environmental changes other than increased temperature, such as ethanol, heavy metals or oxidizing agents, high osmolarity, pollutants, starvation, exposure to low temperature, or interaction with eukaryotic hosts. This is thought to be a generalized stress response in the bacterium. Through a process termed cross protection, this response improves the bacterium's thermotolerance, salt tolerance, tolerance to

heavy metals and UV exposure, and starvation survival. The generalized stress response may be critical for bacterial adaptation to changes in the environment, and is a major link between bacterial ecology and bacterial pathogenesis.

Stress is also thought to cause genomic differences observed among strains of *V. vulnificus*. Genomic differences may be the result of gene rearrangements in the bacterium. Since the bacterium may exist in a rapidly changing ecosystem where major alterations in temperature, salt concentration, UV irradiation, and nutrient availability are routinely encountered, it is possible that such gene rearrangements may increase the chances of survival of the bacterium when it moves from water to oyster to human.

Capsule

Expression of a polysaccharide capsule is necessary for virulence of *Vibrio vulnificus*. The noncapsulated form is nonvirulent. Under laboratory conditions, acapsular variants arise at a fairly high frequency (~1/100), with certain environmental stresses dramatically increasing this switch rate. Once such noncapsulated (translucent) colonies arise, they do not appear able to revert back to the capsule-expressing (opaque) morphology. The mechanism of this capsule switching has not been explained.

Fimbriae

Type IV pili (fimbriae) are required for virulence. Type IV pili are N-methylphenylalanine pili, characteristic of vibrios, that allow the bacteria to adhere to epithelial cells. The receptor has not yet been identified. The N-methylphenylalanine pili of *Vibrio cholerae* utilize N-acetylneurameric acid (sialic acid) as a receptor.

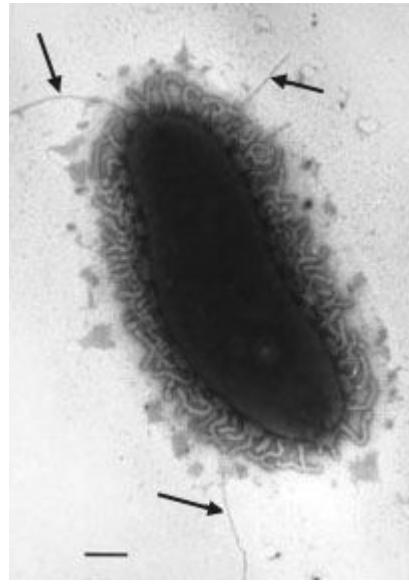


Figure 3. Electron micrograph of *Vibrio vulnificus*. The arrows mark fimbriae (pili) of the bacterium. The laboratory of Dr. Mark Strom at the [NOAA Northwest Fisheries Science Center](#) is studying how the adhesins of the bacterium, which include fimbriae and other cell surface components, influence the course of mammalian colonization and infection, as well as the organism's ability to colonize and persist in shellfish.

LPS

As a Gram negative bacterium, *V. vulnificus* lipopolysaccharide (endotoxin) is expected to play a role in fever and septic shock brought on by infection. On the basis of lipopolysaccharide (LPS) antigens, the species can be organized into three biotypes. Biotype 1 is the predominant human pathogen; biotype 2 is associated with eels; and biotype 3 was recently isolated from fish handlers in Israel. Biotype 2 consists of a homogeneous type of LPS, and although Biotype 1 was originally divided into 5 antigenic subgroups, other subgroups are known to exist. Biotype 1 is almost invariably associated with human disease, and one particular LPS type (1/5) is significantly more prevalent among clinical strains. This suggests that either the presence of this LPS type itself causes increased virulence, or that the LPS type is a marker of more virulent strains.

Besides attachment ability, capsule switching, LPS, and the ability to undergo the stress response, other properties of *V. vulnificus* that have been considered as determinants of virulence include production of alternate (stress) sigma factors, SSR repeats, motility, quorum sensing, production of a siderophore and a hemolysin (cytolysin), and numerous extracellular enzymes, including proteases, collagenase, mucinase, esterase, chondroitinase, hyaluronidase, DNAase and sulfatase.

A recently identified determinant of virulence in *Vibrio vulnificus* is the member of the RTX family of toxins produced by a limited group of Gram-negative pathogens. RTX toxins cause pore formation in red blood cells, necrotic death of Hep2 cells, and depolymerization of actin in HeLa cells.

For an excellent review of the virulence of *Vibrio vulnificus* see [Gulig. et al. Molecular Pathogenesis of Vibrio vulnificus \(2005\)](#).

Treatment

Antibiotics are necessary for treatment of *V. vulnificus* infections. Effective antibiotics include tetracycline, third-generation cephalosporins (e.g., ceftazidime), and imipenem. In case of wound infection, aggressive debridement is necessary to remove necrotic tissue.

For more information on *Vibrio vulnificus*

[CDC - General Information](#)

[CDC - Technical Information](#)

[FDA/CFSAN Bad Bug Book](#)

[FDA/CFSAN Prime Connection](#)

[Clemson University](#)

[Florida State University - Vibrio vulnificus](#)

[Florida State University - Toward Safer Oysters](#)

[NOAA Northwest Fisheries Science Center](#)

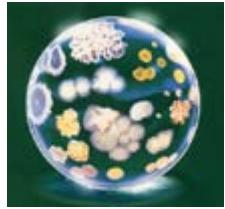
[Southern Medical Association Vibrio vulnificus Infection: case study](#)

[UNC Charlotte - Oliver Research Lab](#)

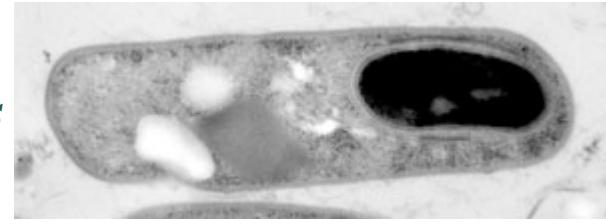
[Molecular Pathogenesis of Vibrio vulnificus](#)

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The Genus *Bacillus*

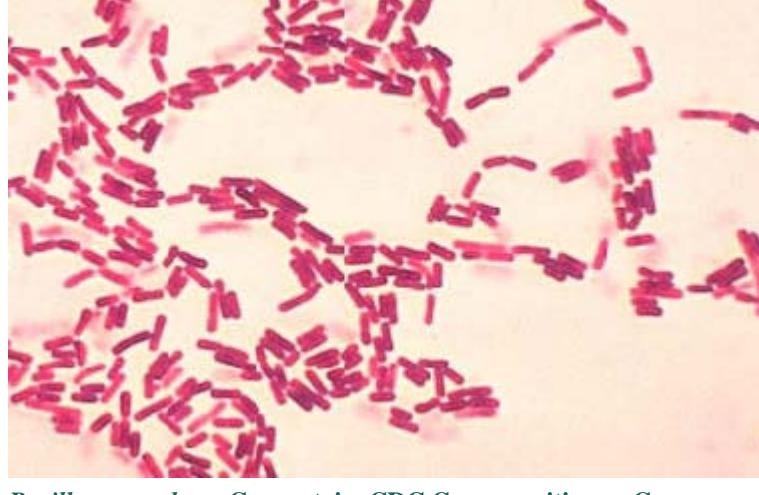
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Introduction

In 1872, Ferdinand Cohn, a student of Robert Koch, recognized and named the bacterium *Bacillus subtilis*. The organism was made to represent a large and diverse genus of Bacteria, *Bacillus*, and was placed in the family *Bacillaceae*. The family's distinguishing feature is the production of **endospores**, which are highly refractile resting structures formed within the bacterial cells. Since this time, members of the genus *Bacillus* are characterized as Gram-positive, rod-shaped, aerobic or facultative, endospore-forming bacteria.

The ubiquity of *Bacillus* species in nature, the unusual resistance of their endospores to chemical and physical agents, the developmental cycle of endospore formation, the production of antibiotics, the toxicity of their spores and protein crystals for many insects, and the pathogen *Bacillus anthracis*, have attracted ongoing interest in the genus since Koch's time.

There is great diversity in physiology among members of the genus, whose collective features include degradation of most all substrates derived from plant and animal sources, including cellulose, starch, pectin, proteins, agar, hydrocarbons, and others; antibiotic production; nitrification; denitrification; nitrogen fixation; facultative lithotrophy; autotrophy; acidophily; alkaliphily; psychrophily; thermophily; and parasitism. Spore formation, universally found in the genus, is thought to be a strategy for survival in the soil environment, wherein the bacteria predominate. Aerial distribution of the dormant spores probably explains the occurrence of *Bacillus* species in most habitats examined.



Bacillus coagulans. Gram stain. CDC. Gram-positive or Gram-negative? The cell wall structure of the bacilli is consistent with that of Gram-positive bacteria, and young cultures stain as expected. However, many sporeformers rapidly become Gram-negative when entering the stationary phase of growth.

Classification and Phylogeny

Early attempts at classification of *Bacillus* species were based on two characteristics: aerobic growth and endospore formation. This resulted in tethering together many bacteria possessing different kinds of physiology and occupying a variety of habitats. Hence, the heterogeneity in physiology, ecology, and genetics, made it difficult to categorize the genus *Bacillus* or to make generalizations about it.

In **Bergey's Manual of Systematic Bacteriology (1st ed. 1986)**, the G+C content of known species of *Bacillus* ranges from 32 to 69%. This observation, as well as DNA hybridization tests, revealed the genetic heterogeneity of the genus. Not only is there variation from species to species, but there are sometimes profound differences in G+C content within strains of a species. For example, the G+C content of the *B. megaterium* group ranges from 36 to 45%.

In **Bergey's Manual of Systematic Bacteriology (2nd ed. 2001)**, phylogenetic classification schemes landed the two most prominent types of endospore-forming bacteria, clostridia and bacilli, in two different "classes" of Gram-positives, "clostridia" and "bacilli". "Clostridia" includes the Order *Clostridiales* and Family *Clostridiaceae* with 11 genera including, *Clostridium*. "Bacilli" includes the Order *Bacillales* and the Family *Bacillaceae*. In this family there are several new genera on the level with *Bacillus*. This explains heterogeneity in G+C content observed in the 1986 "genus" *Bacillus*.

The phylogenetic approach to *Bacillus* taxonomy has been accomplished largely by analysis of 16S rRNA molecules by oligonucleotide sequencing. This technique, of course, also reveals phylogenetic relationships. Surprisingly, *Bacillus* species showed a kinship with certain nonsporeforming species, including *Planococcus*, *Lactobacillus* and *Staphylococcus*.

In one study, 16S rRNA cataloging showed that *B. subtilis* and other ellipsoidal-sporeforming species, *B. cereus*, *B. megaterium*, and *B. pumilus*, formed a coherent cluster, but the round-sporeforming species, *B. sphaericus*, *B. globisporus*, and *B. aminovorans*, did not cluster.

In another 16S rRNA sequencing study, three major *Bacillus* taxonomic cluster groups were defined by determining complete or partial sequences of 16S RNA (exceeding 1,100 nucleotides) on 35 recognized reference strains. These cluster groups were quite different from those previously noted.

For the purposes of differentiation and identification of *Bacillus* species, this article follows the nomenclature in **Bergey's Manual of Systematic Bacteriology (1st ed. 1986)**, which is intended for this purpose.

In **Bergey's Manual of Systematic Bacteriology (1986)** there are **six genera** of endospore-forming bacteria featured. *Bacillus* is distinguished from the other endospore-forming bacteria on the basis of being a strict or facultative aerobe, rod-shaped, and (usually) catalase-positive. Other endospore-forming genera in the are *Sporolactobacillus*, which is microaerophilic and catalase-negative; *Clostridium*, which is anaerobic and does not reduce sulfate; *Desulfotomaculum*, which is anaerobic but does reduce sulfate; *Sporosarcina*, which has a coccoid morphology; and *Thermoactinomycetes*, which while forming endospores, displays typical actinomycete characteristics. These genera are related phenotypically as Gram-positive bacteria that form endospores; they are not related phylogenetically.

In the 1986 edition of Bergey's, there are 40 recognized species in the genus *Bacillus*. The table at the end of this chapter lists the **34 type species** and some of their characteristics. Several valid species of *Bacillus* have been designated since 1986. Some of these are among the more than 200 species of *Bacillus* in the 1986 Bergey's that are in the category "Species Incertae Sedis"; other species are newly-discovered; still others have been moved to new or existing genera of endospore-forming bacteria based on phylogenetic comparison with true *Bacillus* species.

For the most current information on taxonomic flux within the genus *Bacillus* go to this link: [Bacterial Nomenclature up-to-date: the Genus *Bacillus*](#).

Nutrition and Growth

Most *Bacillus* species are versatile chemoheterotrophs capable of respiration using a variety of simple organic compounds (sugars, amino acids, organic acids). In some cases, they also ferment carbohydrates in a mixed reaction that typically produces glycerol and butanediol. A few species, such as *Bacillus megaterium*, require no organic growth factors; others may require amino acids, B-vitamins, or both. The majority are mesophiles, with temperature optima between 30 and 45 degrees, but the genus also contains a number of thermophilic species with

optima as high as 65 degrees. In the laboratory, under optimal conditions of growth, *Bacillus* species exhibit generation times of about 25 minutes.

Bacillus species are easily isolated and readily grown in the bacteriology laboratory. The simplest technique that enriches for aerobic spore formers is to pasteurize a diluted soil sample at 80 degrees for 15 minutes, then plate onto nutrient agar and incubate at 37 degrees for 24 hours up to several days. The plates are examined after 24 hours for typical *Bacillus* colonies identified as catalase-positive, Gram-positive, endospore-forming rods. Although many species contain sporangia and free spores within 24 hours, some cultures must be incubated 5-7 days before mature sporangia, and the size and shape of the endospore contained therein, can be observed. The insect pathogens, *B. larvae*, *B. popilliae* and *B. lentimorbus*, are more fastidious and must be isolated on J-agar (below). Furthermore, they are typically catalase-negative, and they require special media or inoculation into insect hosts for sporulation.



Mucoid-type colonies of an encapsulated *Bacillus* species. CDC.

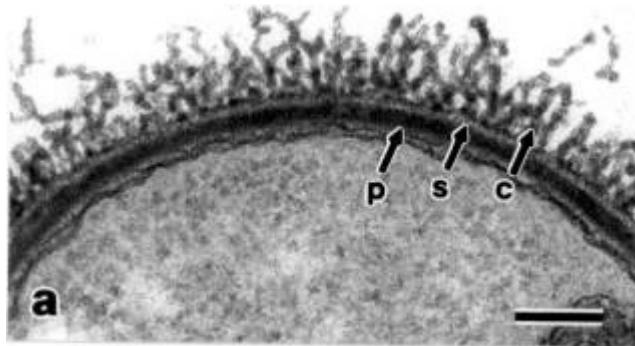
Most *Bacillus* species, except the insect pathogens, *B. larvae*, *B. popilliae* and *B. lentimorbus*, can be grown in defined or relatively-simple complex media. For a few bacilli (e.g. *B. subtilis*, *B. megaterium*), minimal media have been established. Primary isolations can be performed on either nutrient agar (peptone 5g/l, beef extract 3g/l, agar 15g/l, pH 6.8) or plates of J-agar (tryptone 5g/l, yeast extract 15g/l, K₂HPO₄ 3g/l, glucose 2g/l, agar 20g/l, pH 7.4). Stock cultures can be maintained in the laboratory on soil extract agar or on special sporulation media.

Table 1. Minimal medium for the growth of *Bacillus megaterium*.

Component	Amount
sucrose	10.0 g
K ₂ HPO ₄	2.5 g
KH ₂ PO ₄	2.5 g
(NH ₄) ₂ HPO ₄	1.0 g
MgSO ₄ 7H ₂ O	0.20 g
FeSO ₄ 7H ₂ O	0.01 g
MnSO ₄ 7H ₂ O	0.007 g
water	985 ml
pH 7.0	

Surface Structure of *Bacillus*

Like most Gram-positive bacteria the surface of the *Bacillus* is complex and is associated with their properties of adherence, resistance and tactical responses. The vegetative cell surface is a laminated structure that consists of a capsule, a proteinaceous surface layer (S-layer), several layers of peptidoglycan sheeting, and the proteins on the outer surface of the plasma membrane.



Surface of a *Bacillus*. Transmission E.M. C=Capsule; S=S-layer; P=Peptidoglycan. Pasteur Institute.

S-layers

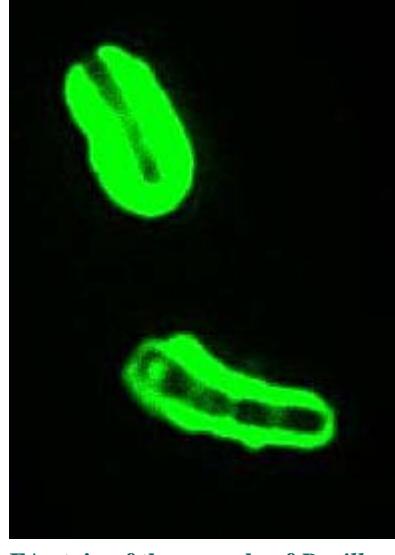
Crystalline surface layers of protein or glycoprotein subunits, called S-layers, are found in members of the genus *Bacillus*. As with S-layers of other bacteria, their function in *Bacillus* is unknown. However, it has been demonstrated that the S-layer can physically mask the negatively charged peptidoglycan sheet in *B. stearothermophilus* and prevent autoagglutination, and it has been proposed that the layer may play some role in bacteria-metal interactions.

Capsules

The capsules of many bacilli, including *B. anthracis*, *B. subtilis*, *B. megaterium*, and *B. licheniformis*, contain poly-D- or L-glutamic acid. Other *Bacillus* species, e.g., *B. circulans*, *B. megaterium*, *B. mycoides*, and *B. pumilus*, produce carbohydrate capsules. Dextran and levan are common, but more complex polysaccharides are produced, as well.

Some of the *Bacillus* polysaccharides cross react with antisera from other genera of bacteria including human pathogens. For example, *B. mycoides* with *Streptococcus pneumoniae* type III; *B. pumilus* with *Neisseria meningitidis* group A; *B. alveli* with *Haemophilus influenzae* type B.

When examined by transmission electron microscopy some polypeptide and complex polysaccharide capsules appear fibrillar in their arrangement on the cell surface. The capsules are easily observed by light microscopy, especially if the bacteria are prepared ahead of time by growth on media that enhance capsule production. Heavily encapsulated strains may form a mucoid or slimy colony on agar.



FA stain of the capsule of *Bacillus anthracis*. CDC



Negative stain (India Ink outline) of the capsule of *Bacillus anthracis*.CDC

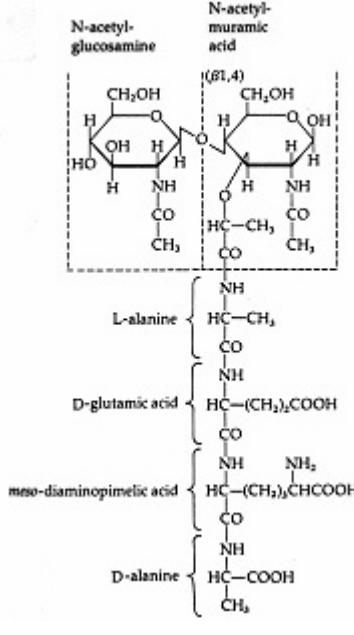
Bacillus megaterium synthesizes a capsule composed of both polypeptide and polysaccharide. The polypeptide is located laterally along the axis of the cell and the polysaccharide is located at the poles and at the equator of the cell.

The capsule of *B. anthracis* is composed of a poly-D-glutamic acid. The capsule is a major determinant of virulence in anthrax. The capsule is not synthesized by the closest relatives of *B. anthracis*, i.e., *B. cereus* and *B. thuringiensis*, and this criterion can be used to distinguish the species.

Cell Walls

The variability of cell wall structure that is common in most Gram-positive bacteria does not occur in the genus *Bacillus*. The vegetative cell wall of almost all *Bacillus* species is made up of a peptidoglycan containing meso-diaminopimelic acid (DAP). The exceptions are *B. sphaericus* and the related species, *B. pasteurii* and *B. globisporus*, that contain lysine, instead. This is the same type of cell wall polymer that is nearly universal in Gram-negative bacteria, i.e., containing DAP as the diamino acid. In some cases, DAP is directly cross-linked to D-alanine, same as in the *Enterobacteriaceae*; in other cases, two tetrapeptide side chains of peptidoglycan are spanned by an interpeptide bridge between DAP and D-alanine, which is characteristic of most Gram-positive bacteria.

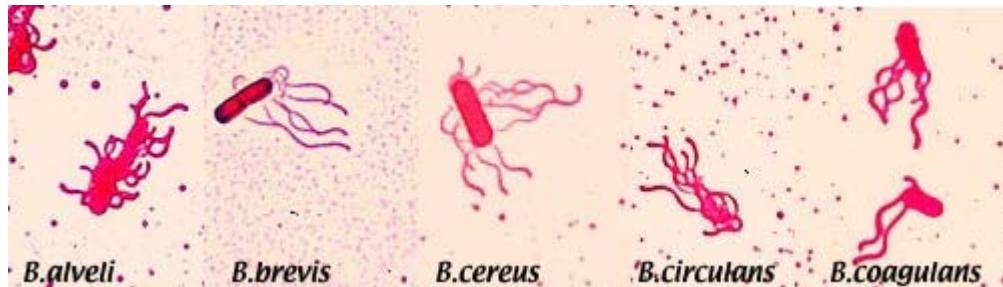
In addition to peptidoglycan in the cell wall, all *Bacillus* species contain large amounts of teichoic acids which are bonded to muramic acid residues. The types of glycerol teichoic acids vary greatly between *Bacillus* species and within species. As in many other Gram-positive bacteria, lipoteichoic acids are found associated with the cell membranes of *Bacillus* species. These compounds are thought to be involved in the synthesis of wall teichoic acids, as regulators of autolytic activity, and as scavengers of bivalent ions for the bacterium.



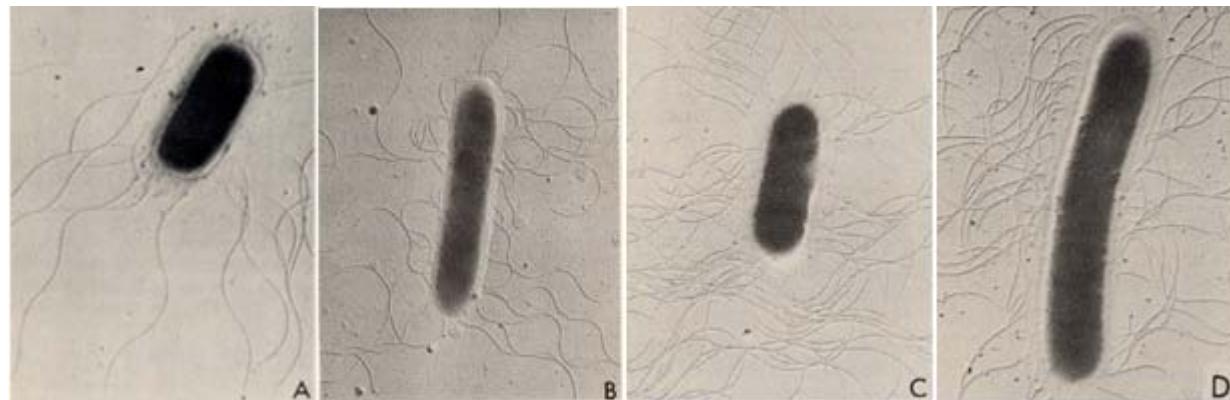
Structure of the muropeptide subunit of the peptidoglycan of *Bacillus megaterium*. In most *Bacillus* species, an interpeptide bridge that connects D-alanine to meso-diaminopimelic acid (DAP) is absent. The peptidoglycan of *B. sphaericus* and *B. pasteurii* contains L-lysine in place of DAP, but all *Bacillus* spores contain this type of muramic acid subunit in the spore cortex.

Flagella

Most *Bacillus* species are motile by means of peritrichous flagella. Chemotaxis has been studied extensively in *B. subtilis*. The flagellar filament of *B. firmus*, an alkaliphile, has a remarkably low content of basic amino acids, thought to render it more stable in environmental pH values up to 11.



Flagellar stains (Leifson's Method) of various species of *Bacillus* from CDC

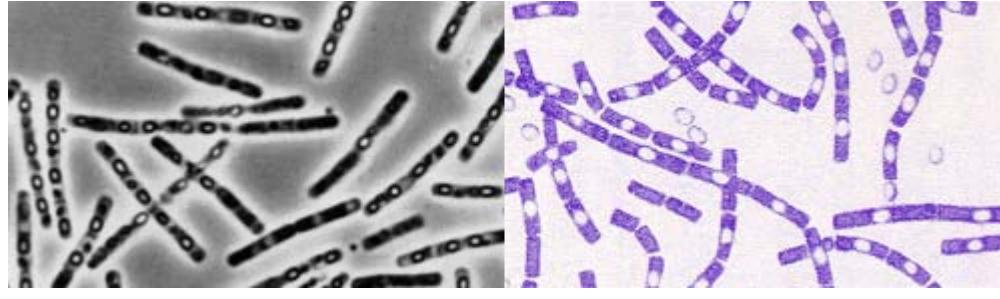


Individual cells of motile *Bacillus* species photographed on nutrient agar. About 15,000X magnification. U.S. Dept. of Agriculture. A. *B. subtilis*; B. *B. polymyxa*; C. *B. laterosporus*; D. *B. alveli*.

Endospores

Endospores were first described by Cohn in *Bacillus subtilis* and later by Koch in the pathogen, *Bacillus anthracis*. Cohn demonstrated the heat resistance of endospores in *B. subtilis*, and Koch described the developmental cycle of spore formation in *B. anthracis*. Endospores are so named because they are formed intracellularly, although they are eventually released from this mother cell or sporangium as free spores. Endospores have proven to be the most durable type of cell found in Nature, and in their cryptobiotic state of dormancy, they can remain viable for extremely long periods of time, perhaps millions of years.

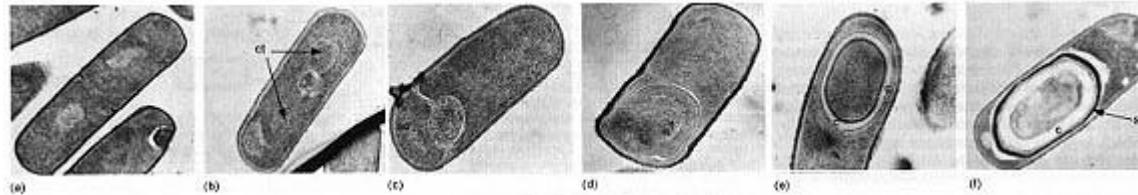
When viewed unstained, endospores of living bacilli appear edged in black and are very bright and refractile. Endospores strongly resist application of simple stains or dyes and hence appear as nonstaining entities in Gram-stain preparations. However, once stained, endospores are quite resistant to decolorization. This is the basis of several spore stains such as the Schaeffer-Fulton staining method which also differentiates the spores from sporangia and vegetative cells.



Left. *Bacillus thuringiensis* phase micrograph. Endospores can be readily recognized microscopically by their intracellular site of formation and their extreme refractility. Right. *Bacillus anthracis* Crystal violet stain viewed by light microscopy. Endospores are highly resistant to application of basic analine dyes that readily stain vegetative cells. Below. Spore stain of a *Bacillus* species. CDC. The staining technique employed is the Schaeffer-Fulton method. A fixed smear is flooded with a solution of malachite green and placed over boiling water for 5 minutes. After rinsing, the smear is counterstained with safranine. Mature spores stain green, whether free or still in the vegetative sporangium; vegetative cells and sporangia stain red.



Endospores do not form normally during active growth and cell division. Rather, their differentiation begins when a population of vegetative cells passes out of the exponential phase of growth, usually as a result of nutrient depletion. Typically one endospore is formed per vegetative cell. The mature spore is liberated by lysis of the mother cell (sporangium) in which it was formed.



The formation of endospores is a complex and highly-regulated form of development in a relatively simple (procaryotic) cell. In all *Bacillus* species studied, the process of spore formation is similar, and can be divided into seven defined stages (0-VI). The vegetative cell (a) begins spore development when the DNA coils along the central axis of the cell as an "axial filament" (b). The DNA then separates and one chromosome becomes enclosed in plasma membrane to form a protoplast (c). The protoplast is then engulfed by the mother cell membrane to form an intermediate structure called a forespore (d). Between the two membranes, the core (cell) wall, cortex and spore coats are synthesized (e). As water is removed from the spore and as it matures, it becomes increasingly heat resistant and more refractile (f). The mature spore is eventually liberated by lysis of the mother cell. The entire process takes place over a period of 6-7 hours and requires the temporal regulation of more than 50 unique genes. Pasteur Institute.

Mature spores have no detectable metabolism, a state that is described as **cryptobiotic**. They are highly resistant to environmental stresses such as high temperature (some endospores can be boiled for several hours and retain their viability), irradiation, strong acids, disinfectants, etc. Although cryptobiotic, they retain viability indefinitely such that under appropriate environmental conditions, they germinate 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.

Below. Drawing of a cross-section of a *Bacillus* endospore by Viake Haas, University of Wisconsin. In cross section, *Bacillus* spores show a more complex ultrastructure than that seen in vegetative cells. The spore protoplast (core) is surrounded by the core (cell) wall, the cortex, and then the spore coat. Depending on the species, an exosporium may be present. The core wall is composed of the same type of peptidoglycan as the vegetative cell wall. The cortex is composed of a unique peptidoglycan that bears three repeat subunits, always contains DAP, and has very little cross-linking between tetrapeptide chains. The outer spore coat represents 30-60 percent of the dry weight of the spore. The spore coat proteins have an unusually high content of cysteine and of hydrophobic amino acids, and are highly resistant to treatments that solubilize most proteins.

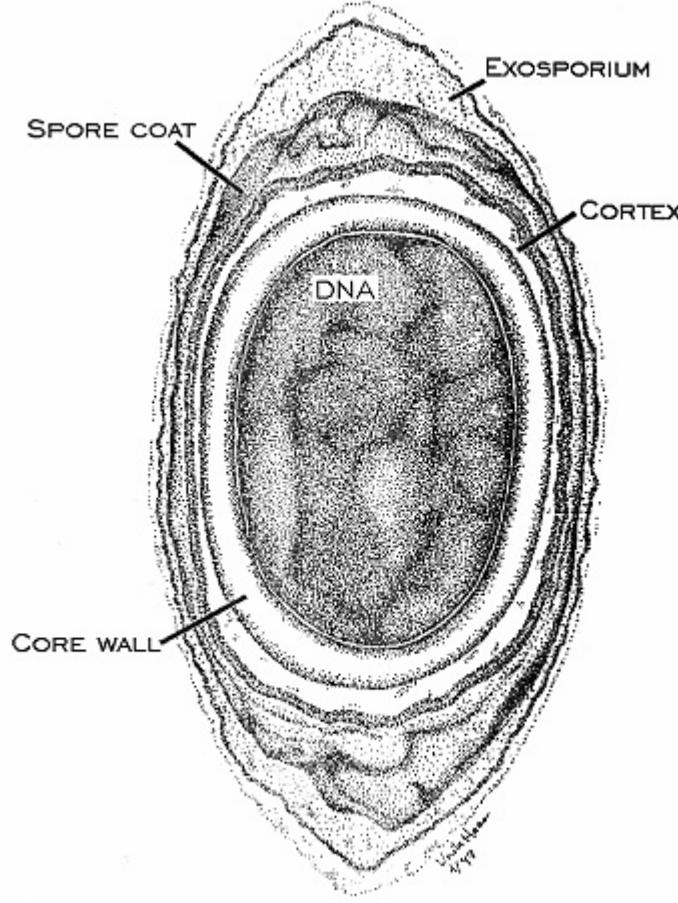


Table 2. Differences between endospores and vegetative cells in *Bacillus* species

Property	Vegetative cells	Endospores
Surface coats	Typical Gram-positive murein cell wall polymer; crystalline S-layer	Thick spore coat, cortex, and unique peptidoglycan core wall; no S-layer
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	Some sensitive; some resistant	Resistant
Sensitivity to dyes and staining	Sensitive	Resistant

Genetics of *Bacillus*

The discovery of **transformation** in a strain of *Bacillus subtilis* in 1958 focused attention on the genetics of the bacterium. This is one of relatively few bacteria in which competence for DNA uptake has been found to occur as a natural part of the bacterium's life cycle. Subsequently, **generalized** and **specialized transduction** was observed in *B. subtilis*, and knowledge of the genetics and chromosomal organization of the bacterium quickly mounted to become second only to that of the enteric bacteria. Furthermore, the identification of numerous genes affecting sporulation in *B. subtilis* has provided a means for analyzing the complex developmental program of sporulation.

Bacteriophages capable of mediating generalized transduction have also been reported in other species of *Bacillus*,

including *B. cereus*, *B. megaterium*, *B. thuringiensis*, *B. anthracis*, and *B. stearothermophilus*.

Conjugative plasmids (fertility plasmids capable of bringing about their own transfer from one bacterium to another) have been described in several species of *Bacillus*. The capacity to produce the insecticidal delta toxin crystal protein in *B. thuringiensis* is encoded in large plasmids. These plasmids can be transferred to plasmid-deficient strains of *B. thuringiensis*, as well as to *B. cereus*, to yield recipients that produce crystal protein. *B. thuringiensis* transfers the pXO11 and pXO12 plasmids to *B. anthracis* and to *B. cereus*. The recipients, in turn, become effective donors, and in the case of those inheriting pXO12, also acquire the ability to produce parasporal crystals. Strains of *B. anthracis* that acquire plasmid pXO12 can subsequently mobilize and transfer nonconjugative plasmids present in the same cell. The *B. anthracis* toxin plasmid pXO1, and the capsule plasmid pXO2 can be transmitted to *B. anthracis* and *B. cereus* recipients lacking these plasmids.

The large *B. anthracis* plasmids are apparently transferred by a process called **conduction**. This involves formation of cointegrative molecules in the donor, and resolution of the cointegrates into pXO12 and the respective *B. anthracis* plasmid in the recipient. Cell-to-cell contact is necessary for plasmid transfer and is resistant to DNase, but little is known about the mechanisms or conjugative structures that may be involved. None of the conjugative plasmids have been found to mobilize and transfer chromosomal markers as is observed with the F plasmid of *E. coli*.

In addition to the naturally occurring transmissible plasmids of *Bacillus*, a **conjugative transposon** (Tn925) has been identified, which transfers from *Enterococcus faecalis* to *B. subtilis*.

Our understanding of the *Bacillus* genome, and their means of DNA transfer, has led to its manipulation. So far, this has resulted in numerous medical, agricultural and industrial achievements, involving the use of the organism or its products.



This e.m. image of a spore-forming *Bacillus* (also at the top of this page) is that of *B. megaterium* which has been cloned with the Bt gene and is expressing Bt in the form of the bipyramidal "parasporal" crystal adjacent to the spore (from faculty.washington.edu/jclaral/410/Micro410Exams.html). Bt is an insecticidal protein produced by *Bacillus thuringiensis*.

Ecology

Due to the resistance of their endospores to environmental stress, as well as their long-term survival under adverse conditions, most aerobic sporeformers are ubiquitous and can be isolated from a wide variety of sources. Hence, the occurrence of sporeforming bacteria in a certain environment is not necessarily an indication of habitat. However, it is generally accepted that the primary habitat of the aerobic endospore-forming bacilli is the soil. The great Russian microbiologist Winogradsky considered them as "normal flora" of the soil.

In the soil environment the bacteria become metabolically-active when suitable substrates for their growth are available, and presumably they form spores when their nutrients become exhausted. This is a strategy used by other microbes in the soil habitat, including the filamentous fungi and the actinomycetes, which also predominate in the aerobic soil habitat. It is probably not a coincidence, rather an example of convergent evolution, that these three dissimilar groups of microbes live in the soil, form resting structures (spores), and produce antibiotics in association with their sporulation process.

Since most *Bacillus* species can effectively degrade a series of biopolymers (proteins, starch, pectin, etc.), they are assumed to play a significant role in the biological cycles of carbon and nitrogen.

From soil, by direct contact or air-borne dust, *Bacillus* spores can contaminate just about anything that is not maintained in a sterile environment. They may play a biodegradative role in whatever they contaminate, and thereby they may be agents of unwanted decomposition and decay. Several *Bacillus* species are especially important as food spoilage organisms.

Ecophysiological groups

Generally, neither morphologic nor phylogenetic criteria adequately distinguish the members of the genus *Bacillus* for discussion or positive identification. An artificial, but convenient, way to organize the members of the genus for this purpose is to place them into **ecophysiological groups**, such as nitrogen-fixers, denitrifiers, insect pathogens, animal pathogens, thermophiles, antibiotic producers, and so on. Such an approach also allows some speculation concerning the natural history and ecology of this important group of bacteria.

Acidophiles: include *B. acidocaldarius*, *B. coagulans*, and *B. polymyxa*.

Alkaliphiles: *B. alkalophilus* and *B. pasteurii*. The optimum pH is 8 and some strains grow at pH 11.

Halophiles: *B. pantothenicus*, *B. pasteurii*. Some strains grow in 10 % NaCl.

Psychrophiles or psychrotrophs: *B. globisporus*, *B. insolitus*, *B. marinus*, *B. macquariensis*, *B. megaterium*, *B. polymyxa*. Two species will grow and form spores at 0° C.

Thermophiles: include *B. acidocaldarius*, *B. schlegelii*, and *B. stearothermophilus*. Acidophiles and Lithoautotrophs are found in this group, too. The upper temperature limit is 65° C.

Denitrifiers: include *B. azotoformans*, *B. cereus*, *B. laterosporus*, *B. licheniformis*, *B. pasteurii*, *B. stearothermophilus* (over half the type species reduce NO₃ to NO₂). Although *Bacillus* species are common in agricultural soils, and they are attributed to participate in wasteful denitrification (conversion of the farmer's paid-for NO₃ fertilizers to volatile NH₃, N₂O or N₂) their exact role in the economy of this process has not been clarified.

Nitrogen-fixers: *B. macerans* and *B. polymyxa*. *B. macerans* is a fairly prominent bacterium in soil and in decaying vegetable material. The bacteria only fix nitrogen under anaerobic conditions because they do not have a mechanism for protection of their nitrogenase enzyme from the damaging effects of O₂. In the same way as the role of the bacilli in denitrification and nitrification, their overall contribution to non symbiotic global nitrogen fixation is not known.

Antibiotic Producers: *B. brevis* (e.g. gramicidin, tyrothricin), *B. cereus* (e.g. cerexin, zwittermicin), *B. circulans* (e.g. circulin), *B. laterosporus* (e.g. laterosporin), *B. licheniformis* (e.g. bacitracin), *B. polymyxa* (e.g. polymyxin, colistin), *B. pumilus* (e.g. pumulin) *B. subtilis* (e.g. polymyxin, difficidin, subtilin, mycobacillin).

Bacillus antibiotics share a full range of antimicrobial activity: bacitracin, pumulin, laterosporin, gramicidin and tyrocidin are effective against Gram-positive bacteria; colistin and polymyxin are anti-Gram-negative; difficidin is broad spectrum; and mycobacillin and zwittermicin are anti-fungal.

As in the case of the actinomycetes, antibiotic production in the bacilli is accompanied by cessation of vegetative growth and spore formation. This has led to the idea that the ecological role of antibiotics may not rest with competition between species, but with the regulation of sporulation and/or the maintenance of dormancy.

Pathogens of Insects: *B. larvae*, *B. lentimorbis*, and *B. popilliae* are invasive pathogens. *B. thuringiensis* forms a parasporal crystal that is toxic to *Lepidoptera*.

B. larvae, *B. lentimorbis* and *B. popilliae* are a related cluster of *Bacillus* species, being insect pathogens, with swollen sporangia, and typically catalase-negative. They also are unable to grow in nutrient broth, probably because it is insufficient in thiamin, which they need as a growth factor. Yeast extract (15g/l) must be added to their media for growth. Also, *B. lentimorbis* and *B. popilliae* are quite similar in their biochemical properties, virulence and host range. They sometimes occur in coinfections.

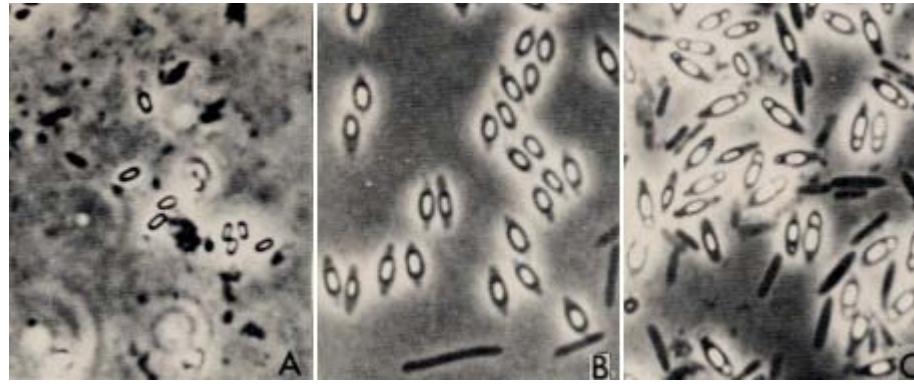
B. larvae is the causative agent of **American foulbrood** of honeybees, which is the most widespread and persistent of the honeybee brood diseases. The organism can be isolated repeatedly from infected brood and honeycomb, usually in a pure culture. It has been noted on many occasions that the natural habitat of the bacterium is remarkably free of contaminants. Presumably, the bacterium can be isolated from soil around the hives of infected bees, but it has not been isolated from other sources. This is indicative of a very close and specific type of host-

parasite interaction between the bacterium and the honeybee.

B. popilliae is the cause of the most widespread of two **milky diseases** of the Japanese beetle, *Popillia japonica*. Their spores, in a swollen sporangium, are frequently accompanied by a parasporal crystal. Interestingly, the bacterium sporulates with ease in the hemolymph of the infected insect, but will not form mature spores in most artificial media. Special media have been designed that induce *B. popilliae* and *B. lentimorbus* to form mature spores. The prospect that *B. popilliae*, together with *B. lentimorbus*, might be used to control or eliminate the Japanese beetle and the European chafer (*Amphimallon majalis*) has drawn attention to these bacteria. *B. popilliae* is encountered in naturally-infected grubs far more frequently than *B. lentimorbus*, which also causes milky disease.

B. lentimorbus is similar in most ways to *B. popilliae*. The most obvious difference is that *B. lentimorbus* does not form a parasporal body. The bacteria also differ morphologically and culturally. *B. lentimorbus* likewise causes one of two milky diseases in the Japanese beetle. The bacterium can only be isolated from the hemolymph of scarabaeid beetles, although it most certainly exists in soil inhabited with infected larvae.

The principal interest in *B. lentimorbus* arises from its ability to cause disease of Japanese beetle and European chafer larvae, which together cause millions of dollars in damage each year to a variety of plants. *B. lentimorbus* is more widespread than *B. popilliae*, which also causes milky disease in the same hosts. The reason the infections are called "milky disease" is that as the disease develops, the larvae become milky in appearance. This is caused by the prolific production of spores in the hemolymph.



Spores of the the insect pathogens seen by phase microscopy. U.S. Dept. of Agriculture. A. *B. larvae* spores from a comb infected with American foulbrood; B. *B. lentimorbus* spores from hemolymph of infected Japanese beetle larvae; C. Spores of *B. popilliae* from hemolymph of infected Japanese beetle larvae.

Bacillus thuringiensis is a variety of *B. cereus* and is therefore considered in the *B. cereus-B. anthracis-B. thuringiensis* group. *B thuringiensis* is distinguished from *B. cereus* or *B. anthracis* by its pathogenicity for lepidopteran insects and by production of an intracellular parasporal crystal in association with spore formation. The bacteria and protein crystals are sold as "Bt" insecticide, which is used for the biological control of certain garden and crop pests.

Pathogens of Animals: *B. anthracis*, and *B. cereus*. *B. alvei*, *B. megaterium*, *B. coagulans*, *B. laterosporus*, *B. subtilis*, *B. sphaericus*, *B. circulans*, *B. brevis*, *B. licheniformis*, *B. macerans*, *B. pumilus*, and *B. thuringiensis* have been isolated from human infections.

The Genus *Bacillus* includes two bacteria of significant medical importance, *B. anthracis*, the causative agent of anthrax, and *B. cereus*, which causes food poisoning. Nonanthrax *Bacillus* species can also cause a wide variety of other infections, and they are being recognized with increasing frequency as pathogens in humans.

Anthrax

Anthrax is primarily a disease of domesticated and wild animals, particularly herbivorous animals, such as cattle, sheep, horses, mules, and goats. Humans become infected incidentally when brought into contact with diseased animals, which includes their flesh, bones, hides, hair and excrement. In the United States, the incidence of naturally-acquired anthrax is extremely rare (1-2 cases of cutaneous disease per year). Worldwide, the incidence is unknown, although *B. anthracis* is present in most of the world's soils.

The most common form of the disease in humans is **cutaneous anthrax**, which is usually acquired via injured skin

or mucous membranes. A minor scratch or abrasion, usually on an exposed area of the face or neck or arms, is inoculated by spores from the soil or a contaminated animal or carcass. The spores germinate, vegetative cells multiply, and a characteristic gelatinous edema develops at the site. This develops into papule within 12-36 hours after infection. The papule changes rapidly to a vesicle, then a pustule (malignant pustule), and finally into a necrotic ulcer from which infection may disseminate, giving rise to septicemia. Lymphatic swelling also occurs within seven days. In severe cases, where the blood stream is eventually invaded, the disease is frequently fatal.

Another form of the disease, **inhalation anthrax** (woolsorters' disease), results most commonly from inhalation of spore-containing dust where animal hair or hides are being handled. The disease begins abruptly with high fever and chest pain. It progresses rapidly to a systemic hemorrhagic pathology and is often fatal if treatment cannot stop the invasive aspect of the infection.

Gastrointestinal anthrax is analogous to cutaneous anthrax but occurs on the intestinal mucosa. As in cutaneous anthrax, the organisms probably invade the mucosa through a preexisting lesion. The bacteria spread from the mucosal lesion to the lymphatic system. Intestinal anthrax results from the ingestion of poorly cooked meat from infected animals. Gastrointestinal anthrax is rare, but may occur as explosive outbreaks associated with ingestion of infected animals.

The pathology of anthrax is mediated by two primary determinants of bacterial virulence: presence of an antiphagocytic capsule, which promotes bacterial invasion, and production of a powerful lethal toxin, the anthrax toxin.

For more information on anthrax, including use and detection of *Bacillus anthracis* as an agent of bioterrorism, please see the chapter on [*Bacillus anthracis* and Anthrax](#).



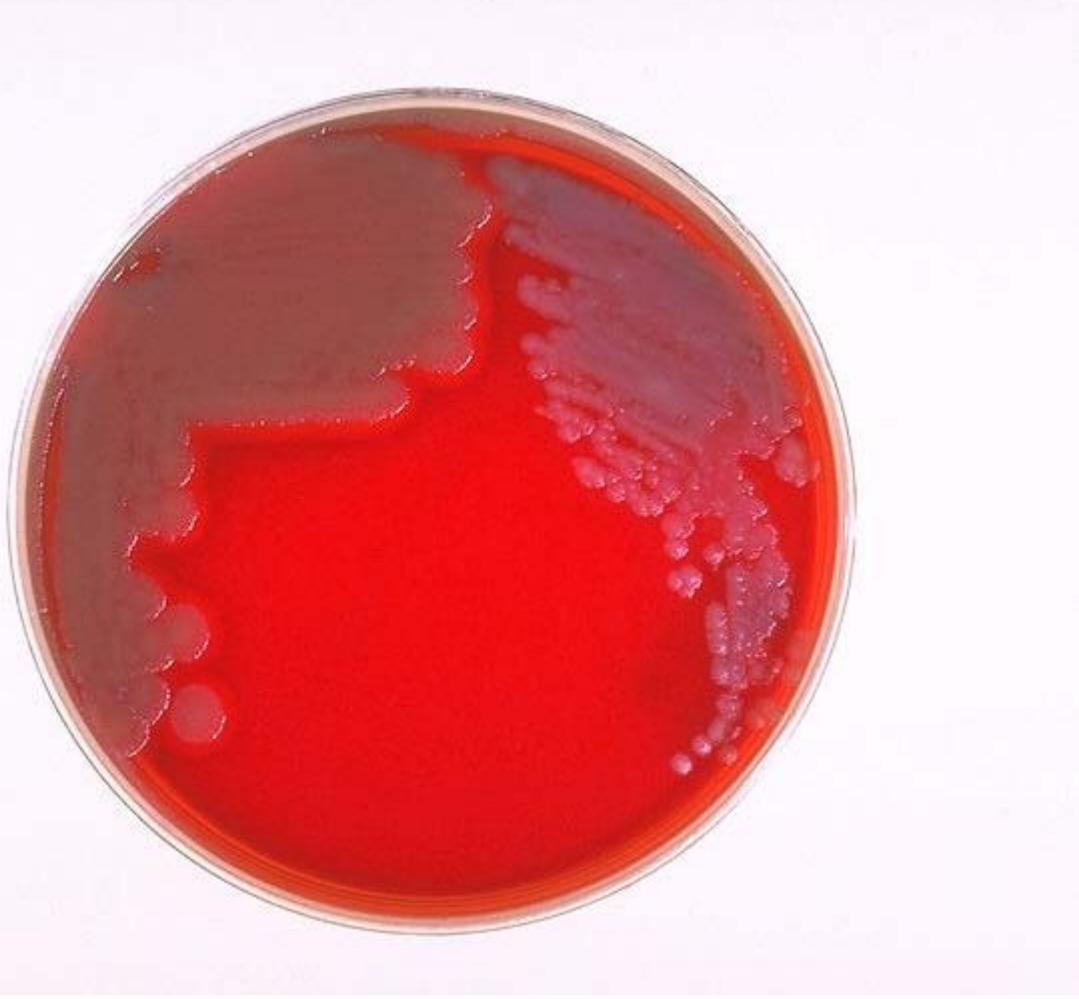
Bacillus anthracis Gram stain. CDC.

Bacillus cereus food poisoning

Bacillus cereus causes two types of **food-borne intoxications**. One type is characterized by nausea and vomiting and abdominal cramps and has an incubation period of 1 to 6 hours. It resembles *Staphylococcus aureus* food poisoning in its symptoms and incubation period. This is the "short-incubation" or **emetic form** of the disease. The second type is manifested primarily by abdominal cramps and diarrhea with an incubation period of 8 to 16 hours. Diarrhea may be a small volume or profuse and watery. This type is referred to as the "long-incubation" or **diarrheal form** of the disease, and it resembles more food poisoning caused by *Clostridium perfringens*. In either type, the illness usually lasts less than 24 hours after onset.

The short-incubation form of disease is caused by a preformed heat-stable enterotoxin. The mechanism and site of action of this toxin are unknown. The long-incubation form of illness is mediated by a heat-labile enterotoxin which activates intestinal adenylate cyclase and causes intestinal fluid secretion.

This bacterium is dealt with separately in the medical section of the text at [*Bacillus cereus* and Food Poisoning](#).



Colonies of *Bacillus anthracis* (right) and *Bacillus cereus* (left) on a plate of blood agar. CDC.

Characteristics of Type Species of the Genus *Bacillus*

B. acidocaldarius Thermoacidophile. Limits of temperature for growth are 45° and 65° C degrees. Limits of pH for growth are 2 and 6. Found in hot acidic environments. Spores have surprisingly weak thermal resistance.

B. alkalophilus Alkaliphile. Tolerant to alkaline conditions and does not grow at pH 7. Capable of growth at pH >10.

B. alvei Isolated from soil and from honeybee larvae suffering from European foulbrood disease. Not classified as an insect pathogen.

B. anthracis The causative agent of anthrax in humans and in animals. Spores persist for long periods on contaminated materials.

B. azotoformans. Has a negative Gram reaction. Can respire anaerobically using NO₃, NO₂, SO₄ or fumarate as a final electron acceptor. A vigorous denitrifying bacterium in soils, it converts NO₃, NO₂ and N₂O to large amounts of N₂.

B. badius Forms a distinct colony with rhizoid outgrowths. Has been isolated from feces, dust, marine sources,

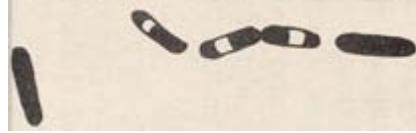
foods and antacids.



B. brevis Has been isolated chiefly from soils and foods. Requires a mixture of amino acids without vitamins for growth.

B. cereus A close relative of *B. anthracis*, *B. mycoides* and *B. thuringiensis*. Spores are widespread in soil and air. Usually observed multiplying in foods such as cooked rice and may lead to food poisoning. Produces antibiotics.

B. circulans Some strains are cellulolytic. Has a distinct rhizoid colony.



B. coagulans Includes acidophilic strains. Spores are relatively sparse in soils. May multiply in acid foods such as canned tomato juice and silage. Found in medicated creams and antacids

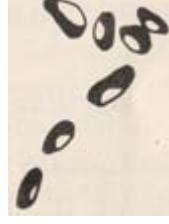
B. fastidiosus Uses only uric acid, allantoic acid or allantoin as an energy source. Isolated from soil and from poultry litter.

B. firmus Isolated chiefly from soil. Pigmented strains occur in salt marshes.

B. globisporus Forms spherical spores and is related to *B. sphaericus*. Found in soil and river water.

B. insolitus Growth and sporulation occur at 0 degrees. Vegetative cells are short and stout. Found in Arctic soils.

B. larvae Causes American foulbrood in honeybees.



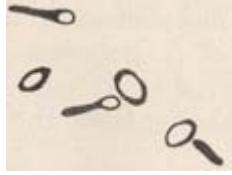
B. laterosporus Produces a canoe-like body attached to the side of spore forcing the spore into a lateral position in the sporangium. Rarely isolated, but has been found in dead honeybee larvae, soil, water and antacids.

B. lenticimorbus More fastidious nutritionally and more widespread than *B. popilliae*, it also infects the Japanese beetle and the European chafer. Isolated from diseased larvae or infected honeycombs.

B. lenthus Similar to *B. firmus*, but more nutritionally-versatile. Isolated from soil, food and spices.



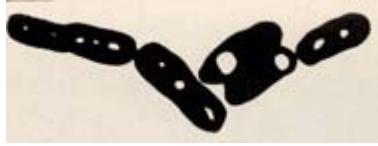
B. licheniformis Produces same type of poly-D-glutamate capsule as *B. anthracis*. Red pigment produced by many strains. Spores occur in soil. Growth in foods, especially if held between 30 and 50 degrees. Industrial source of bacitracin, a medically useful antibiotic.



B. macerans Most strains fix N₂ under anaerobic conditions. Degrades pectin and plant polysaccharides. Some strains moderately thermophilic. Also has been found in canned fruit at pH 3.8.

B. macquariensis Grows and sporulates at 0 degrees. Otherwise similar to *B. circulans*.

B. marinus Grows at 5-30 degrees but not at 37 degrees. Has an obligate requirement for Na⁺. Isolated routinely from marine sediments.

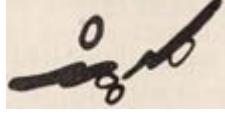


B. megaterium "megaterium" means "big beast". The largest cell diameter of any aerobic spore former (1.2 - 1.5 micrometers). Grows in minimal medium without any added growth factors. Spores are common in soil. Subject of many basic studies of Gram-positive bacteria in the laboratory.

B. mycoides Similar to *B. cereus* but non motile, and forms distinctive rhizoid colonies. High degree of relatedness with *B. anthracis*, *B. cereus* and *B. thuringiensis*.

B. pantothenticus Has a growth factor requirement for pantothenic acid, apparently unique to the genus *Bacillus*. Has been isolated from soils and antacids.

B. pasteurii Converts urea to ammonium carbonate more actively than any known bacterium. Requires alkaline medium (pH 9) for growth. Isolated from soil, water, sewage and encrustations on urinals.



B. polymyxa Colonies are mucoid, slimy and tend to spread. Synthesizes profuse levan capsule from sucrose. Spores have longitudinal surface ridges so are star-shaped in cross-section. Degrades pectin and plant polysaccharides. Nitrogen fixed under anaerobic conditions. Spores are widespread. Multiplication occurs chiefly in decaying vegetation. Often isolated from foods. Found in medicated creams and antacids. Source of the antibiotic

polymyxin. A very versatile and widespread *Bacillus*.

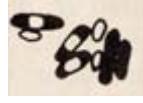
B. popilliae Pathogen of scarabeid beetles that causes (one variety of) milky disease in the Japanese beetle. Together with *B. lentinoribus*, it is a biological agent for the Japanese beetle and the European chafer. The larvae become milky white because of the prolific production of spores in the insect hemolymph. Forms a distinctive parasporal crystal that distinguishes it from *B. lentinoribus*. Isolated from hemolymph of Japanese beetle grubs.

B. pumilus Spores are ubiquitous; occurs in soil more frequently than those of *B. subtilis*.

B. schlegelii Thermophile, similar to *B. acidocaldarius* and *B. sphaericus* in its high G+C content, but differentiated from the latter because it is a facultative lithoautotroph. The bacterium can derive energy from the oxidation of H₂ or CO while obtaining carbon from either CO₂ or CO. Isolated from lake sediments and sugar factory sludge.

B. sphaericus Isolated from soil, marine and fresh water sediments, milk and foods.

B. stearothermophilus Grows at 65° C and has tolerance to acid. Occurs in soil, hot springs, desert sand, Arctic waters, ocean sediments, food and compost.

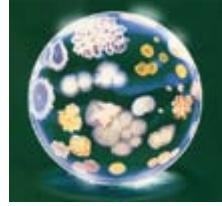


B. subtilis Grows as a unicellular rod, seldom as chains. Degrades pectin and polysaccharides in plant tissues, and some strains cause rots in live potato tubers. Grows in a minimal defined medium with no added growth factors. Endospores are widespread. Vegetative organisms take part in various stages in the early breakdown of materials of plant and animal origin. Grows in non acid food under aerobic conditions. Causative agent of ropy (slimy) bread. This bacterium is the "E. coli" of Gram-positive bacteria. Much of the information we have on the biology, biochemistry and genetics of the Gram-positive cell, indeed, of bacteria in general, has been derived from the study of *B. subtilis*.

B. thuringiensis Distinguished from *B. cereus* by pathogenicity for lepidopteran insects, and production of a parasporal crystal in association with spore formation. In the larval gut, the protein (crystal) is toxic. The spores and crystals are marketed in garden centers as BT, for biological control of lepidopterans that attack garden and crop plants.

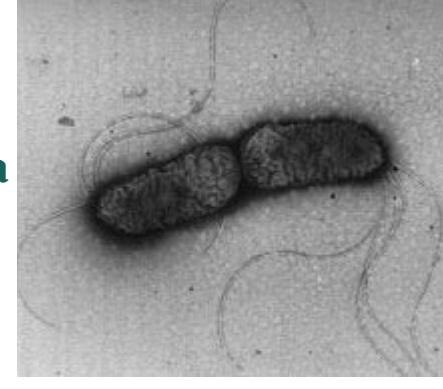
Bt is encoded on a plasmid which can be spontaneously transferred to *B. cereus*, endowing it with the ability to produce the toxic crystal. Some taxonomists have argued that this is evidence of such a close genetic relationship between the two bacteria that *B. thuringiensis* should be considered a variant subspecies of *B. cereus*. The same argument has been made for the relationship between *B. anthracis*, the plasmid-encoded anthrax toxin, and *B. cereus*.

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Pseudomonas and Related Bacteria



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Pseudomonas and Pseudomonas-like Bacteria

The biological identity of the genus **Pseudomonas** has changed dramatically in recent years during the transition between artificial classification based on phenotypic properties (e.g. Bergey's Manual of Systematic Bacteriology, 1st ed., 1986) and revisionist classification based on genotypic (phylogenetic) properties (e.g. Bergey's Manual of Systematic Bacteriology, 2nd ed., 2001). However, in either scheme, the genus comprises a relatively large and important group of Gram-negative bacteria. Members of the genus are found abundantly as free-living organisms in soils, fresh water and marine environments, and in many other natural habitats. They may also be found in associations with plants and animals as normal flora or as agents of disease. For the purposes of this article, the term "**pseudomonad**" refers to a bacterium with ecophysiological properties similar to members of the genus **Pseudomonas**. Some of these bacteria were formerly in the genus **Pseudomonas** but have been moved to other genera, families, or orders among the **alpha Proteobacteria** because of their phylogenetic distance from *Pseudomonas*. This article will use both old and new taxonomy to identify the **pseudomonads**, until this period of confusion has passed.

Morphologically, members of the genus *Pseudomonas* (as well as most other pseudomonads) may be described as Gram-negative, non-spore forming, straight or slightly curved rods. They are typically motile by means of one or more polar flagella. These basic morphological characteristics, however, are common to many families of bacteria and so are of little value in the positive identification or diagnosis of a member of the genus *Pseudomonas*.

Generally, common to all constituent species of the genus *Pseudomonas* are certain physiological properties such as chemoorganotrophic nutrition, aerobic metabolism, absence of fermentation, absence of photosynthesis, inability to fix nitrogen, and capacity for growth at the expense of a large variety of organic substrates.

There are, of course, a few exceptions to these standardized criteria of definition or identification:

-The traditional description of a short rod-shape body does not always fit the cell morphology of all *Pseudomonas* species. In some of them, the cells can be extremely short, while in others (e.g. certain strains of *Pseudomonas putida*, and *P. syringae*) they may be very long. Occasionally, particularly in old cultures, the cells can be of such unusual shapes and sizes that a casual microscopic observation may cast doubt on the purity of the population.

-Some defining physiological characteristics have not been critically tested in all members of the family, and as a consequence, occasional reports of exceptional strains have been noted. Thus, nitrogen fixation has been shown to occur in *Pseudomonas stutzeri*, and *Pseudomonas aeruginosa* is capable of anaerobic respiration utilizing NO_3^- as a final electron acceptor (denitrification), and it can grow anaerobically, albeit slowly, with arginine and small amounts of yeast extract.

Pseudomonads are important in the balance of nature and also in the economy of human affairs. Pseudomonads are globally active in aerobic decomposition and biodegradation, and hence, they play a key role in the carbon cycle. *Pseudomonas* species are renowned for their abilities to degrade compounds which are highly refractory to other

organisms, including aliphatic and aromatic hydrocarbons, fatty acids, insecticides and other environmental pollutants. Apparently, the only organic compounds that these pseudomonads can't attack are teflon, styrofoam and one-carbon organic compounds (methane, methanol, formaldehyde, etc.). Pseudomonads are also a regular component of microbial food spoilage in the field, in the market place, and in the home.

Pseudomonas and certain other pseudomonads include species pathogenic for humans, domestic animals, and cultivated plants. *Pseudomonas* species, as well as species included in the newly-created genera *Burkholderia* and *Ralstonia* (ex-*Pseudomonas*) are among the most important bacteria that are pathogens of plants. They cause economically significant crop disease and crop loss world-wide. *Pseudomonas aeruginosa* infects both plants and animals and has evolved into one of the most common and refractory nosocomial pathogens of the post-antibiotic era.

A close relative of *Pseudomonas* is *Xanthomonas*. *Xanthomonas* includes both phytopathogenic species and saprophytic strains. Another pseudomonad, *Zoogloea* is ecologically more restricted, but it has an extremely active oxidative metabolism in its natural habitat and is an important participant in the carbon cycle as a component of the microflora of activated sludge. Differentiation of the three is possible (though often not clear cut) by certain structural, physiological or ecological characteristics, some of which are summarized in the table below.

Table 1. Selected characteristics of diagnostic value for the differentiation of three genera of bacteria considered pseudomonads. This scheme of internal subdivision is reasonably consistent with separation of the genera on the basis of phylogenetic criteria.

Genus	Characteristic
<i>Pseudomonas</i>	Usually motile and oxidase-positive. Capable of growth in simple minimal media at the expense of a large variety of low-molecular-weight organic compounds. Organic growth factors are not required.
<i>Xanthomonas</i>	Water-insoluble yellow pigments (xanthomonadins) produced by the plant pathogenic species. Growth on nutrient agar inhibited by 0.1% triphenyltetrazolium chloride. Weak or negative oxidase reaction. Organic growth factors required.
<i>Zoogloea</i>	Cells actively motile when young. Production of dendritic masses of growth attaching to solid detritus in natural waters and sewage.

The Genus *Pseudomonas*

The bacteriological criteria that distinguish the members of the genus *Pseudomonas* are given below in Table 2.

Table 2. General characteristics of the genus *Pseudomonas*

Gram-negative

Rod-shaped, 0.5-0.8 um x 1-3 um

Strictly aerobic; the only anaerobic activities may be denitrification and arginine degradation to ornithine

Motile by polar flagella; some strains also produce lateral flagella

Oxidative, chemoorganotrophic metabolism

Catalase-positive

Usually oxidase-positive

No organic growth factors are required

Diffusible and/or insoluble pigments may be produced

GC content of the DNA: 58-68 mol%

Classification and Taxonomy

Note: The classification and taxonomy of *Pseudomonas* has been in flux since the advance of techniques for comparative alignment of small subunit rRNA sequences, and also in between editions of *Bergey's Manual* and *The Prokaryotes*. This article, "The genus *Pseudomonas*", is mainly about bacterial species that have existed historically as members of the genus *Pseudomonas*. These bacterial species exemplify the notion of a "pseudomonad" even though they are not related on phylogenetic grounds.

Previously, according to Palleroni and his colleagues at the University of California, *Pseudomonas* species were classified into one of five natural clusters based on ribosomal RNA homology, called “**RNA similarity groups**” (Table 3). There were about forty species, not counting biovars among fluorescent pseudomonads and serovars among phytopathogenic species. In more recent times, beginning in 1990, the members of group I were held in the genus *Pseudomonas*, but the members of groups II, III, IV and V are (to be) moved into new or previously-existing genera. Their new generic assignments are shown in () where appropriate for all bacterial names used below Table 3.

Table 3. Species of *Pseudomonas* and *Xanthomonas* assigned to the various rRNA similarity groups, according to Palleroni, et al. 1973. Groups II, III, and IV are currently placed in new genera among the alpha proteobacteria based on 16S rRNA nucleotide sequencing. This might have been predicted based on Palleroni's "RNA homology groups", and in a sense, he was the first to use RNA homology as a tool to classify bacteria.

RNA similarity group	Constituent Species
I	<i>P. aeruginosa</i> , <i>P. fluorescens</i> (several biovars), <i>P. putida</i> , <i>P. chlororaphis</i> , <i>P. syringae</i> (many pathovars), <i>P. cichorii</i> , <i>P. stutzeri</i> , <i>P. mendocina</i> , <i>P. alcaligenes</i> , <i>P. pseudoalcaligenes</i> , <i>P. agarici</i> , <i>P. angulata</i> , <i>P. fragi</i> , <i>P. synxantha</i> , <i>P. taetrolens</i> , <i>P. mucidolens</i> , <i>P. oleovorans</i> , <i>P. resinovorans</i>
II	<i>P. cepacia</i> , <i>P. gladioli</i> , <i>P. caryophylli</i> , <i>P. pseudomallei</i> , <i>P. mallei</i> , <i>P. solanacearum</i> , <i>P. pickettii</i> , <i>P. pyrrocinia</i> , <i>P. andropogonis</i>
III	<i>P. (Comamonas) acidovorans</i> , <i>P. (Comamonas) testosteroni</i> , <i>P. saccharophila</i> , <i>P. facilis</i> , <i>P. delafieldii</i> , <i>P. alboprecipitans</i> , <i>P. palleronii</i>
IV	<i>P. diminuta</i> , <i>P. vesicularis</i>
V	<i>Xanthomonas</i> spp. including <i>X. (Pseudomonas) maltophilia</i> , <i>P. geniculata</i> , <i>P. gardneri</i>

Group I is the largest of the groups. It includes **fluorescent species** such as *P. aeruginosa*, *P. fluorescens* and the plant pathogens, *P. syringae* and *P. cichorii*. It also includes several important **nonfluorescent species**, such as *P. stutzeri* and *P. mendocina*. The current taxonomic genus *Pseudomonas* has contracted into this group.

P. aeruginosa is the **type species** of the genus. It is one of the most sharply defined and the easiest to identify of the members of the genus. It is also the species of the genus best known from a genetic standpoint, and of course, its genome has now been sequenced. Among the species of *Pseudomonas*, *P. aeruginosa* is the most important as an opportunistic pathogen of humans. As such, *Pseudomonas aeruginosa* is presented in another section of the textbook ([Opportunistic Infections caused by *Pseudomonas aeruginosa*](#)).

In contrast to *P. aeruginosa*, *P. fluorescens* is a remarkably heterogeneous species that can be subdivided by various taxonomic criteria into subspecies referred to as **biotypes** or **biovars**. On purely phylogenetic grounds, some of these subspecies may warrant a new *Pseudomonas* species designation.

In the laboratory, the plant-pathogenic pseudomonads, *P. cichorii* and *P. syringae*, are distinguished by an inability to utilize arginine through possession of an arginine dihydrolase system. They also represent a branch phylogenetically separated from the other fluorescent bacteria of group I. The oxidase reaction differentiates the two species, *P. cichorii* (oxidase positive) and *P. syringae* (oxidase negative). Like *P. aeruginosa*, *Pseudomonas syringae* is actually represented by many different strains presently classified as **pathovars**. These strains were originally described as separate species, based on their host (plant) specificity. What these strains have in common, that is uncommon in *Pseudomonas*, is a lack of a specific cytochrome c oxidase in their respiratory electron transport chain which renders a negative oxidase reaction. Consequently, the *Pseudomonas syringae* taxon became the dumping ground for oxidase-negative phytopathogenic *Pseudomonas* species.

The second rRNA similarity group, **group II**, is mainly composed of pathogens. One of the most important species of the group is *Pseudomonas (Burkholderia) cepacia*, which is a plant pathogen as well as an animal pathogen, and which includes the most versatile strains of the genus with regard to nutritional properties. Two other group II species, *Pseudomonas (Burkholderia) pseudomallei* and *Pseudomonas (Burkholderia) mallei*, the agents of the animal diseases melioidosis and glanders, respectively, are also very versatile organisms.

The third RNA similarity group, **group III**, is represented by five species. Two of the species, *P. (Comamonas)*

acidovorans and *P. (Comamonas) testosteroni*, have been shown to be so distantly related to other *Pseudomonas* species that a new genus, *Comamonas*, has been proposed.

The two genera in **Group IV**, *P. diminuta* and *P. vesicularis*, have also been challenged as bona fide taxonomic members of the genus. In their properties, these bacteria more closely resemble strains of *Gluconobacter*.

Group V, the fifth RNA similarity group, consists of *P. (Xanthomonas) maltophilia* and species of *Xanthomonas*. While the strains of the latter are considered to be universally plant pathogenic, *P. (Xanthomonas) maltophilia* can be found in many natural habitats living as a saprophyte, and it is also occasionally isolated from clinical specimens.

Nutrition and Growth

Pseudomonas species have very simple nutritional requirements. In the laboratory they grow well in media with some organic matter in solution, at neutral pH, and at temperatures in the mesophilic range. One of the handiest media for culturing *Pseudomonas* in the laboratory is King's B medium, the formulation of which is given below (Table 4). Most *Pseudomonas* species grow in chemically-defined media without added growth factors.

Pseudomonas species are respiratory and never fermentative. All species respire aerobically, and some respire anaerobically with NO_3^- as a final electron acceptor. *Pseudomonas* species dissimilate sugars through the Entner-Doudoroff pathway. Some *Pseudomonas* species can utilize more than 150 different organic compounds as a sole source of carbon and energy and are remarkable for their catabolic diversity.

Saprophytic species of *Pseudomonas* can be directly isolated by streaking source materials on plates of common bacteriological media such as nutrient agar. A simple enrichment procedure involves plating the organisms onto media with special carbon sources (e.g. nicotine, toluene, octane, etc.) that can be used by the desired organism. Fluorescent species of the genus *Pseudomonas* can be isolated from soils by the use of selective media that contain antibiotics and/or metals which enhance pigment formation.

Denitrifying pseudomonads can be isolated by specific enrichment procedures, essentially in medium containing NO_3^- under anaerobic conditions. Denitrification enrichments with various carbon sources carried out at 37 - 40°C can yield *P. aeruginosa*, *P. stutzeri*, and *P. mendocina*. Lower incubation temperatures (around 30°C) enriches for the denitrifying biotypes of *P. fluorescens*.

Pigments

A common characteristic of the fluorescent pseudomonads is the production of pigments that fluoresce under short wave length (254 nm) ultraviolet light, particularly after growth under conditions of iron limitation. Some of these pigments and/or their derivatives are known to play a role as siderophores in the iron uptake systems of the bacteria., and hence, their production is markedly enhanced under conditions of iron deficiency. The production of pigments is readily demonstrated by culturing the bacteria in media such as King's Medium B, which contains no added iron. The medium is also recommended to demonstrate the production of the nonfluorescent blue pigment, pyocyanin, characteristically produced by most strains of *P. aeruginosa*.

Table 4. Composition of King's Medium B. The mineral salts of this medium, as well as the exclusion of iron, enhance the production of pigments. In many laboratories, King's B Medium is used as a general medium for routine cultivation of *Pseudomonas*.

Proteose Peptone no. 3	2 g
Glycerol	1 g
K_2HPO_4	0.15 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.15 g
Agar	1.5 g
Distilled water	100 ml
Adjust to	pH 7.2

Temperature of Incubation

In clinical laboratories it is usual to maintain the temperature of incubators at 37°C. This is optimal for the growth

of *P. aeruginosa*, the most likely species to be encountered in medical specimens, but all the known species of *Pseudomonas* grow quite well at 28-30°C. Indeed, this temperature is more appropriate for some of the species of group I. By lowering the temperature of incubation to within this range, strains which only grow marginally at body temperature may not be missed in isolations from clinical materials.

Motility and Flagella

Most *Pseudomonas* species are motile by means of one or more polar flagella. In wet mounts of pond water or hay infusions they are likely to be the rapidly motile rod-shaped bacteria that dart across the microscope field. Chemotaxis and aerotaxis have been observed, and motility is clearly a selectively useful trait for existence in the aquatic environment.

In the laboratory, both the composition of the medium and the temperature of incubation affect motility. Young cultures in the active stages of growth are best for observation of motility. In dense cell suspensions in wet mounts, motility rapidly ceases except in cells that are close to a bubble or the edges of the coverslip, suggesting that the cells must find O₂ as a respiratory electron acceptor in order to maintain flagellar rotation. Arginine can prolong motility under anaerobic conditions in strains that possess the arginine dihydrolase system.

Plasmids

Most importantly, *Pseudomonas* plasmids confer resistance to many antibiotics and antibacterial agents. However, in comparison with clinical strains of other species, the proportion of strains of *P. aeruginosa* carrying transmissible extrachromosomal determinants of resistance is fairly low. The species is also naturally resistant to deleterious agents, including many antibiotics. Such natural resistance results from its inherent Gram-negative cell wall structure, and its propensity to construct protective biofilms in medical settings.

Thirteen compatibility groups of plasmids have been identified in *Pseudomonas*. One group, P-2, contains over half the transmissible plasmids identified in the species. In addition to a variety of markers determining resistance to various antibiotics and simple chemicals (e.g. mercury, borate), all P-2 plasmids carry determinants of resistance to tellurite and tellurate, which makes these agents useful for selective isolation. The largest *Pseudomonas* plasmids (some exceeding 300 kbp) are in this group.

The plasmids of group P-9 are typically catabolic plasmids, and they contribute significantly to the nutritional diversity of some *Pseudomonas* species. For example, plasmids NAH, SAL, and TOL, carry genes involved in the degradation of naphthalene, salicylate, and toluene, respectively.

In addition to resistance to toxic agents, some plasmids of *Pseudomonas* determine various other important properties, such as fertility and resistance to physical agents, bacteriophages and bacteriocins. Some *Pseudomonas* plasmids are capable of mobilizing the bacterial chromosome and have been used extensively in the study of *Pseudomonas* genetics.

Group P-1 contains "wide host-range plasmids" which are capable of transfer to strains of practically any Gram-negative species. Derivatives of these plasmids can be isolated from various Gram-negative species by means of complementation of auxotrophic markers in *Pseudomonas* species. The transfer in nature of *Pseudomonas* resistance markers to other Gram-negative species of medical importance is a matter of some concern.

Ecology

Pseudomonads are truly ubiquitous organisms, even though some pathogenic species are ecological specialists found in specific types of environments. The ubiquity of the pseudomonads would seem to be a consequence of their meager nutritional requirements, the range of carbon compounds they can utilize, and the diversity of their metabolism which reaches into autotrophy, lithotrophy and anaerobic modes of respiration. Having said this, *Pseudomonas* species predominate in soils and water under aerobic, mesophilic and neutral conditions. They do not ferment, they are not abundant in anaerobic environments, and they do not occur in thermophilic or acidophilic habitats.

Pseudomonas and its relatives occupy a prominent position in nature for their active participation in the carbon cycle. *Pseudomonas* metabolism has been the subject of intensive biochemical research, and many of the unique

catabolic pathways found in pseudomonads have been described. Studies on the degradation of natural and artificially synthesized compounds by pseudomonads has been exploited in approaches to solution of environmental pollution problems.

The conditions under which pseudomonads flourish in soil are also favorable for growth of aerobic actinomycetes of the genus *Streptomyces*, among many other types of bacteria. It has been suggested that soil dwelling pseudomonads may preferentially grow in association with the streptomycetes. The streptomycetes, which are masters of aerobic decomposition of organic compounds the soil could provide pseudomonads with monomeric carbon sources which they require. Also, associations between *Pseudomonas* and *Streptomyces* in Nature may explain the notorious resistance of *Pseudomonas* to streptomycete antibiotics in a medical setting.

Strains of *Pseudomonas* are often carriers of plasmids containing genes that confer the capacity for antibiotic resistance, and if the notion that R factors originated in antibiotic producing organisms is correct, it is possible that *Pseudomonas* strains were among the first groups of organisms to have received these factors from streptomycetes as a consequence of their association in the same ecological niches.

In nature, *Pseudomonas* species exist as saprophytes and as parasites. Those that are parasites, with one exception, apparently have a saprophytic mode of existence as well. After all, the ultimate distinction between a parasite and a saprophyte is whether or not the organism is growing at the expense of "living" or "dead" organic matter.

Many phytopathogenic pseudomonads, which also includes members of the genus *Xanthomonas*, can only be found on diseased plants. In these special ecological niches they appear as practically homogeneous populations when the pathological lesions are young, which makes their isolation in pure culture a relatively straightforward process. The distribution of many of these pathogens outside their host plants is poorly known at present, although many of them seem to be able to exist as saprophytes.

Animal pathogens are far less host specific than the phytopathogens, and most are considered to be opportunistic pathogens. One species, *P. (Burkholderia) mallei*, is an exception to this rule. This bacterium is the agent of glanders in horses, mules, and donkeys. *P. (Burkholderia) mallei* is not found living saprophytically, and it is considered to be the only pseudomonad with a specialized parasitic animal niche.

Pathogens of Humans and Animals

Pseudomonas species are widespread saprophytes in Nature, particularly in soil and water, but some are also pathogens of plants and animals. Consequently, some species are medically important and frequently can be isolated from a variety of clinical specimens. Most species, however, are classified as opportunistic pathogens. Only *Pseudomonas (Burkholderia) mallei* and *P. (Burkholderia) pseudomallei* are regarded as primary pathogens. Even so, *P. (Burkholderia) pseudomallei* can be found in nature as a free-living organism.

P. aeruginosa is by far the dominating human and animal pathogen. Interestingly, it is also a pathogen of plants, which illustrates the fuzzy boundary between phytopathogenic and medical pseudomonads.

In a disease context, the *Pseudomonas* pathogens of humans and animals are characterized as having a "low virulence", i.e., they are opportunistic and do not produce disease unless the animal's constitutive or immune defenses are compromised. This is certainly not to say that they lack any determinants of virulence. Considering the number of structural, biochemical, and genetic properties of *P. aeruginosa* that contribute to its virulence, it ranks with leading pathogens such as *Staphylococcus* or *Streptococcus*.

Pathogen Habitats

Pathogenic *Pseudomonas* may be isolated from infections at almost any body site, most commonly of the urinary tract, respiratory tract, wounds, and the blood.

Strains of *Pseudomonas* can be extremely versatile from a nutritional standpoint, and they can grow in very simple nutritional environments without any organic growth factors. They can remain viable for long periods of time in many different habitats. Many different materials used in medical practice may be contaminated with pseudomonads. This even includes solutions of antiseptics and disinfectants, but more commonly, water, saline solutions, utensils, and medical instruments. The bacteria have been found in pharmaceuticals, cosmetics, and

preparations involving plant materials.

In hospitals, *P. aeruginosa* can be spread through fecal material. Also *Pseudomonas* has been isolated from many natural and manufactured foods, and therefore, foods have been implicated as sources of infection in hospitals. In addition, visitors can transport the bacteria to the hospital as contaminants in foods, plants, flowers and presents. Health personnel may also be involved.

The widespread habitat of *P. aeruginosa* in nature, which includes soil, water, food, and the surfaces of plants and animals, makes it very difficult to control the organism in a hospital setting. Prevention of contamination is practically impossible. The main danger is the infection of patients who are immunologically compromised, or in burn units, neonatal units and cancer wards. When conditions are favorable, *P. aeruginosa* and other pseudomonads can infect wounds, burnt areas, and the urinary and respiratory tracts, and may also be involved in pneumonia, endocarditis, meningitis, and various other pathological conditions.

Pseudomonas aeruginosa

Among pseudomonads, *P. aeruginosa* has attracted the most attention from general and clinical microbiologists, geneticists, and biochemists. The list of materials from which this species can be isolated is almost endless, so that from a practical point of view, one can assume that the bacterium is present everywhere. Most strains of the species can be easily identified by a number of phenotypic characteristics never found in the same combination in other species. Most important among these are production of pigments, including pyocyanin, the ability to denitrify, and the ability to grow at 41°C.



Gram stain of *Pseudomonas aeruginosa* cells

The colonies of *P. aeruginosa* are flat, grayish, with irregular edges, and with time they tend to spread on the surface of the agar. Mucoid colonies frequently appear among isolates from the respiratory tract of patients with cystic fibrosis. The mucoid extracellular substance is alginic acid.

P. aeruginosa is capable of producing several pigments, of which the most characteristic is **pyocyanin**. As far as is known, this blue pigment is an absolute diagnostic character, since no other species has been found to produce it. **Pyoverdin** is the fluorescent pigment most often produced, but the bacteria may be able to produce several additional pigments, including a reddish pigment, **pyorubrin**, and a brown pigment, **pyomelanin**. This pigment, in common with other melanins, is produced from aromatic amino acids such as tyrosine or phenylalanine, while pyorubrin production is enhanced by the addition of glutamate to the medium. Besides pyoverdin, which acts as a siderophore, the function of these pigments is obscure.



Pseudomonas aeruginosa colonies on agar

P. aeruginosa has long been known as an opportunistic pathogen, especially dreaded in the hospital environment. Early reports pointing to infection with this organism described a “blue pus” associated with wound infections. *P. aeruginosa* has been isolated from wounds in almost all locations in the human or animal body, as well from purulent infections of the urinary and respiratory tracts. *P. aeruginosa* associated with pneumonia, enteritis, vaginitis, mastitis, and endometritis in animals is abundantly recorded in the literature.

Since the tissue invasiveness of the organism is very limited, *P. aeruginosa* usually uses accidental ports of entry (burns, wounds, intravenous and urinary catheterization, surgical procedures, etc.) to gain access to its compromised host.

Several extracellular products (proteases, elastase, etc.) help the invasion and dissemination of *P. aeruginosa*. Most isolates of the species produce exotoxin A, which is induced under the conditions of iron limitation that characterize many animal tissues. The target of this toxin is one of the elongation factors in translation during protein synthesis. Strains incapable of producing exotoxin A have reduced virulence. A general discussion of virulence and disease caused by *P. aeruginosa* is presented in another section of the text ([Opportunistic Infections caused by *Pseudomonas aeruginosa*](#)).

For epidemiological investigations the strains of *P. aeruginosa* can be divided into “types,” which are taxonomic categories below the species level. Various typing methods can be used: biotyping, antibiograms, serotyping, bacteriocin typing, and phage typing. As far as serotyping is concerned, there has been subdivision into 12 somatic groups.

P. fluorescens* and *P. putida

These species were described a few years after the description of *P. aeruginosa*. In human and veterinary medicine, while *P. aeruginosa* has historically been the most significant pathogenic species, it is now becoming evident that other species of the genus may be serious opportunistic pathogens. This includes the fluorescent pseudomonads, herein described, and certain nonfluorescent species which will be discussed below. Both *P. fluorescens* and its close relative, *P. putida*, have a natural history similar to *P. aeruginosa*. Two phenotypic characteristics of *P. fluorescens* that distinguish it from *P. putida* are its ability to grow at 4°C, and its ability to hydrolyze gelatin. These characteristics help explain its frequent involvement in spoilage of refrigerated food, in particular chicken and processed meats. If it's fluorescent, get rid of it!

Bacteria in the *P. fluorescens*-*P. putida* complex have been isolated from lizards, insects and mammals. Clinical sources from which strains of these species have been isolated include respiratory tract specimens, pleural fluid, urine, cerebrospinal fluid, feces, blood, and a variety of other materials. However, from a medical point of view, the clinical importance of *P. fluorescens* and *P. putida* is debatable. The main property that conspires against their becoming important opportunistic pathogens is their inability to grow at body temperature. Indeed, they are rarely pathogenic for humans, even though they have been found associated with empyema, urinary tract infections, septicemia, and various other episodes. In any event, although their virulence may be low, *P. fluorescens* and *P. putida* should be considered as potentially pathogenic.

Nonfluorescent *Pseudomonas* pathogens

Note: Among the nonfluorescent members of the genus, only those assigned to Palleroni's group I are designated to remain in the genus. Groups II, III, IV and V are slated for new genus names, and hence, are formally moved out of the genus *Pseudomonas*. Group I survivors are considered here.

Among the the nonfluorescent *Pseudomonas* species, those which have been encountered in clinical laboratories include *P. stutzeri*, *P. mendocina*, and *P. alcaligenes*.

P. stutzeri is a strong denitrifier that usually can be identified by the appearance of the colonies, which are wrinkled, coherent, hard, and of a dark brown color due to their high cytochrome c content. *P. stutzeri* is a common soil inhabitant and can also be found in plant materials and in water. Therefore, reports of its isolation from various clinical materials are not surprising. Moreover, the organism can grow at body temperature. However, its pathogenic properties have not been clearly demonstrated.

P. mendocina is related to *P. stutzeri*, and it can be isolated similarly from denitrification enrichments. The colonies are flat and smooth, and have a yellowish color due to the presence of carotenoid pigments. *P. mendocina* has rarely been isolated from materials of clinical origin, but it has not been incriminated as a cause of infection in humans. However, strains of *P. mendocina* have been found to produce alginate, same as *Pseudomonas aeruginosa*. In the latter species, alginate-producing strains can be isolated from cystic fibrosis patients, where they become selected through the protecting action of the exopolysaccharide against phagocytosis by alveolar macrophages.

P. alcaligenes is only found occasionally associated with infection in humans, and it can, at best, be considered as a rare opportunistic pathogen.

Other Pathogenic Pseudomonads

The genus *Pseudomonas* has become restricted to members of of Palleroni's group I, based on nucleic acid sequence similarity studies. This puts aside many species of bacteria formerly assigned to the genus that are pathogenic for humans or other animals. Some of these are briefly discussed here as "pseudomonads" or bacteria with a phenotypic and ecophysiological similarity to members of the genus *Pseudomonas*.

Most of the species of *Pseudomonas* of Palleroni's group II (Table 3) are pathogenic for plants, animals or humans. These species have now been placed in the genus *Burkholderia*. *B. mallei* is the agent of glanders, a zoonosis in equines; *B. pseudomallei* causes a similar condition, melioidosis. *B. cepacia* and *B. gladioli*, in addition to *B. multivorans*, and *B. vietnamensis*, have been found associated with lung infection, bacteremia, endocarditis, septic arthritis, and cystitis. Their role in cystic fibrosis patients is unclear. *Burkholderia* species are resistant to aminoglycosides.

A species that has attracted some attention is *B. cepacia*, originally a poorly known plant pathogen, *Pseudomonas cepacia*. Of all the species originally assigned to the genus *Pseudomonas*, this is by far the most versatile from the point of view of its nutrition. Many different compounds can serve individually as sole sources of carbon and energy when added to minimal media, including penicillin G, which can be used both as a carbon and a nitrogen source.

B. cepacia has been isolated from a large number of different sources, including habitats that normally are unfavorable for most other prokaryotes. These include antiseptic solutions and crystal violet solutions. A penicillinase has been characterized, in addition to which natural resistance to various antibiotics, including chloramphenicol, aminoglycosides, beta-lactams and polymyxin, has been reported.

B. cepacia has been found associated with many different infections of nosocomial origin. In recent years, *B. cepacia* has been found associated with cases of cystic fibrosis. Adherence of cells to respiratory epithelial cells was found to be enhanced by the presence of *Pseudomonas aeruginosa*, another opportunistic pathogen found in infections of the respiratory tract.

B. mallei is the etiologic agent of glanders, a serious disease of equines, and *B. pseudomallei* causes a related disease. Glanders can be transmitted from equines to animals of other groups and also to humans. The disease can start in the respiratory tract or as an ulcerative process, rapidly spreading to the lymph nodes with fatal consequences. The organism is not found living freely in Nature, and normally passes from animal to animal.

In contrast to *B. mallei*, *B. pseudomallei* can be found in soil and water. But its occurrence is mostly restricted to

tropical regions. Animals and humans are highly susceptible. The organism usually enters the host through wounds. *B. pseudomallei*, and to a lesser degree, *B. mallei*, are also nutritionally-versatile organisms, analogous to many other pseudomonads.

Pseudomonas pickettii was described as an organism resembling the plant-pathogenic species, *P. (Ralstonia) solanacearum*. *P. pickettii* has been found in hospital samples, including urine, abscesses, wounds, and various other clinical sources. Although the pathogenicity of the bacterium is not clear, it was once incriminated in an epidemic bacteremia in Australia. *P. pickettii* also might be moved into the genus *Ralstonia*.

Phytopathogenic and Plant-Associated Pseudomonads

The phytopathogenic pseudomonads are a very diverse group of bacteria with respect to their genetics, ecology, and the kinds of diseases they cause. Currently, they are grouped into approximately 25 species in three distinct genera. One species, *Pseudomonas syringae*, contains over 50 pathovars, most of which attack different hosts. There also are multiple pathovars in other species.

Phytopathogenic pseudomonads are worldwide in distribution and cause diseases of most major groups of higher plants. Some of the world's most serious plant diseases are caused by pseudomonads, such as *Pseudomonas (Ralstonia) solanacearum*. Disease symptoms in plants range from necrotic lesions, spots, cankers and blights, to hyperplasias (galls, scabs), tissue maceration (rots), and vascular infections (wilts). As mentioned earlier, some pseudomonads, for example, *P. aeruginosa* and *P. (Burkholderia) cepacia*, appear to infect both plants and animals.

Besides parasitic associations with plants, some pseudomonads exist in various other associations with plants. For example, some pseudomonads affect plant growth through their interactions with fungal plant pathogens or by their direct effect on the roots, possibly because of hormone production. These bacteria may colonize plant parts, and in some cases, they appear to damage the plant. Here, again, there is no clear demarcation between parasitism and saprophytism.

Pseudomonas, Ralstonia, Burkholderia and Acidovorax

The evidence suggesting that the *Pseudomonas* phytopathogens belong to at least three different genera is derived primarily from studies of rRNA-DNA homologies. The three groups containing plant pathogens are the ***P. fluorescens* group** (called the “fluorescents”), the ***P. (Ralstonia) solanacearum*—*P. (Burkholderia) cepacia* group**, and the ***P. (Acidovorax) avenae* group**. The latter two groups are usually lumped together as the “nonfluorescents.”



Bacterial fruit blotch of watermelon caused by *Acidovorax avenae* biovar *citrulli*. Photograph by Tom Kucharek, UF-IFAS. [DOACS-Plant Pathology Section-Acidovorax avenae subsp. *citrulli*](#). The bacterium can be introduced into watermelon fields by infested seed, infected transplants, natural spread from alternate hosts or from volunteer watermelon. The bacterium can be a surface contaminant of seed harvested from infected watermelon. Bacterial fruit blotch disease development is favored by warm wet weather. The naturally occurring waxy layer that develops on maturing watermelon hinders bacterial invasion and infection unless the fruit rind becomes injured and the protective wax layer is compromised.

Xanthomonas

This pseudomonad is differentiated from members of the genus *Pseudomonas* (here, to include *Ralstonia*, *Burkholderia* and *Acidovorax*) by production of water-insoluble yellow pigments (**xanthomonadins**), and a requirement of certain organic growth factors. *Xanthomonas* is a legitimate genus in the family *Pseudomonadaceae* (see Table 1) and it, along with *Pseudomonas (Xanthomonas) maltophilia*, is the primary constituent member of Palleroni's RNA homology group V (see Table 3).

As a phytopathogen, only one species of *Xanthomonas* is important, *X. campestris*. However, *X. campestris* represents at least 100 pathovars based on characteristics of their pathogenicity, i.e. the species of plant which is their host. Hence the pathovars are named for the specific plant that becomes infected. Thus, *X. campestris* pv. *cannabis* infects *Cannabis sativa*; *X. campestris* pv. *eucalyptae* infects *Eucalyptus citrodora*; and so on. The original isolates of *Xanthomonas campestris* caused a vascular disease of *Brassica campestris*, its host plant. But now the species includes and is subdivided into a large number of pathovars that cause diseases in many plants.



Common Blight of beans caused by *Xanthomonas campestris* pv. *phaseoli*. Bacterial [Bights Fact sheet, Cornell University, Department of Plant Pathology](#). Characteristic leaf symptoms of common blight consist of irregular areas of brown dry tissues surrounded by a narrow lemon yellow border. These lesions frequently occur at the leaf margins. Pods have sunken circular spots with a reddish brown narrow border. The bacteria invade the seeds and remain dormant until germination begins. Even a trace of infected seed when planted can initiate severe infection of entire fields. The bacteria exude in the leaf and pod spots and are spread mainly by splashing and blowing rain. Warm, humid conditions favor development of the disease.

Toxins

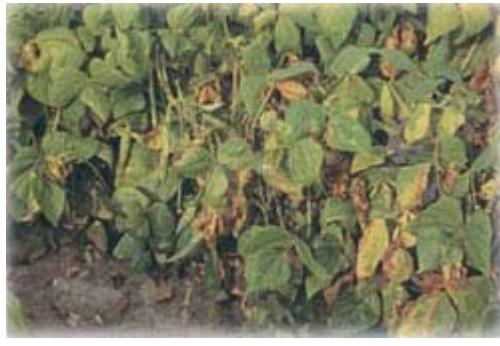
Several pathovars of *P. syringae* produce **phytotoxins**. Chemical structures have been established for four of these toxins, as well as rhizobitoxin which is produced by *Pseudomonas andropogonis*. Sites of action of bacterial phytotoxins include amino acid biosynthetic pathway enzymes (e.g. glutamine synthetase and ornithine carbamoyltransferase for the active moieties of tabtoxin and phaseolotoxin, respectively), chloroplast RNA polymerase for tagetitoxin, cystathionase for rhizobitoxin, and certain protein kinases of plant and other eukaryotic cells for syringomycin. Several of these toxins are capable, in purified form, of reproducing one or more aspects of disease symptomatology attributed to them, usually chlorosis or necrosis. The causal relationships between the effect of the toxin on the target site and expression of disease symptoms are less clear.

In contrast to the phytotoxins produced by fungal pathogens, which are host specific, the *Pseudomonas* phytotoxins are active against plants on which their producers do not cause disease. Some of these toxins are also active against other microorganisms.

Whether these phytotoxins constitute “pathogenicity” or “virulence” factors is actively debated among plant pathologists because much of the experimental information is equivocal. For example, mutants of *P. s.* pv. *phaseolicola* that do not produce phaseolotoxin show identical growth kinetics in the plant as do wild type strains. Other *Pseudomonas* toxins that have been examined in this respect (e.g. coronatine, syringotoxin) appear to play a role in pathogen multiplication in the plant, since tox⁻ mutants of *P. s.* pv. *tomato* and *P. s.* pv. *syringae* (citrus strain) show reduced virulence.

Two *Pseudomonas* toxins, syringomycin and syringotoxin, are cyclic peptides containing one or more nonprotein amino acids. They are presumed to be synthesized by enzyme systems analogous to those involved in the synthesis of cyclic peptide antibiotics by *Bacillus* species. Two other toxins, tabtoxin and phaseolotoxin, are synthesized and secreted from the cells as linear peptides but are hydrolyzed in the plant to nonpeptide forms. Cleavage by plant

peptidases potentiates the action of these toxins.



Halo Blight caused by *Pseudomonas phaseolicola*. Bacterial Blights Fact sheet, Cornell University, Department of Plant Pathology.

Halo blight is one of the most important bacterial diseases of beans. The most characteristic symptoms occur on the bean leaves. Small, water-soaked spots, resembling pinpricks, develop on the undersides of the leaves. These spots soon turn reddish brown, and the tissues surrounding the spots gradually become yellow green. Severe infections resulting from seed contamination may give internal systemic infection, exhibited by yellowing and stunting. These plants defoliate, wilt, and die early, but serve as important reservoirs of bacteria to be spread to neighboring plants.

Phytohormones

In addition to phytotoxins, phytohormones represent another group of low-molecular-weight substances which play a role in pathogenesis by *Pseudomonas*. A useful conceptual distinction between a phytotoxin and a phytohormone is that the latter are also produced by the host. A role for phytohormones is usually suspected when pathogens induce growth abnormalities in the host, examples being the crown gall, hairy root and olive knot diseases. However, other pathogens that do not cause hypertrophic symptoms on their hosts also produce these substances.

Nine different plant pathogenic pseudomonads produce indole acetic acid (IAA) and additional indole compounds in media that have been supplemented with tryptophan. *P. (Ralstonia) solanacearum* and *P. s. pv. syringae* also synthesize exceptionally high amounts of IAA both in the presence and absence of exogenous tryptophan. A role of hormones in either vascular wilt or in the brown spot disease has not been established. IAA has numerous other effects on plant cells which may favor bacterial multiplication in the absence of hypertrophy.

There is definitive evidence for the involvement of IAA in the interaction between *P. s. pv. savastanoi* and two of its hosts. The pathogen synthesizes the hormone (from tryptophan in two steps catalyzed by tryptophan monooxygenase and indoleacetamide hydrolase), which incites hyperplasias on oleander and olive.



Bacterial galls on oleander stem and leaves caused by *Pseudomonas savastanoi* (*Pseudomonas syringae* pv. *savastanoi*) Photo by Jack Kelly Clark. [UC Management Guidelines for Olive Knot on Olive](#). Olive knot appears as rough galls or swellings about 0.5 to 2 inches in diameter on twigs, branches, trunks, roots, leaves, or fruit stems. Galls also form at trunk or limb wounds. Olive knot does not kill

trees, but it does reduce productivity by destroying twigs and branches, and the flavor of fruit from infected trees may be affected. Bacteria survive in the knots and are readily spread by water at all times of the year. Infection occurs at low temperatures, usually in fall or spring. Openings are necessary for penetration of bacteria, and these are provided by leaf scars, pruning wounds, or bark cracks made by freezing. Damage can be severe when weather favors disease.

P. s. pv. savastanoi and *P.(Ralstonia) solanacearum* have been shown to produce cytokinins. There is diversity in both the types of cytokinins and the amounts produced by different strains. The role of these substances in symptom expression and the basis of variations has not been established.

Ice Nucleation

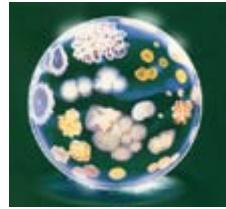
Three *Pseudomonas* species are efficient ice nucleators at temperatures above -10°C: *P. syringae* (various pathovars), *P. fluorescens* (biotype G), and *P. viridiflava* (several strains). The study of bacterial ice nucleation has received attention in recent years because of the role of ice-nucleating bacteria, particularly *P. syringae*, as agents of frost injury to plants.

It is now firmly established that a family of large and unusual proteins (ice nucleation proteins, mw 118 kDa, or larger) are a key component of bacterial ice nuclei. Predicted amino acid sequences of two such proteins from *P. syringae* and *P. fluorescens*, respectively, have revealed a consensus octapeptide with alternative periodicities of 16 and 48 amino acids. Although not unique among proteins, the repeat structure suggests itself as being central to the ice nucleation function. Several repeat domains may collectively form a catalytic surface of some critical size which effectively orders water molecules in an ice-like lattice.

Site-directed deletion of the ice nucleation gene from competent nonpathogenic strains of *P. syringae* and *P. fluorescens* ("ice-minus" mutants) have been generated as biological control agents of frost injury to plants, representing the first genetically-engineered bacteria to be released outside the laboratory under Environmental Protection Agency (EPA) permit in the USA.

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The Enteric Bacteria

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Introduction to the Family *Enterobacteriaceae*

Enterobacteriaceae are Gram-negative, oxidase-negative, rod-shaped bacteria, 0.3-1.0 x 1.0-6.0 um. Typically, they are motile by peritrichous flagella. They are facultative anaerobes, being chemoorganotrophs that exhibit both respiratory and fermentative metabolism. Most grow well between 22 and 35°C on media containing peptone or beef extract. They also grow on MacConkey's agar which may be used for their selective isolation. Most grow on glucose as a sole carbon source, although some require vitamins and/or amino acids for growth. They produce mixed acids and often gas from fermentation of sugars. With very few exceptions they are catalase-positive, and most strains reduce nitrate to nitrite.

Escherichia coli is the type species. *E. coli* is considered the most thoroughly studied of all species of bacteria, and the family *Enterobacteriaceae*, as a whole, is the best studied group of microorganisms. Among the reasons for their popularity are their medical and economic importance, ease of isolation and cultivation, rapid generation time, and their ability to be genetically manipulated.

Enterobacteriaceae are distributed worldwide. They are found in water and soil and as normal intestinal flora in humans and many animals. They live as saprophytes, as symbionts, epiphytes, and parasites. Their host range includes animals ranging from insects to humans, as well as fruits, vegetables, grains, flowering plants, and trees.

Economic and Medical Importance

As stated above, one of the reasons that the enterobacteriaceae have been so widely studied is due to their obvious impact on human and animal health and on agricultural practice. The enterobacteriaceae include agents of food poisoning and gastroenteritis, hospital-acquired infections, enteric fevers (e.g. typhoid fever) and plague. They also cause infections in domestic, farm and zoo animals and include an important group of plant pathogens. Some of these bacteria are discussed below.

Plant Pathogens

Many species of *Enterobacteriaceae* are responsible for significant economic losses in agriculture. *Erwinia* species cause blight, wilt, or soft-rot in numerous trees, flowers, and crops, often destroying substantial amounts of crops. Among the plants affected are walnut and oak trees, rose, orchid and chrysanthemum flowers, and crops such as corn, wheat, potato, carrot, sugar beet, sugar cane and pineapple.

Animal Pathogens

Enterobacteriaceae cause disease in all sorts of animals, ranging from nematodes and insects through primates. *Salmonella* alone has been associated with disease in more than 125 species. Infections frequently cause problems in zoos, often in snakes and lizards. In regional primate centers in the United States, the most frequently diagnosed diarrheal diseases were caused by *Enterobacteriaceae*, most often by *Shigella*, *E. coli* and *Salmonella*. *Klebsiella pneumoniae* is a frequent cause of respiratory disease in primates, and *Yersinia pseudotuberculosis* is associated with enterocolitis and peritonitis.

Pets and farm animals are affected by a variety of enterobacterial diseases. Cats and dogs are susceptible to cystitis and other urogenital infections caused by *E. coli*. *Proteus* species cause other diseases in cats and dogs, and these animals can be carriers of *Salmonella*. *Salmonellae*, especially *S. typhimurium*, *S. newport*, and *S. anatum*, cause

enteritis with high fatality and septic abortion in horses, and *K. pneumoniae* causes metritis in mares and pneumonia in foals.

Septicemia caused by *E. coli* is an important cause of death in chickens. Serotypes of *Salmonella enterica* are pathogenic and highly fatal for turkeys and other poultry, causing a characteristic diarrheal syndrome. Pullorum disease, caused by *Salmonella pullorum*, is highly fatal to eggs and chicks. Fowl typhoid, a septicemic disease of poultry, especially chickens, is caused by *Salmonella gallinarum*. Both pullorum disease and fowl typhoid can be largely eradicated if infected adult birds are slaughtered. Nearly 200 *Salmonella* serotypes had been isolated from fowl in the United States. The distribution of salmonellosis in poultry is worldwide. As in human disease, certain serotypes are prevalent in some regions and absent in others. A mortality rate of 10-20% is normal in young birds, mostly in the first two weeks after hatching.

Sheep suffer from a variety of illnesses caused by *Enterobacteriaceae*. Infant diarrhea in lambs, is usually caused by strains of *E. coli* producing a heat-stable enterotoxin. Most of these strains also contain the K-99 fimbrial adhesin. *Salmonella* abortion is usually caused by *Salmonella abortusovis*, *S. typhimurium*, or *S. dublin*, which also cause stillbirths and wool damage.

Calves are susceptible to both systemic colibacillosis and neonatal diarrhea (calf scours), which are usually fatal if not promptly treated. Specific heat-stable enterotoxigenic *E. coli* serotypes containing K99 fimbrial adhesin are the causative agents. Bovine mastitis has become a very prevalent disease since the advent of antibiotics. The most prevalent causative agents are *E. coli* and *Serratia* species, and less often, *Klebsiella* species and *Citrobacter freundii*. Salmonellosis is frequent in cattle. Most cases are due to *Salmonella dublin* and *S. typhimurium*, although more than 100 serotypes have been isolated. As with other animal infections, *Salmonella* is frequently introduced through contaminated feed.

Swine are subject to infection with several species of *Enterobacteriaceae*. *E. coli* infection may present as diarrhea in piglets, or as edema preceded by mild diarrhea. Both forms are acute and highly fatal. As in sheep and cows, the causative strains produce a heat-stable enterotoxin, but they may also produce a heat-labile enterotoxin. Swine strains usually possess a K88 fimbrial adhesin, which is antigenically distinct from K99. Sows are susceptible to mastitis and metritis caused by *K. pneumoniae*, and to enteritis and lymphadenitis caused by *Yersinia enterocolitica*. More than 100 *Salmonella* serotypes have been isolated from pigs. However, only two serotypes, *S. choleraesuis* and *S. typhisuis*, have pigs as their primary host. *S. choleraesuis* has a wide host range, including humans, but *S. typhisuis* is rarely pathogenic to animals other than pigs. *Salmonella typhimurium* and *S. derby* are also frequently isolated from porcine salmonellosis.

Substantial losses in fishing industries are caused by enterobacterial diseases. *Yersinia ruckeri* is the cause of outbreaks of redmouth disease in salmon and trout hatcheries. *Edwardsiella tarda* is pathogenic for eels, catfish, and goldfish, and *Edwardsiella ictaluri* is pathogenic for catfish.

The host range for species of *Enterobacteriaceae* varies greatly. For example *Proteus myxofaciens* has been isolated only from larvae of gypsy moths and *Escherichia blattae* has been isolated only from the hindgut of cockroaches. Shigellae are seen only in primates. Others, including *E. coli*, many salmonellae, and yersinia, infect or are carried by hosts ranging from insects to humans.

Human Pathogens

Enterobacteriaceae as a group were originally divided into pathogens and nonpathogens based on their ability to cause diarrheal disease of humans. The pathogenic genera were *Salmonella* and *Shigella*. However, it is now known that *E. coli* causes at least five types of gastrointestinal disease in humans. Pathogenicity in *E. coli* strains is due to the presence of one or more virulence factors, including invasiveness factors (invasins), heat-labile and heat-stable enterotoxins, verotoxins, and colonization factors or adhesins. Pathogenic strains are usually identified by detection of a specific virulence factor or of a serotype associated with a virulence factor. The most recently identified *E. coli* disease is hemorrhagic colitis caused by strains of serotype 0157:H7. The disease, characterized by painful abdominal cramping and bloody diarrhea, is caused by strains that produce verotoxin, and the same strains are associated with hemolytic uremic syndrome (HUS).

Yersinia enterocolitica causes diarrhea, probably by a combination of invasiveness and the presence of a heat-stable enterotoxin. Strains of *Klebsiella pneumoniae* and *Enterobacter cloacae* isolated from patients with tropical sprue contained a heat-stable enterotoxin. *Edwardsiella tarda* and *Citrobacter* strains are occasionally associated

with diarrhea and have been shown to produce heat-stable or heat-labile enterotoxin.

Foodborne and waterborne disease outbreaks in the U.S. are frequently associated with *Enterobacteriaceae*. According to the Centers for Disease Control (CDC), 40-45% of such outbreaks are caused by *Enterobacteriaceae*, the overwhelming majority by *Salmonella*. Meats, milk and milk products, and eggs are the most common vehicles of transmission. Such figures represent only a small fraction of total foodborne disease, since the etiologic agent is identified in only about one-third of the outbreaks, and many outbreaks are undetected or are not reported to the Centers for Disease Control. For *Salmonella*, it is estimated that each reported case represents about 100 total cases. The largest outbreak of salmonellosis in the United States occurred in 1985 in Illinois and Wisconsin, where an estimated 170,000 to almost 200,000 persons were infected with *Salmonella typhimurium* transmitted in pasteurized milk from a single dairy plant.

The incidence and recognition of rheumatoid disease occurring secondary to foodborne and waterborne diarrheal disease have also increased. These diseases include reactive arthritis, Reiter's syndrome, ankylosing spondylitis, septic and aseptic arthritis, ulcerative colitis, Crohn's disease, and Whipple's disease. *Y. enterocolitica*, *Y. pseudotuberculosis*, *Shigella flexneri*, *Shigella dysenteriae*, various salmonellae, *E. coli*, and *K. pneumoniae* have been associated with these chronic conditions.

Waterborne disease outbreaks due to *Enterobacteriaceae* are usually due to contaminated wells. Cases of shigellosis due to a contaminated wells have been reported; even typhoid fever has occurred fairly recently in community water systems contaminated with human sewage.

Enterobacteriaceae not normally associated with the GI tract or diarrheal disease may still be pathogens of humans. Most notably, *Yersinia pestis*, which does not have an intestinal habitat, is the etiologic agent of plague a highly fatal disease that has disseminated whole populations of individuals at several times in the history of civilization. Furthermore, most, if not all, *Enterobacteriaceae* are opportunistic pathogens. Once established, they can cause a variety of infections, including urinary tract disease, pneumonia, septicemia, meningitis, and wound infection.

According to the CDC, *Enterobacteriaceae* are responsible for 40-50% of nosocomial infections occurring in the United States. *E. coli* is the worst offender, followed by *Klebsiella*, *Proteus-Providencia-Morganella*, *Serratia*, and *Citrobacter*. The compromised host is particularly susceptible to nosocomial infections. Catheterized patients, patients on immunosuppressants, burn patients, cancer patients, and elderly patients are all especially vulnerable to opportunistic pathogens. To make matters worse, many of these organisms acquired in the hospital setting are multiply drug resistant.

Taxonomy and Classification of Enteric Bacteria

In artificial classification schemes (e.g. *Bergey's Manual of Systematic Bacteriology*, 1st edition, 1986) *Enterobacteriaceae* is a family of bacteria in Section 8 - **Gram-negative facultatively anaerobic rods**. Because of the large number and broad range of phenotypic properties that solidify the group, these traits being a reflection of their genetic relatedness, these bacteria have remained unified in modern phylogenetic schemes based on 16S ribosomal RNA comparison. Thus, *Citrobacter*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Escherichia*, *Klebsiella*, *Proteus*, *Providencia*, *Salmonella*, *Serratia*, *Shigella*, and *Yersinia* (along with several other genera, including *Hafnia*, *Morganella*, *Photorhabdus*, and *Xenorhabdus*) are presently classified in the subclass **Gammaproteobacteria**, order *Enterobacterales*, family *Enterobacteriaceae* in *Bergey's Manual of Systematic Bacteriology*, 2nd Edition, 2001.

The classic definition of an **enteric** bacterium is one that is found in the intestinal tract of warm-blooded animals in health and disease, but bacteriologists reserve the term for reference to *E. coli* and its relatives, even though some of the relatives of *E. coli* rarely or never are found growing in the GI tract. But in the end, this is one of the most close-related and cohesive groups of bacteria that can be brought together for discussion.

The Genus *Escherichia*

Theodor Escherich first described *E. coli* in 1885, as *Bacterium coli commune*, which he isolated from the feces of neonates. It was later renamed *Escherichia coli*, and for many years the bacterium was simply considered to be a commensal organism of the large intestine. It was not until 1935 that a strain of *E. coli* was shown to be the cause

of an outbreak of diarrhea among neonates.

Most investigations of enteric organisms at the turn of the 20th century were concerned with the problems of being able to distinguish the "typhoid bacillus" and other types of *Salmonella* from non-*Salmonella* organisms. Early workers also demonstrated that there were a number of types and subtypes of these organisms, which could easily be distinguished from the typhoid bacillus and *E. coli*. Thus, the biochemical techniques that have become the basis for most taxonomic studies came into being during the early 1900s. These studies led to the modern taxonomy of the group, which in principle is still valid today.

Initially, the family *Enterobacteriaceae* was created by Rahn in 1937, for the genus *Enterobacter*, and despite some debate about nomenclature among bacteriologists, the family name was maintained with the type genus becoming *Escherichia*. The family currently comprises Gram-negative, nonsporeforming, rod-shaped bacteria that are often motile by means of peritrichous flagella. The majority of strains grow well on the usual laboratory media in both the presence and absence of oxygen, and metabolism can be either respiratory or fermentative. The fermentation products of glucose and other carbohydrate substrates include mixed acids and (usually) detectable gas. Most strains are oxidase-negative and are able to reduce nitrate to nitrite.

The taxonomic distinctiveness of *Escherichia* has been confirmed by rRNA-DNA heteroduplex studies. On the basis of DNA-DNA relatedness studies, the genera of enteric bacteria are placed into a series of groupings, with *Escherichia* and *Shigella* forming a close group distinct from their nearest group, which includes the genera *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Salmonella*.

Although most investigations of the genus *Escherichia* have centered on various aspects of the *E. coli* species, it should not be forgotten that a number of other species have been described, including *E. blattae*, *E. fergusonii*, *E. hermanii*, and *E. vulneris*. These species can be differentiated on the basis of a large battery of biochemical tests.

For many years it has been realized that there exists a close relationship between two genera of enterics, *Escherichia* and *Shigella*. This is true for their biochemical characteristics as well as various other phenotypic traits. Also, studies of certain *E. coli* antigens have shown a close relationship ("cross reactivity") with *Shigella* antigens. The "O" antigens of virtually all serotypes of *Shigella* are either identical with or closely related to those of *E. coli*. The discovery that the characteristic "invasiveness" of *Shigella* strains is also possessed by certain types of *E. coli*, which have become known as enteroinvasive *E. coli* (EIEC), also suggests a close relationship. EIEC can cause dysentery-like symptoms clinically indistinguishable from those caused by strains of *Shigella*. Furthermore, antigens of *E. coli* strain O124 are shown to have a very close relationship to *Shigella dysenteriae* type 3 antigens, and a serological relationship between *E. coli* strain O129 and *S. flexneri* type 5 is also known. Finally, enterohemorrhagic strains of *E. coli* (EHEC), specifically *E. coli* O157:H7 produce the shiga (vero) toxin which is identical to the toxin produced by *Shigella dysenteriae*. The point being that it has become apparent that the line dividing these two genera of enteric bacteria is exceedingly thin, and it should be remembered that on the basis of DNA relatedness alone, *Shigella* and *E. coli* have been considered one genus.

Detection and Isolation of *Escherichia coli*

***E. coli* as an Indicator of Fecal Pollution**

For most of the 20th century, *E. coli* has been used as the principal indicator of fecal pollution in both tropical and temperate countries. *E. coli* comprises about 1% of the total fecal bacterial flora of humans and most warm-blooded animals. Sewage is always likely to contain *E. coli* in relatively large numbers. In addition, *E. coli*, being a typical member of the *Enterobacteriaceae*, is presumed to have survival characteristics very similar to those of the well-known pathogenic members of the family, *Salmonella* and *Shigella*. Thus, *E. coli* has been used world-wide as an indicator of fecal microbiological contamination. As such an indicator organism, its value is significantly enhanced by the ease with which it can be detected. and cultured.

Tests to identify isolates as *E. coli* have, of necessity, been simple tests designed predominantly to differentiate them from organisms normally associated with uncontaminated water. Since full biochemical analyses are not generally performed, the term "coliform" has been coined to describe *E. coli*-like organisms that satisfy these limited tests. As a result, regulations are promulgated throughout the world defining standards for water based on the so-called "coliform count." For example, in the U.S., according to a regulation published in the Federal Register (1986), there is a requirement that there be 0 coliforms/100 ml drinking water, as determined by any

method for any sampling frequency. Since not all organisms which meet the criteria of a coliform are associated with the intestinal tract (some may be saprophytic), a further distinction must be made between "fecal coliforms" (*E. coli*) and "nonfecal coliforms" (e.g. *Klebsiella* and *Enterobacter*).

Early attempts to distinguish strains of *E. coli* from other related *Enterobacteriaceae* centered on being able to distinguish them from the various pathogenic groups, since *E. coli* was initially not considered to be a pathogen. When *E. coli* was recognized to be a useful marker for fecal pollution, it similarly became important to distinguish it from related species likely to be found naturally in the environment. The realization that strains of *E. coli* generally ferment lactose, while those of *Salmonella* and *Shigella* do not, led to an early method of preliminary differentiation. The **IMViC tests** were developed in order to distinguish strains of *E. coli* from related species that also produced acid and gas from the fermentation of lactose. IMViC is an acronym in which the capital letters stand for **I**ndoole, **M**ethyl red, **V**oges-Proskauer, and **C**itrate.) The IMViC set of tests examines: the ability of an organism to (1) produce **I**ndoole; (2) produce sufficient acid to change the color of **M**ethyl red indicator; (3) produce acetoin, an intermediate in the butanediol fermentation pathway (a positive result of the **V**oges-Proskauer test); and (4) the ability to grow on **C**itrate as the sole source of carbon. Lactose fermenters are considered *E. coli* if they are positive in the first two tests and negative in the second two.

Detection of *E. coli* in Food

The International Commission on Microbiological Specifications for Foods (ICMSF, 1978) has adopted a set of standard techniques for the enumeration of *E. coli* in food products, accepted by the International Standards Organization (ISO, 1984). This method employs the use of lauryl sulfate tryptose broth at 35 or 37°C as a mildly selective-enrichment medium. This is followed by growth in EC broth containing 0.15% bile salts at 45°C as a second selective step. The ability to produce indole from tryptophan (in tryptone broth) at 45°C defines the strains as *E. coli*. These tests miss some types of *E. coli*, such as those most closely related to the *Shigella* group, but it is the detection of possible fecal contamination that is important in these tests rather than the presence of specific types.

Detection of *E. coli* in Water

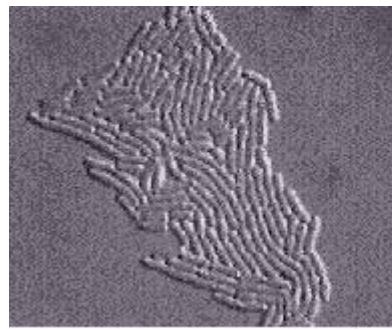
There is no method for the detection of *E. coli* in water that is accepted throughout the world. In the US, a standard method using membrane filter enumeration for both total and thermotolerant coliforms has been established (American Public Health Association (1986). Further IMViC tests on selected isolates can then be performed.

In the UK, the definition of *E. coli* in water microbiology is also based on the ability to produce gas from lactose and produce indole from tryptophan at 44°C. A method for enumeration employs a standard multiple tube test with a modified glutamate synthetic medium at 37°C as a first selective step, followed by further cultivation in standard media at 44°C.

Detection of *E. coli* in Clinical Specimens

While large numbers of *E. coli* will be found in fecal specimens or specimens contaminated with feces or intestinal contents, most other clinical specimens are usually not contaminated with *E. coli*. The major exception is urine, which requires special attention in the clinical situation. From those specimens in which *E. coli* is likely to be present in large numbers, direct plating on media such as MacConkey agar or Eosin Methylene Blue (EMB) agar is sufficient. If the number of *E. coli* is likely to be very low or the amount of specimen is limited, enrichment in a rich nutrient medium such as brain heart infusion broth may be used. A number of different commercially available kits are generally used to identify the isolates as *E. coli*.

From specimens likely to contain only a few viable *E. coli* cells, such as blood from patients suspected of having *E. coli* bacteremia, various enrichment procedures are used. Identification follows standard bacteriological techniques.



Left: *Escherichia coli* microcolony. **Right:** *E.coli* colonies on EMB Agar.

Rapid Methods for Detecting *E. coli*

A fluorogenic detection method has been developed based on the cleavage of methylumbelliferyl-D-glucuronide (MUG) to the free methylumbelliferyl moiety, which fluoresces a blue color after irradiation with long-wave ultraviolet radiation. Although strains of *E. coli* are generally positive in this test, some strains of *Salmonella*, *Shigella*, and *Yersinia* are also capable of splitting MUG; the latter two genera are usually not present in food. A disadvantage is that enterohemoragic *E. coli* (EHEC) strains are generally negative in this test. MUG can be added to various selective media, so there is a great potential in its use for detecting *E. coli*.

Automated or semi-automated systems are also being used for the detection of *E. coli* as part of the detection methods for *Enterobacteriaceae*. Techniques involving impedance measurements have shown promise. Other techniques such as immunoassays and nucleic acid hybridization studies can also be used to enumerate *E. coli*, and DNA probes directed at a number of genes have also been developed.

Physiology of *E. coli*

Physiologically, *E. coli* is versatile and well-adapted to its characteristic habitats. In the laboratory it can grow in media with glucose as the sole organic constituent. Wild-type *E. coli* has no growth factor requirements, and metabolically it can transform glucose into all of the macromolecular components that make up the cell. The bacterium can grow in the presence or absence of O₂. Under anaerobic conditions it will grow by means of fermentation, producing characteristic "mixed acids and gas" as end products. However, it can also grow by means of anaerobic respiration, since it is able to utilize NO₃ or fumarate as final electron acceptors for respiratory electron transport processes. In part, this adapts *E. coli* to its intestinal (anaerobic) and its extraintestinal (aerobic or anaerobic) habitats.

In the ecological niches that *E. coli* occupies, its abilities to grow both aerobically and anaerobically are important. *E. coli* is well adapted to its intestinal environment as it is able to survive on a relatively limited number of low-molecular weight substances, which may only be available transiently and at relatively low concentrations. The generation time for *E. coli* in the intestine is thought to be about 12 hours. The type of nutrients available there to *E. coli* consist of mucus, desquamated cells, intestinal enzyme secretions, and incompletely digested food. Given the absorption capacity and efficiency of the intestine, there are probably only small amounts free carbohydrates or other easily absorbable forms of nutrients, and there is competition from hundreds of other types pf bacteria. A similar situation probably also applies to sources of nitrogen.

In its natural environment, as well as the laboratory, *E. coli* can respond to environmental signals such as chemicals, pH, temperature, osmolarity, etc., in a number of very remarkable ways considering it is a single-celled organism. For example, it can sense the presence or absence of chemicals and gases in its environment and swim towards or away from them. Or it can stop swimming and grow fimbriae that will specifically attach it to a cell or surface receptor. In response to changes in temperature and osmolarity, it can vary the pore diameter of its outer membrane porins to accommodate larger molecules (nutrients) or to exclude inhibitory substances (e.g. bile salts). With its complex mechanisms for regulation of metabolism the bacterium can survey the chemical content its environment in advance of synthesizing any enzymes necessary to use these compounds. It does not wastefully produce enzymes for degradation of carbon sources unless they are available, and it does not produce enzymes for synthesis of metabolites if they are available as nutrients or growth factors in the environment.

Escherichia coli in the Gastrointestinal Tract

The commensal *E. coli* strains that inhabit the large intestine of all humans and warm-blooded animals comprise about 1% of the total bacterial biomass. This *E. coli* flora is in constant flux. One study on the distribution of different *E. coli* strains colonizing the large intestine of women during a one year period (in a hospital setting) showed that 52.1% yielded one serogroup, 34.9% yielded two, 4.4% yielded three, and 0.6% yielded four. The most likely source of new serotypes of *E. coli* is acquisition by the oral route. To study oral acquisition, the carriage rate of *E. coli* carrying antibiotic-resistance (R) plasmids was examined among vegetarians, babies, and nonvegetarians. It was assumed that nonvegetarians might carry more *E. coli* with R factors due to their presumed high incidence in animals treated with growth-promoting antimicrobial agents. However, omnivores had no higher an incidence of R-factor-containing *E. coli* than vegetarians, and babies had more resistant *E. coli* in their feces than nonvegetarians. No suitable explanation could be offered for these findings. Besides, investigation of the microbial flora of the uninhabited Krakatoa archipelago has shown the presence of antibiotic-resistant *E. coli* associated with plants.

Infections Caused by Pathogenic *E. coli*

E. coli is responsible primarily for three types of infections in humans: **urinary tract infections, neonatal meningitis, and intestinal diseases**. These conditions depend on a specific array of pathogenic (virulence) determinants possessed by the organism. Pathogenic *E. coli* are discussed elsewhere in the text in more detail at [Pathogenic *E. coli*: Gastroenteritis, Urinary tract Infections and Neonatal Meningitis](#).

Urinary Tract Infections

Uropathogenic *E. coli* cause 90% of the urinary tract infections (UTI) in anatomically-normal, unobstructed urinary tracts. The bacteria colonize from the feces or perineal region and ascend the urinary tract to the bladder. Bladder infections are 14-times more common in females than males by virtue of the shortened urethra. The typical patient with uncomplicated cystitis is a sexually-active female who was first colonized in the intestine with a uropathogenic *E. coli* strain. The organisms are propelled into the bladder from the periurethral region during sexual intercourse. With the aid of specific adhesins they are able to colonize the bladder.

The adhesin that has been most closely associated with uropathogenic *E. coli* is the P fimbria (or pyelonephritis-associated pili [PAP] pili). The letter designation is derived from the ability of P fimbriae to bind specifically to the P blood group antigen which contains a D-galactose-D-galactose residue. The fimbriae bind not only to red cells but to a specific galactose disaccharide that is found on the surfaces uroepithelial cells in approximately 99% of the population.

The frequency of the distribution of this host cell receptor plays a role in susceptibility and explains why certain individuals have repeated UTI caused by *E. coli*. Uncomplicated *E. coli* UTI virtually never occurs in individuals lacking the receptors.

Uropathogenic strains of *E. coli* possess other determinants of virulence in addition to P fimbriae. *E. coli* with P fimbriae also possess the gene for Type 1 fimbriae, and there is evidence that P fimbriae are derived from Type 1 fimbriae by insertion of a new fimbrial tip protein to replace the mannose-binding domain of Type 1 fimbria. In any case, Type 1 fimbriae could provide a supplementary mechanism of adherence or play a role in aggregating the bacteria to a specific mannosyl-glycoprotein that occurs in urine.

Uropathogenic strains of *E. coli* usually produce siderophores that probably play an essential role in iron acquisition for the bacteria during or after colonization. They also produce hemolysins which are cytotoxic due to formation of transmembranous pores in host cells. One strategy for obtaining iron and other nutrients for bacterial growth may involve the lysis of host cells to release these substances. The activity of hemolysins is not limited to red cells since the alpha-hemolysins of *E. coli* also lyse lymphocytes, and the beta-hemolysins inhibit phagocytosis and chemotaxis of neutrophils.

Another factor thought to be involved in the pathogenicity of the uropathogenic strains of *E. coli* is their resistance to the complement-dependent bactericidal effect of serum. The presence of K antigens is associated with upper urinary tract infections, and antibody to the K antigen has been shown to afford some degree of protection in experimental infections. The K antigens of *E. coli* are "capsular" antigens that may be composed of proteinaceous organelles associated with colonization (e.g., CFA antigens), or made of polysaccharides. Regardless of their

chemistry, these capsules may be able to promote bacterial virulence by decreasing the ability of antibodies and/or complement to bind to the bacterial surface, and the ability of phagocytes to recognize and engulf the bacterial cells. The best studied K antigen, K-1, is composed of a polymer of N-acetyl neuraminic acid (sialic acid), which besides being antiphagocytic, has the additional property of being an antigenic disguise.

Neonatal meningitis

Neonatal meningitis affects 1/2,000-4,000 infants. Eighty percent of *E. coli* strains involved synthesize K-1 capsular antigens (K-1 is only present 20-40% of the time in intestinal isolates).

E. coli strains invade the blood stream of infants from the nasopharynx or GI tract and are carried to the meninges.

The K-1 antigen is considered the major determinant of virulence among strains of *E. coli* that cause neonatal meningitis. K-1 is a homopolymer of sialic acid. It inhibits phagocytosis, complement, and responses from the host's immunological mechanisms. K-1 may not be the only determinant of virulence, however, as siderophore production and endotoxin are also likely to be involved.

Epidemiologic studies have shown that pregnancy is associated with increased rates of colonization by K-1 strains and that these strains become involved in the subsequent cases of meningitis in the newborn. Probably, the infant GI tract is the portal of entry into the bloodstream. Fortunately, although colonization is fairly common, invasion and the catastrophic sequelae are rare.

Neonatal meningitis requires antibiotic therapy that usually includes ampicillin and a third-generation cephalosporin.

Intestinal Diseases

As a pathogen, *E. coli*, of course, is best known for its ability to cause intestinal diseases. Five classes (virotypes) of *E. coli* that cause diarrheal diseases are now recognized: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC). Each class falls within a serological subgroup and manifests distinct features in pathogenesis.

Enterotoxigenic *E. coli* (ETEC)

ETEC are an important cause of diarrhea in infants and travelers in underdeveloped countries or regions of poor sanitation. The diseases vary from minor discomfort to a severe cholera-like syndrome. ETEC are acquired by ingestion of contaminated food and water, and adults in endemic areas evidently develop immunity. The disease requires colonization and elaboration of one or more enterotoxins. Both traits are plasmid-encoded.

ETEC adhesins are fimbriae which are species-specific. For example, the K-88 fimbrial Ag is found on strains from piglets; K-99 Ag is found on strains from calves and lambs; CFA I, and CFA II, are found on strains from humans. These fimbrial adhesins adhere to specific receptors on enterocytes of the proximal small intestine.

Enterotoxins produced by ETEC include the LT (heat-labile) toxin and/or the ST (heat-stable) toxin, the genes for which may occur on the same or separate plasmids. The LT enterotoxin is very similar to cholera toxin in both structure and mode of action. It is an 86kDa protein composed of an enzymatically active (A) subunit surrounded by 5 identical binding (B) subunits. It binds to the same identical ganglioside receptors that are recognized by the cholera toxin (i.e., GM1), and its enzymatic activity is identical to that of the cholera toxin.

The ST enterotoxin is actually a family of toxins which are peptides of molecular weight about 2,000 daltons. Their small size explains why they are not inactivated by heat. ST causes an increase in cyclic GMP in host cell cytoplasm leading to the same effects as an increase in cAMP. STA is known to act by binding to a guanylate cyclase that is located on the apical membranes of host cells, thereby activating the enzyme. This leads to secretion of fluid and electrolytes resulting in diarrhea.

Symptoms ETEC infections include diarrhea without fever. The bacteria colonize the GI tract by means of a fimbrial adhesin, e.g. CFA I and CFA II, and are noninvasive, but produce either the LT or ST toxin.

Enteroinvasive *E. coli* (EIEC)

EIEC closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. EIEC

penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes a dysentery-like diarrhea with fever. EIEC apparently lack fimbrial adhesins but do possess a specific adhesin that, as in *Shigella*, is thought to be an outer membrane protein. Also, like *Shigella*, EIEC are invasive organisms. They do not produce LT or ST toxin and, unlike *Shigella*, they do not produce the shiga toxin.

Enteropathogenic *E. coli* (EPEC)

EPEC induce a watery diarrhea similar to ETEC, but they do not possess the same colonization factors and do not produce ST or LT toxins. They produce a non fimbrial adhesin designated intimin, an outer membrane protein, that mediates the final stages of adherence. Although they do not produce LT or ST toxins, there are reports that they produce an enterotoxin similar to that of *Shigella*. Other virulence factors may be related to those in *Shigella*.

Adherence of EPEC strains to the intestinal mucosa is a very complicated process and produces dramatic effects in the ultrastructure of the cells resulting in rearrangements of actin in the vicinity of adherent bacteria. The phenomenon is sometimes called "attaching and effacing" of cells. EPEC strains are said to be "moderately-invasive" meaning they are not as invasive as *Shigella*, and unlike ETEC or EAEC, they cause an inflammatory response. The diarrhea and other symptoms of EPEC infections probably are caused by bacterial invasion of host cells and interference with normal cellular signal transduction, rather than by production of toxins.

Some types of EPEC are referred to as Enteroadherent *E. coli* (EAEC), based on specific patterns of adherence. They are an important cause of traveler's diarrhea in Mexico and in North Africa.

Enteroaggregative *E. coli* (EAEC)

The distinguishing feature of EAEC strains is their ability to attach to tissue culture cells in an aggregative manner. These strains are associated with persistent diarrhea in young children. They resemble ETEC strains in that the bacteria adhere to the intestinal mucosa and cause non-bloody diarrhea without invading or causing inflammation. This suggests that the organisms produce a toxin of some sort. Recently, a distinctive heat-labile plasmid-encoded toxin has been isolated from these strains, called the EAST (EnteroAggregative ST) toxin. They also produce a hemolysin related to the hemolysin produced by *E. coli* strains involved in urinary tract infections. The role of the toxin and the hemolysin in virulence has not been proven. The significance of EAEC strains in human disease is controversial.

Enterohemorrhagic *E. coli* (EHEC)

EHEC are represented by a single strain (serotype O157:H7), which causes a diarrheal syndrome distinct from EIEC (and *Shigella*) in that there is copious bloody discharge and no fever. A frequent life-threatening situation is its toxic effects on the kidneys (hemolytic uremia).

EHEC has recently been recognized as a cause of serious disease often associated with ingestion of inadequately cooked hamburger meat. Pediatric diarrhea caused by this strain can be fatal due to acute kidney failure (hemolytic uremic syndrome [HUS]). EHEC are also considered to be "moderately invasive". Nothing is known about the colonization antigens of EHEC but fimbriae are presumed to be involved. The bacteria do not invade mucosal cells as readily as *Shigella*, but EHEC strains produce a toxin that is virtually identical to the Shiga toxin. The toxin plays a role in the intense inflammatory response produced by EHEC strains and may explain the ability of EHEC strains to cause HUS. The toxin is phage encoded and its production is enhanced by iron deficiency.



E. coli O157:H7 Transmission EM. American Society for Microbiology

Biotechnological Applications of *E. coli*

The advances in molecular biology, genetics and biochemistry during the past four decades have led to an enormous development in the field of biotechnology. Studies with *E. coli* have played a major role in these developments, and the bacterium has been in the forefront of many technological advances.

In the early days of biotechnology (1960s), emphasis was placed on improvements of established procedures of bioprocessing, such as the production of yeasts, vaccines, and antibiotics. These investigations stimulated genetic research of microbes to increase their potential to produce a wide variety of products in the service of humanity. Although much was being learned about *E. coli* and its genetics, the direct use of the bacterium in the industry was limited. The industrial production of the amino acid threonine by *E. coli* mutants, begun in 1961, is an exception. At this time, organisms were generally subjected to mutagenic agents, which produced a series of random mutations, from which the specifically required mutants were selected.

In the last two decades, procedures have evolved which permit the preparation of strains that have very specific productive capabilities. As the genetic structure of *E. coli* was well known, and it is an organism which can grow on simple media (mineral salts and glucose) under aerobic and anaerobic conditions, the bacterium became the basis for most developments in genetic manipulations leading to genetic engineering.

The basic principle of these genetic manipulations is gene cloning, which enables the isolation and replication of individual DNA fragments. This consists of a series of linked steps, involving the isolation of the desired gene as double-stranded DNA (dsDNA), insertion of the gene into a suitable vector, and using the vector to introduce the DNA into a cell which will express the desired genetic information. In the case of cloning a gene in *E. coli*, first the DNA of suitable character is isolated, then it is joined to the DNA of a suitable vector producing a series of recombinant molecules. Then the recombinant molecules are introduced into the bacterium in which the target gene becomes established. Recombinants are selected in various ways with the purpose of expressing the desired genetic information.

The source for DNA cloning can be genomic DNA fragments, cDNA fragments produced by the action of reverse transcriptase on mRNA molecules, chemically synthesized oligonucleotides, or amplified DNA from the products of the polymerase chain reaction (PCR). Plasmids, phages, and cosmids have all been successfully used as vectors, and transformation, transfection, and transduction have all been used to introduce the foreign DNA into the *E. coli* cell. Plasmids are among the most widely used vectors for the insertion of foreign DNA into an *E. coli*. Plasmids lend themselves very well as vectors since they are independent replicons which are stably inherited in an extrachromosomal state and can be made to carry easily identifiable phenotypic markers such as antibiotic resistance or sugar fermentation.

An example of the use of plasmids to introduce a foreign gene into *E. coli* in order to produce a useful product is illustrated by the use of the *E. coli* plasmid pBR322 to clone the gene for production of the human growth hormone, somatostatin. In this case, the gene for the small polypeptide hormone was produced by synthetic means. The double-stranded DNA coding for the 15 amino acids of somatostatin was synthesized with the addition of a translation stop signal at the end. The synthetic gene was then recombined with the plasmid within the beta-galactosidase structural gene and introduced into *E. coli*. In this way, the production of the somatostatin peptide could be controlled by the lac operon. In a similar manner, the genes for human insulin production were inserted into *E. coli* which was then able to synthesize the human hormone.

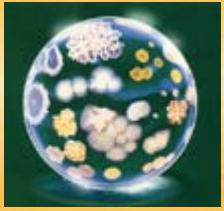
Such general techniques of molecular biology and bacterial genetics are now being applied within research laboratories and industry to produce a wide variety of strains of genetically engineered *E. coli* from which a number of useful products can be produced. Likewise, the problems associated with the expression of eukaryotic DNA by a prokaryotic promoter in *E. coli* were solved by construction of a fusion gene. In this system, the control region and the N-terminal coding sequence of an *E. coli* gene are ligated to a eukaryotic sequence so that translation of the chimeric protein can occur. The only condition is that the eukaryotic sequence must be in the correct reading frame. The desired protein is then enzymatically or chemically cleaved from the *E. coli* product.

E. coli strains have been genetically engineered to produce a variety of mammalian proteins, especially products of medical or veterinary interest including enzymes and vaccine components. *E. coli* has also been used to manufacture other substances including enzymes that are useful in the degradation of cellulose and aromatic compounds and enzymes for ethanol production. There may be no limit to what *E. coli* can produce through recombinant DNA technology as long as the substance is a natural product for which a genetic sequence can be found.

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Dedication

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This work is dedicated to Hans Zinsser, a great American microbiologist of the early 20th century, who rightfully claims the name of the original **Textbook of Bacteriology**.

Hans Zinsser was born November 17, 1878, in New York.

Zinsser obtained his doctorate from Columbia University in 1903. From 1903 to 1910 he was a bacteriologist at Roosevelt Hospital, an assistant pathologist at St. Luke's Hospital, and instructor for bacteriology at Columbia University. In 1910 he became associate professor at Stanford University, then full professor at Columbia University in 1913, and professor at Harvard University in 1923.

Zinsser made major contributions to bacteriology and public health. In 1906 he developed a medium and a simple method to plate anaerobic organisms. He did extensive work on typhus, and in 1934 he developed a vaccine of killed rickettsias that would protect against typhus.

Zinsser was an assistant to bacteriologist Philip Hanson Hiss (1868-1913), and was his co-writer on six editions of the **Textbook of Bacteriology**, published from 1910 until 1928.

Zinsser published **Rats, Lice and History** in 1935 "being a study in biography, which, after 12 preliminary chapters indispensable for the preparation of the lay reader, deals with the life history of typhus fever".

"We have chosen to write the biography of our disease because we love it platonically — as Amy Lowell loved Keats — and have sought its acquaintance wherever we could find it. And in this growing intimacy we have become increasingly impressed with the influence that this and other infectious diseases, which span — in their protoplasmic continuities — the entire history of mankind, have had upon the fates of men."

Hans Zinsser died in 1940.

Adapted from Ole Daniel Enersen, [Hans Zinsser \(www.whonamedit.com\)](http://www.whonamedit.com)

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