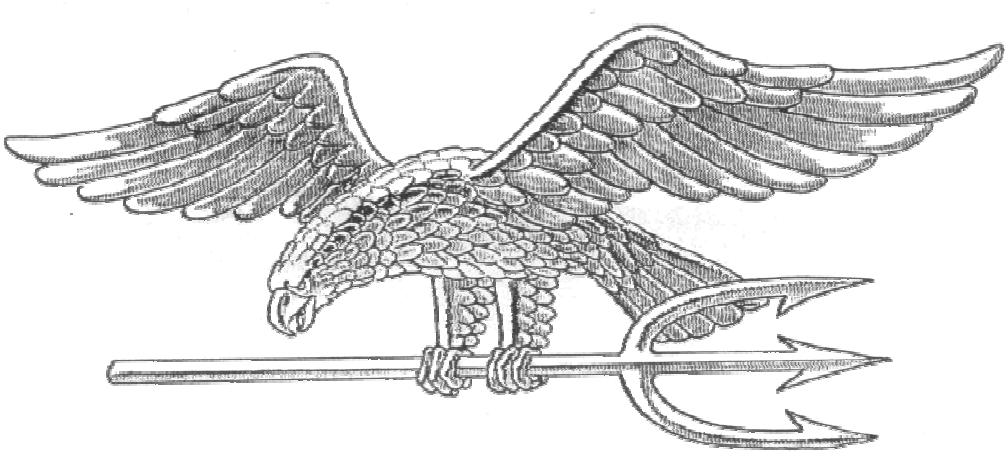
Scientific Principles of Improvised Warfare & Home Defense

Advanced Biological Weapons Series



Volume 6-E Modified Bacteria Weapons

Germ Attacks are the "Gift That Keeps on Giving"
President William Clinton, 21 Jan 1999 in an interview with New York Times reporters

Timothy W. Tobiason Author, 15 Nov 2001

This book is dedicated to the many Americans who have stood up to the government of the United States, only to be subjected to professional psychological operations, harassment, dirty tricks, and entrapments that have imprisoned most of them and ruined many others. The unaccountability of this governments secret and dirty operations will never be brought to light in the pretend democracy of the United States.

By being able to use biological markers to identify and unmask the governments dirty little operators, and by equipping the public with the knowledge to fight back on an invisible battlefield on equal terms with this government, real justice and reform may one day be possible.

I am also dedicating this book to the Omaha World Herald who refused to publish, or even listen to the first accounts of the attempted murder of this author on the way home from a gun show or the deployment of military personnel to my home afterwards. When a story was finally produced in the aftermath of the Anthrax attacks, the OWH ignored the fact that I had published and hand delivered to every member of the US Senate the instructions on how to conduct postal warfare with anthrax months earlier. I had been subsequently interviewed by the US Secret Service and FBI about it, with written details and photographs taken during the interview.

The OWH reporter framed his news story in such as fashion as to be insulting to me personally and to denigrate, discredit, belittle and ridicule my own story and published works. In a personal conversation with him afterwards, I asked him if the FBI had written the story. After a pause, he said that he had. I told him that I doubted that such a story could ever be considered a serious work of journalism given the background he had been provided. The obvious answer was that it was never intended to inform the public of the serious issues and charges that I had made and bear personal witness to.

The way to fight back against lies and crooked government is with the solid application and use of science in all its forms, including the application of military sciences. A well armed and completely informed public is the only protection citizens have against the tyranny of the self interested, pretend democratic state we live under.

Timothy W. Tobiason November, 2001

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Preface

The expression “knowledge is power” has always translated directly in the application of sciences for weapons production. Those early humans who knew how to make bows and arrows could slay enemies who did not at a distance and use this power to kill and conquer. Those civilizations that did not adapt this new weapon found themselves at a military disadvantage and in some cases found themselves extinct.

The United States became a free nation because its citizens knew how to make firearms and arm themselves sufficiently to fight an army. Modern day biological sciences can be applied to do likewise.

By learning the couple of hundred new words in volume 6-A, a person acquires the ability to grow his own microorganisms from any soil sample or other source he wishes. He has a rudimentary understanding of disease and can use it to inflict disease upon an enemy or understand it to protect himself and his family.

Volume 6-B and 6-C covers a few hundred more new words, most of which are plant and mold names. Some new categories of toxins are described but the material adds considerably to a persons repertoire. Volume 6-D can be read without having to understand what any new words mean. The ideas and concepts are the most significant here. These ideas allow you to take an understanding of the words and use them in war (and peace).

This volume adds several hundred new words to your vocabulary. Some of it may come slowly but the rewards are great. Understanding what these words mean can insure that a government can never disarm its citizens by overcoming a single disease organism. These words can confer upon an educated individual the power to create new forms of life that do not exist today and to harness these new forms in war and peace. Knowledge of these biological sciences translates directly to enormous political and military power.

This type of power does not lead to any progress or accomplishment if used solely for killing. It does not confer institutional controls over people. It does not win their hearts and minds, or support. It may well only frighten them into surrendering their autonomy and rights to anyone in control of the institutions.

The application of biological weapons must be combined with a working knowledge of the appropriate revolutionary and political sciences that would accompany any revolution. Support must be gained from followers and these followers must believe in the cause enough to fight for it. They must know how to translate the fight into control of institutions and the media in order to control what people know. [People only know what things they are told about. They cannot know about things beyond their experience and education]. Those that control the educational and broadcasting institutions exert control as thought and knowledge police. Even in a free nation as the United States, we are not free of this. This is the reason for the existence of this book!

Introduction

The reader of this book should first study Volume 6-D, The Organization & Conduct of Biological Warfare and Volume 6-A, Bacteria Based Weapons to obtain some practice growing, handling and understanding bacteria cultures. This will greatly aid the reader in building a good foundation of skills and understanding for the material that will be published in this book.

This book is written so that ordinary citizens without advanced education or lab equipment, can learn, understand and be able to produce modified bacteria weapons for use in war and home defense. It will provide a basic understanding of the sciences involved and is not intended to substitute for college degrees in the applied biological sciences.

This public record of the United States government and practically all other governments in history is one of lying to its citizens, hiding or omitting the truth, and serving its own self interests. The public cannot vote based on information it doesn't have, and this author intends that the citizens of this nation and all nations have all information possible, even if governments deem that information to be dangerous or undesirable.

This author has had, over the course of three years, the US Army outside his house and has had to live with the daily and weekly harassment, dirty tricks, Psy Ops, threats, intimidation, and the burglary (while under federal surveillance) of his home. Attempted murder, and numerous acts of property damage and electronic warfare also make the list of notable grievances. This has been described in another work (Identifying Undercover Activity & Agents). It is in the spirit of these events that this author has produced this work.

This book should produce an appropriate balance of power between the US government & its citizens. Its entire contents originate from the University of Nebraska library and it is clearly public domain material that is intended to be available to all citizens (even the pain in the rear ones the government doesn't like).

Chapter 1
Bacteria Biology

The largest single killing force in the history of mankind is the group of organisms we call bacteria. About thirty of these single cell life forms can account for the majority of the loss of human life in all of recorded human existence. Medical advances in the last 100 years have enabled society to mitigate disease produced by bacteria and greatly prolong human life spans. The advances in biology, most notably genetic engineering, now offer the promise of producing millions of new bacteria species and bacteria derived forms of life that will arm and equip the armies of the future. These weapons will be invisible, unique and easily transferred to the bulk of human population's overnight within hours or days of their invention via the mail or other forms of delivery or distribution.

An understanding of bacteria biology is essential to the warrior of the future. These are the bullets that will be used in the future. They will be fired from disguised and invisible guns that will take the form of letters (the anthrax attacks of 2001), key chains in pockets, humidifiers, car exhausts and so on. Knowing how the bullets are produced (cultured), maintained, delivered and infect is essential in becoming expert at this type of warfare.

Bacteria History & Basics

In the 4th century BC, Aristotle observed that small insects would arise from substances that were not living like wood and soil with decaying vegetable matter. He formed his theory called spontaneous generation in which he believed that living things could rise spontaneously from lifeless substances. The idea that very small living things could reproduce sexually like man or asexually was not appreciated because they were too small to be observed. This idea would govern scientific thinking for more than 1,100 years until a new method could be developed to observe tiny life forms.

In the late 1600's, a Dutch merchant Anton van Leeuwenhoek invented a single lens microscope that had sufficient magnification to see the invisible bacteria. He learned to grind glass lenses with flawless accuracy and this enabled him too create magnifications of over 200 times. Some bacteria would move under his new microscope so he knew that these tiny specks were alive and not just specks of dirt. It would take almost two centuries until someone would be able to take the tiny living things and find a way to grow or culture them in pure form.

In 1655, the English Scientist Robert Hooke using his microscope, first used the word cell to describe compartments in cork that reminded him of small jail cells. These cells were empty because they had died and their contents had drained away. He soon studied living cells that he found were filled with fluid and the name cell was soon applied to all tiny living organisms with a wall holding its contents inside. They also soon discovered that all living cells come from the division of preexisting cells.

The job of growing bacteria in pure culture fell to a number of scientists in the 1800's. Louis Pasteur was the most famous and he demonstrated that some of the microbes could live without air, some were responsible for the fermenting of beer and cheese while others caused disease.

Over the years, Pasteur examined wine samples that had gone sour under a microscope and discovered oval bodies (yeast cells). He then examined grape juice, boiled it too kill the cells, and found that it failed to ferment. By adding back living yeast, he observed that the juice would now ferment. His study allowed the wine industry to sterilize grape juice and then select the desired yeast strains to produce the best fermenting properties.

During these experiments with yeast, he discovered that "sour" wines consistently had small microscopic rods present in the samples. He reasoned that if these small "bacteria" were removed from the juice , the wine might not become sour. He removed the bacteria by boiling the juice and killing them. The yeast were added back into the juice and the container sealed so new bacteria could not float in from the air and contaminate the sample. The juice fermented and produced the characteristic taste and aroma of the wine.

Physicians of this time had observed similar bacteria in the blood of the dead, but they thought that the presence of bacteria was the result of cells feeding off the dead tissue rather than being the cause of illness and death. Pasteur would develop the germ theory of disease and he would use this new knowledge as a basis for developing protection against infection such as rabies and other vaccines. He was unable to isolate the bacteria basic to his theory but his work would allow future scientists to isolate many more microbes and to study their biochemical and pathogenic properties. The history and science of identifying these properties of each important species is found in Volume 6A on Bacteria based weapons.

In the late 1800's, Robert Koch, a young country doctor from Germany, discovered food formulas for selectively isolating, growing and subsequently testing colonies of bacteria. He was the first to isolate a pure culture of a disease causing bacteria and he developed Koch's postulates for distinguishing disease causing organisms from the other bacteria around them. The basic method was to isolate pure colonies from the infection site and then introduce them in pure form to healthy animals (or people) to see if they would produce the same infection.

In the 1870's, Koch became interested in a deadly disease that turned the blood of dead animal's black and killed large numbers of sheep, cattle and goats. Often entire herds were lost. Koch conducted a now famous experiment. He took a syringe of blood from a dead animal and examined it under a microscope. He saw the sticks and rods known to physicians as bacteria and he sprayed the blood sample with these bacteria onto a plate of boiled beef broth that had been mixed with gelatin and solidified (Jell-O). He incubated the plate at body temperature (37C) and in two days he observed visible masses

or colonies growing ever larger on the surface. All the colonies consisted of only one pure type of bacteria.

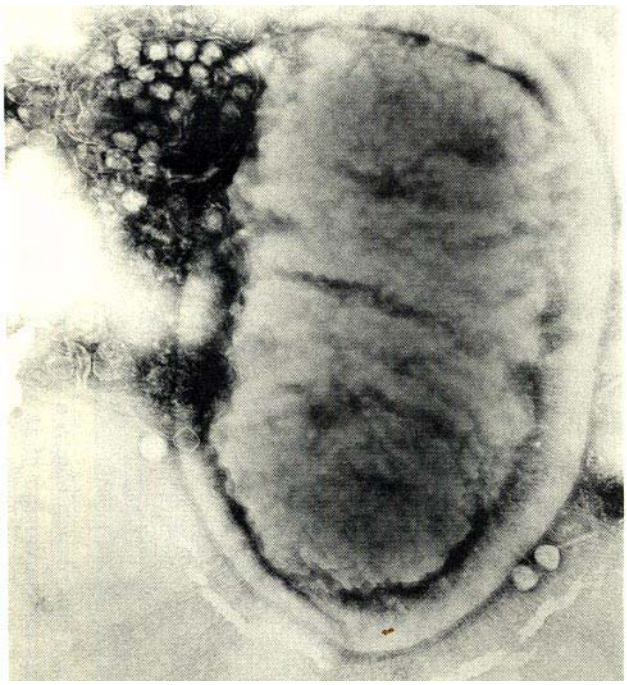
Koch had accomplished what Pasteur had not. He had isolated a disease causing organism in pure culture. Koch now took one of the colonies and injected it into a healthy mouse and in seven days the mouse died with the identical symptoms. On autopsy, the mouse had the same black blood as the original cow. He now took a sample of the black blood from the mouse and grew the bacteria again. He observed that the bacteria were the same under the microscope and on the gelatin plate. The disease that he could now reproduce was known as Anthrax. It was the first one to ever be isolated and identified in pure culture.

This led to "Kochs Postulates" which were the procedures from this experiment. This allowed any scientist anywhere to grow disease organisms and correctly identify them as the cause by using the suspect colonies to cause the same disease in other animals. In 1876, Koch presented his findings to the University of Breslau and his audience of scientists were astonished. He had finally proven Pasteurs germ theory of disease and this now allowed scientists from all over the world to isolate disease causing organisms and deal with epidemics. The sciences of microbiology and advanced medicine had begun.

Even as recent as 1977, new disease causing bacteria were still being discovered from nature with the outbreak of Legionnaires disease, and new strains of deadly E-coli which infected meat supplies.

Most bacteria feed on dead or decaying organic matter while a handful act as parasites and feed on living animal tissues. Both types can cause disease.

The bacteria are so small that the internal structures and functions of bacteria were still invisible to the best light microscopes. Viruses were much smaller still and could not be easily studied except when they infected bacteria. The observation of viral multiplication in bacteria cells led to many discoveries and new understanding. A virus that could infect a bacteria is called a **bacteriophage**. The working name of bacteria viruses has been shortened to **phage.** It appears that every bacteria in nature can act as a host for one or more phages. Some do not kill the host bacteria and are called **temperate phages**. These often cause the bacteria to have new properties after infection. Those that do kill the host usually take over the cellular machinery and force it to reproduce numerous of copies of itself. These copies are released when the bacteria die and the cell wall decays away (lysis).



A photo of a bacteria releasing numerous virus particles through its cell walls after infection, cell death and cell lysis.

Bacteria are identified and classified in a family tree of sorts. This permits their identification in groups with similar properties that can be sorted out using growth media and added “foods” which act as indicators of the bacteria biology. In general, all bacteria share almost all of the same internal organs such as ribosomes, mesosomes, and chromosomes which will have similar chemistry.

There is a great variation in the outside surface of the bacterial cell walls. Some bacteria produce capsules which often allow them to infect other living organisms (the capsule forms a protective shell-spores are a separate form of protective shell). The chemical composition of this wall will have different sugars, amino acids, and some will have flagella which allow them to move around like fish with flippers. These bacteria are called **motile**.

Almost all bacteria on earth do not cause disease and most are beneficial or necessary to the ecosystem. Although bacteria generally will only consume a few chemical forms of substances around them, all bacteria and fungi put together can use every physical, naturally produced substance on earth, which is why we do not have mountains of leaves, tree branches, animal bones and so on piled up miles high. These become bacteria and fungi food over time. The byproduct from consuming these materials is CO2 and N2 gas. Plants then use these gases to grow and produce oxygen as a by product and this is an important part of the ecosystem on earth.

Bacteria Reproduction

Bacteria reproduce asexually. This means that they grow physically larger and then divide in two making a perfect copy, or **clone** of itself. If the copy of itself is not perfect, then it is considered to be a **mutation** or **mutant**. It will have some new physical property that makes it different from its parent in some way. Most mutations are harmful and some prevent further division resulting in cell death.

From a single parent cell, a "pile" of cells, or a colony is formed. If the food is adequate, such as prepared Jell-O or agar with a nutrient soup mixed in, the colony grows so large that it can be seen with the naked eye. Colonies this size will contain billions of daughter cells all descended from the same parent. Each daughter continues to divide and produce more copies at the same time as the parent which allows the colony to grow exponentially. In a day, you might see a single dot on the agar which represents the billions of accumulated cells. In another day, if the cells are motile, they may swarm over the entire plate and completely cover it.

If the bacteria are grown in liquid mediums, they may produce chains or aggregates which clump together. Pure cultures are maintained by taking colonies from one plate and sub-culturing them onto another. Some bacteria divide every 20 minutes and the colonies can be seen in 10-24 hours. Others take days to divide and are not visible to the naked eye for as long as a month or more (These can be seen under a microscope however). Colonies of bacteria can be counted by taking a small sample of the colony, diluting it in a large volume of water, and then spreading the water out over another plate. The dilution can be a gallon of water to a square millimeter of colony. This can be diluted further if needed until the bacteria are spread out enough on the plates so they do not cover the plate and run together while growing.

In this way, bacteria can be counted and the percentage of **viable** counts is determined. Cells that reproduce themselves are viable or living. A sample taken every day of a colony from an original dish will show lower counts each day as the bacteria die off. Some bacteria may be hardier and live longer. Others, like anthrax may produce spores and yield viable counts years later. By refrigerating the original plate, the bacteria will die off much more slowly and by freeze drying, many bacteria can be preserved for decades.

Most bacteria grow on simple soups from beef hearts, blood or other animal tissues. Yeast extracts or soups also enhance growth of most bacteria. These soups are added to solid mediums like Jell-O or agar so that solid colonies can be grown. Each bacteria has different foods it prefers and produces distinct physical shapes that help identify them. These are described in detail in Volume 6A. Special substances are often added to produce color or physical changes to allow for easy and quick identification of a particular bacteria species.

In 1953, Watson and Crick discovered DNA. This material contains the instructions inside of every living thing that allows it to make copies of itself. All bacteria species contain DNA that are unique to each species. Each species can have small differences in its DNA which cause them to be called different strains. One strain of E-coli can infect humans and make them ill while other strains, lacking this tiny bit of DNA will not. When the DNA differences are large, they change the way the bacteria live and these are considered different species. By studying the DNA of bacteria (and all other living things) a greater understanding of replication, evolution, mutation, gene repair and expression has been achieved.

It was soon discovered that virus DNA can integrate itself into the DNA of a host bacteria and change its genetic instructions. It was also found that blocks of DNA from one bacteria can be exchanged with other bacteria by a vehicle called a **plasmid**. Plasmids have carried DNA blocks (called **genes**) from one species to another unrelated species which is how much of evolution takes place. By this method many new living things are created in nature. By using this knowledge man can speed up nature and by controlling it, can invent many new life forms.

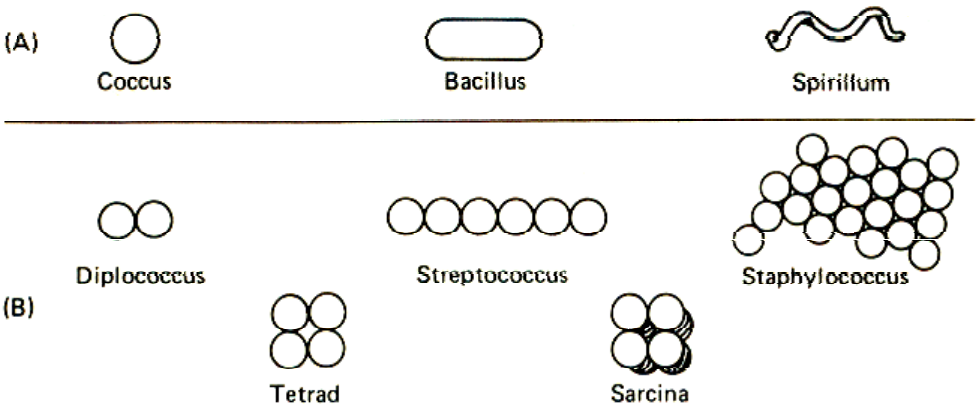
In the 1970's, scientists discovered that every gene in every living thing could be separated as a single block from the rest of the DNA. These genes could be separated by using an enzyme unique to that gene only. The discovery of these enzymes, one by one, allows scientists to separate every major gene sequence in every living thing. By growing cells without a gene sequence, you can discover what the gene does be seeing what the cell no longer can do. By inserting the same gene in another living organism, you can also discover what the gene will do.

Genes can be removed from one living organism and placed in another one. Using these methods, the gene to produce anthrax toxin can be removed from B. anthracis and inserted into an E-coli to make an E-coli version of anthrax that colonizes the human intestines and kills the host.

Bacteria Structures

Bacteria come in many different shapes and sizes. Anthrax is considered large measuring about 1 micron wide to 5 microns long. Just the right size to infect lower lung tissue as single cells. Haemophilus influenza is much smaller (.3 x 1 micron). Viruses are much smaller still. A large virus like smallpox is a bit smaller than Haemophilus while the polio virus is 100 times smaller. The size is similar to that of a cell phone (virus) to a small compact car (bacteria).

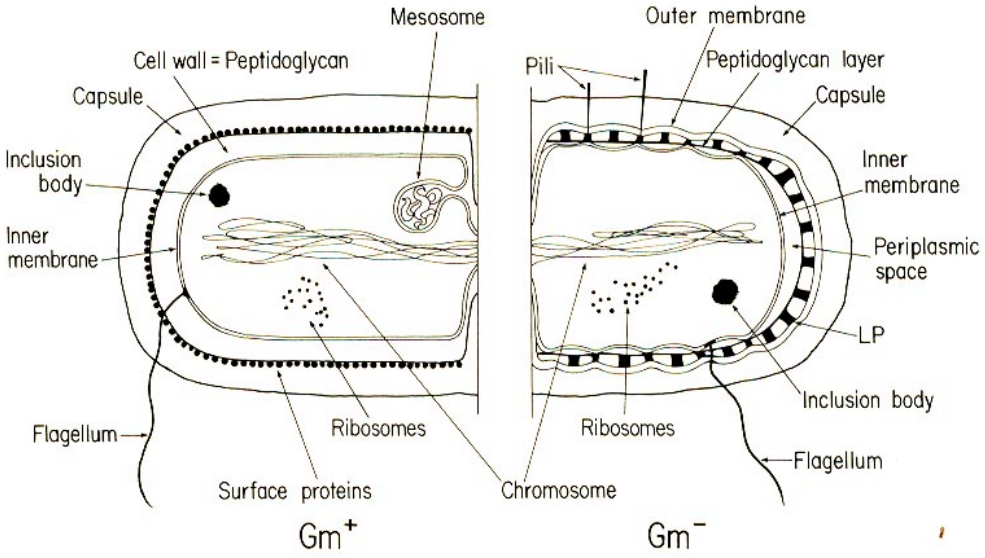
Bacteria in a species vary in size depending on many factors. A cell about to divide is about twice as large as both cells immediately after separation. Bacteria assume a variety of shapes. Some species are rods, others are spheres, while others form aggregates like pairs, chains, clusters and so on. These aggregates reflect the axes of cell division.



(A) Various shapes of bacteria. (B) Various arrangements of cocci.

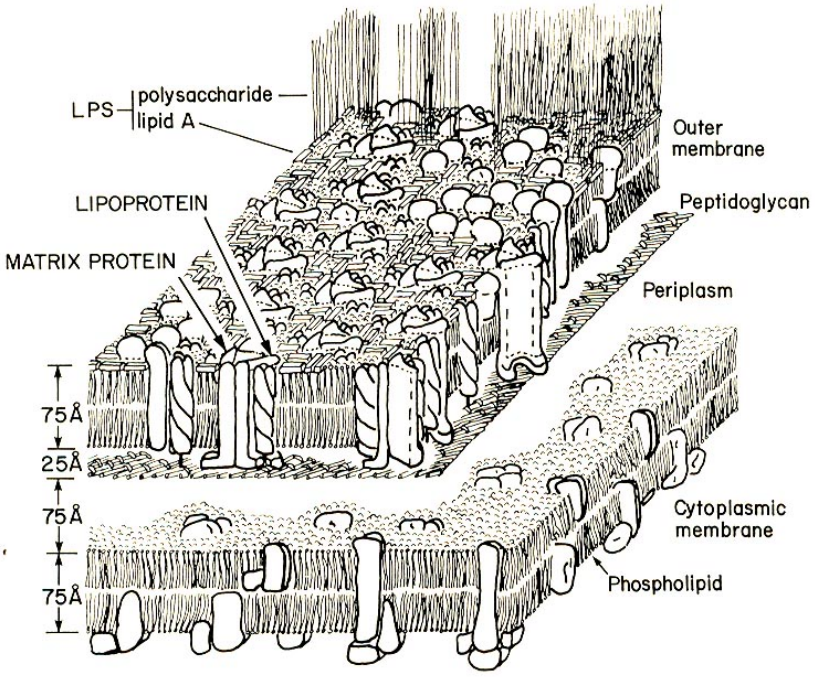
All bacteria have a single large piece of DNA called a chromosome. (Humans have many large pieces of chromosomes in pairs) There is no nuclear membrane or protective shell around the chromosome like human beings have. The cells of humans and other living things with a nuclear membrane surrounding the DNA are called **eukaryotes** while bacteria and other life without the membrane are called **prokaryotes**. The bacterial DNA makes up about 2% of the weight of the bacteria.

Also found inside of bacterial cells are **ribosomes.** These are pockets of proteins and RNA which synthsize proteins for use by the cell. The RNA are essentially transcripts of sections of the DNA which provide the genetic blueprint for the cell.



The inner skin layer is called the cytoplasmic membrane. It is composed of proteins and fatty acids that vary with temperature so that the membrane is mostly fluid and exists in a partly melted state. This allows water and small or select molecules to enter and exit while holding all the main parts and fluid inside. This cell membrane contains molecules of proteins arranged as globules as well as phospholipid molecules which move freely within the membrane. Materials that dissolve in lipid are dissolved by these molecules and allowed to pass through the membrane with the water.

The next layer is a rigid net known as the peptidoglycan layer. It is similar in all bacteria. It is composed of a polymer that is unique to bacteria. The building blocks of this polymer are called mucopeptides or mureine. Many antibiotics target the formation of this layer. Since human cells do not have this protective wall they are unaffected by the chemicals used to attack it. Penicillin was the first antibiotic discovered to prevent peptidoglycan synthesis. All antibiotics that inhibit peptidoglycan synthesis cause **lysis** of the cells. Lysis means that the cell leaks out the fluids from its insides. Some bacteria produce toxins that stay inside of its cell until the cell dies and lysis, or release of its internal fluids and other substances occurs. Lysis can come before cell death and causes the cells to die in those instances.

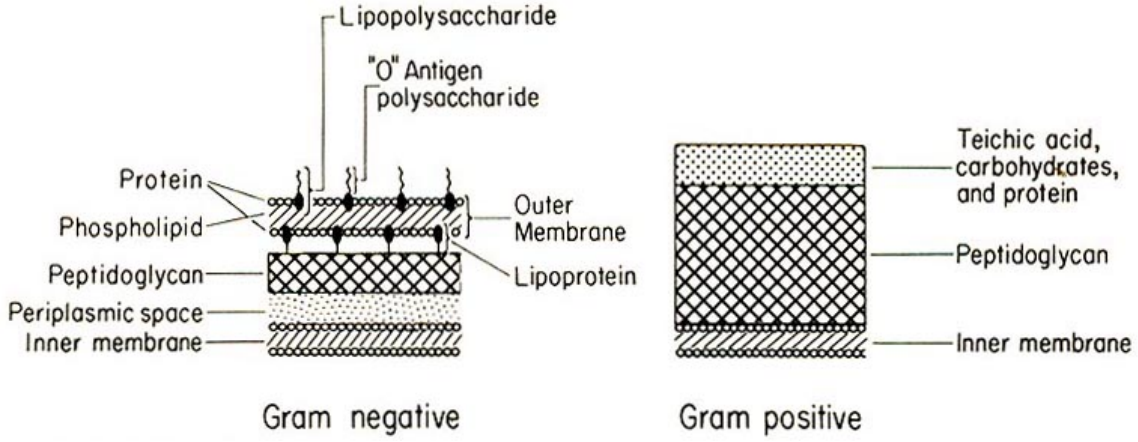


The cell wall has many more layers and all these layers together are known collectively as the cell envelope. Grams stain was developed to make the tiny bacteria more visible in light microscopy and it was discovered that differences in the cell envelope would cause some bacteria to stain blue while the other bacteria cell walls would stain red.

When using gram stains, bacteria are allowed to dry on a microscope slide and then "fixed" by heating the slide in a flame. A drop of blue dye (crystal violet) is added for a minute and then iodine is added to "fix" the dye to the stainable tissues. Alcohol is used to wash away the unfixed dye. A red counterstain (safranin) is then added an blotted to soak up free stain. A cover slip is put over the sample on the slide and then it is examined under the microscope.

Blue cells seen under the microscope are called gram positive while cells that are destained by the alcohol and become red from safranin are called gram negative. A few bacteria have an unusual cell envelope and do not stain either color. Mycobacteria which cause tuberculosis and leprosy are an example. They have a cell wall with a high content of unusual lipids including waxes containing huge fatty acids. These are stained using an acid fast method such as heating over a steam bath with the dye carbol fuchsin.

The peptidoglycan layer is typically much thicker in gram positive bacteria then it is in gram negative bacteria. A group of compounds make up the outer layer of gram positive bacteria and include teichoic acids. Teichoic acids are found only in gram positive bacteria and act as surface **antigens**. Antigens are compounds that interact with **antibodies** produced by humans and animals to ward off infection.



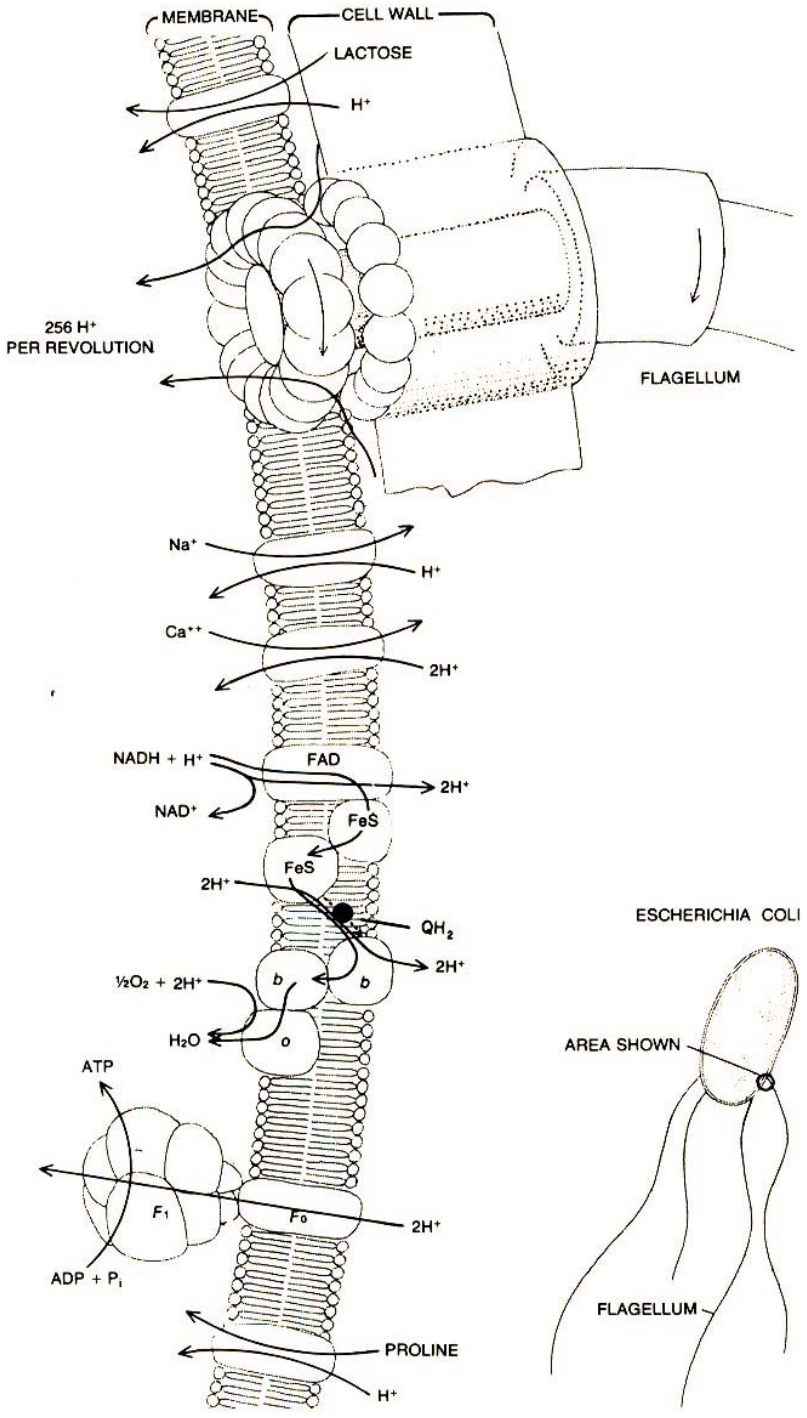
As you can see in the diagram, gram negative bacteria have a more complex cell wall. There is a **periplasmic space** between the inner membrane and peptidoglycan layer that is not found in gram positive bacteria. Gram negative bacteria also have a second **outer membrane** which contains a unique component called **lipopolysaccharide** or **LPS**.

The LPS molecules have three main parts. The first is **Lipid A** which is embedded in the outer membrane. It is the toxic component of **endotoxin** that is produced on the outside of bacteria and it is the susbtance that causes illness and death in infected animals or people. The endotoxins produced by the bacteria are usually different with each species and can be different with different serotypes within each species. This is why some E-coli are harmless to people, others cause mild food poisoning while others can be lethal. The LPS lipid A is different in each type.

The two other parts of LPS are core polysaccharides which are similar in all gram negative bacteria and then, on the outermost edge, attached to the core, are "**O" antigen polysaccharide** side chains. Like the surface antigens in gram positive bacteria, these also react with antibodies in humans and these reactions help determine which strain of bacteria a person is infected with.

Pores in the outer membrane allow small molecules of water and other substances to pass through it. The LPS and surface proteins selectively determine which other substances can pass through the membrane and they are effective at preventing many antibiotics from passing through these layers. When bacteria exchange genetic information, the instructions for making different proteins in the outer membrane can be exchanged. A bacteria that could be killed by an antibiotic can now become resistant if the new proteins can prevent the antibiotic from entering the cell.

[Penicillin affects bacteria by interfering with maintenance and synthesis of the cell wall. Some bacteria produce penillinase, an enzyme that converts penicillin to penicilloic acid which is harmless to the bacteria. These bacteria are **resistant** or immune to the antibiotics effect and the ability to produce penicillinase can be passed between bacteria species by plasmid exchange and other methods.]



The periplasmic space contains water soluble proteins which break down larger molecules and bind to them for transport inside the cytoplasm. These proteins are too large to escape through the outer membrane pores.

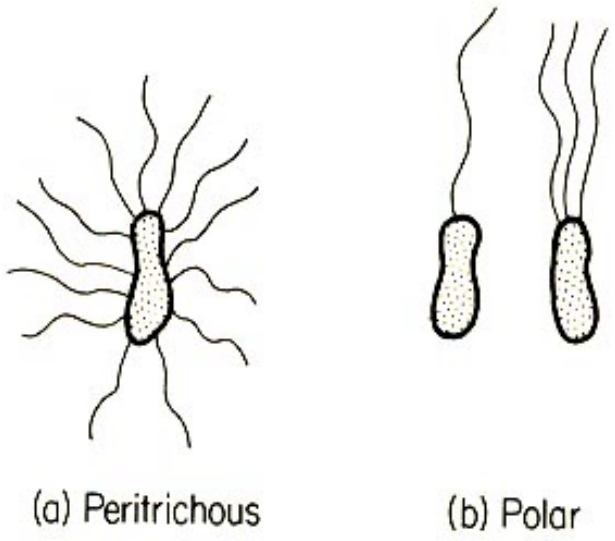
The outside layer of many gram positive and gram negative bacteria is often a capsule. The capsule is usually a loose gelatinous layer that is very thick and often larger than the bacteria cell itself. It is often so large that it can be seen without a gram stain using india ink as a negative stain on a microscope slide. The capsule often protects the bacteria from the bodies defenses which aids in its ability to infect. It impedes **phagocytosis** (white blood cells attacking and consuming bacteria) unless specific antibody to it is present.

Most capsules are made up of simple polysaccharides or polypeptides. In the case of anthrax, it is known that it must not only produce toxin, but must have a special protein capsule as well to be virulent and infect humans. [This capsule is not to be confused with spore formation which is a different outer protective shell that anthrax forms to effectively "hibernate" until surroundings contain food and a temperate environment.]

Capsules are important for infection and they are also important in medicine because they are antigenic. When an antibody is produced by the human immune system for a particular capsule, it is specific only for that capsule and no others. The bacteria species *Streptococcus pneumonia* has more than 80 capsules with different surface antigens. When a specific antibody reacts with the same specific capsular antigen, swelling occurs on this layer of the capsule and it becomes "refractile" and is visible under a light microscope. This is called the "quellung reaction".

Many bacteria also have **pili** which protrude from the cell wall and are composed of protein. Many of these antigenic as well. Mats of pili in some bacteria act as adhesive allowing the bacteria to attach itself to certain tissues like the intestinal wall.

Bacteria may also have **flagella** which allow the bacteria to "swim" around their environment. The flagella can be all over the cell (peritrichous) or just at one end of the cell (polar).



Bacteria Nutrition and Metabolism

All bacteria need food and water like human beings. Different species of bacteria will have different food that they can break down and utilize or grow on. Most bacteria can grow in soups of beef heart or boiled yeast because these contain all the simple proteins, sugars and trace minerals needed to survive and grow.

Some bacteria need very specific trace minerals energy and carbon sources to grow. *Stretococcus faecalis* requires the following specific growth factors in its diet –

Isoleucine	Tryptophan	Glutamate	Arginine	Uracil
Leucine	Tryrosine	Proline	Lysine	Pant. acid
Valine	Phenylalinine	Glycine	Histidine	Riboflavin
Cysteine	Alanine	Serine	Adenine	Thiamine
Methionine	Aspartate	Threonine	Guanine	Nicotinic acid
Pyridoxine	p-Aminobenzoic acid	Biotin	Folic acid	

This is a long list of proteins, B vitamins and other ingredients and most of these would be present in animal tissue soups. Some might not be and if a single item on the list is not present in the medium around the bacteria, it will not grow at all. All these ingredients are found in feed mills since most of these are required by all livestock to grow most efficiently. The proteins are usually found in soybean meal, blood meal, meat meal and so on. Boiled soups from these meals contain most of the proteins necessary for bacterial growth. Blood meal contains enrichment factors that are preferred for species like anthrax.

The cell composition of all bacteria is similar but bacteria vary in their ability to take in certain foods and break them down into needed molecules. E-coli for example can build all 20 of its needed amino acids by breaking down whatever proteins are available. [Proteins are made up of amino acids] It has all the metabolic pathways needed to make all the amino acids from different proteins and in some cases, bacteria can use inorganic nitrogen like ammonia to make up its amino acids.

E-coli and human cells have the machinery inside them to make **nucleic acid** bases while the *S. faecalis* with its long list of specific needs above does not. Many bacteria produce enzymes that allow it to break down food from its surroundings and take in the broken down components. These components can then be rebuilt to meet specific needs of the bacteria. Many bacteria can also synthesize their own vitamins if the necessary minerals are present.

These metabolic pathways are numerous and so complex that we will not go into them in detail here. They are important to understand because they let scientists make special media up of specific food ingredients so that bacteria can be selectively grown. By using special diets on culture plates, only some types of bacteria will grow while others cannot. This is also important in understand human infections.

[Anthrax does not have the cellular machinery to turn human skin, mucus, and the tissues in the upper lung linings into food. Therefore it cannot grow and infect in these areas. In the lower lung tissues-the alveoli, and in scratches in the skin where the underlying "meat" tissues are exposed, the anthrax can grow because these tissues meet its food requirements and contains essential germinating proteins that the anthrax cell walls detect. In order to infect a host, the bacteria must have all the essential food requirements met in its surroundings. This is why the use of food to pre-germinate and start infections is useful in bio-weapons and has been described in Volume 6-D.

This is also one of the more intriguing aspects of the potential of modified bacteria weapons. By inserting genes into anthrax from colonizing bacteria of the lungs, throat and nose, anthrax, and other prime candidates for weapons can be made communicable. Anthrax that can live on lung mucus and accumulated debris in the nostrils can grow & reproduce there while sending cells into the lower lungs and onto other individuals as they are expelled by coughing and sneezing. The mechanisms for using these new food sources are the small stretches of DNA, or genes that tell the cells to produce enzymes that make them into food. What anthrax may lack, an E-coli or Streptococcus can provide.]

Bacteria also need carbon as part of its food source. Most often this is met by the CO2 in the air (.02%) or by adding baking soda to the medium. If the air is inadequate, a candle is burned inside the sealed jar that the culture is grown in until it burns out. This raises the CO2 to several percent in the air. Some bacteria can use hundreds of different compounds to meet its sugar, protein and mineral requirements while others can only use a few. This is a reflection of the **catabolic pathways** that the organism uses in its digestion of the food. These pathways determine what food sources can be degraded to the basics and it is the difference in these pathways that determines if a bacteria can use human tissues as food. These pathways, as will be seen, can be exchanged between bacteria to give them new life properties. Methods which we will use to teach how this can be done include **mutation** by various methods, **cell fusion** or **hybridization**, **plasmid exchange** and **genetic engineering**.

Catabolic Pathways

Catabolic pathways are the machinery inside of cells that take chemicals in foods and break them down into very basic and simple sugars, amino acids and so on that the cells can use directly in breathing, growth and reproduction. These pathways can be very simple like converting maltose to glucose (simple sugars) or very complex and long as in conversion of glucuronic acid to pyruvate.

Many bacteria can switch pathways and use different foods. If a bacteria is growing on a simple carbon-sugar diet and is switched to a more complex diet, there is a delay of usually 30 minutes or more while the bacteria machinery adjusts to the new food and then begins to use it using a different pathway. This is called a lag phase or period.

During this period, the bacteria produces new enzymes that let it break down the new and more complex food chemicals (if it can).

The first step in the catabolic pathway is to break down food outside the cell into small enough molecules so that they pass through the cell membrane. This is usually accomplished by excreting enzymes into the surroundings that split the food into parts. These enzymes are specific for the chemistry of the food. If the surrounding food is made up of cellulose, the correct cellulase enzymes are excreted to break them down. If they are proteins, then correct proteinase enzymes are excreted.

[Think of the chemicals in the food as large appliances being sent into a recycling center. In order to use the parts of the appliances you use specific tools to loosen and remove various nuts and bolts so that the motor, moving parts, lids and so on can be removed and used further down the line. The cell uses different enzymes as its different size wrenches to break apart the food chemicals that it uses.]

Water and simple salts pass in and out of the cell by a process called **diffusion**. This can be demonstrated by watching water poured through a handkercheif and filling up a bowl. The water can pass back and forth through a handkercheif if you place it in the bowl. Smaller substances dissolved in the water will pass with it. The pores in the cell wall are so tiny that large molecules dissolved in the water cannot pass through. They require special transports into the cell. These transports are built by the cell and require energy to make them and use them to move chemicals in and out of the cell.

When some food chemicals such as simple sugars enter the cell, they are reacted with a substance that prevents their leaving the cell and escaping by diffusion. Some of the systems whereby bacteria draw in large amounts of food from the surroundings involve **periplasmic binding proteins** which bind to substances and have strong extracting powers for drawing the correct food molecules into the cells. The diagram on page 10 shows some of the important membrane proteins for E-coli.

There are many systems of transport and internal pathways used by bacteria to move and process food. The many different pathway combinations are often unique to each species and are used to help identify them. That is why some bacteria will grow on one type of food or soup and not on others. Gram positive bacteria do not have an outer membrane and therefore do not have transport systems like the E-coli. They use different methods for drawing sugars like maltose into the cell. Some do not need periplasmic proteins to concentrate maltose and may use assisted diffusion to draw in the sugar.

There are many transports used to bind and move minerals such as iron in E-coli. Iron is nearly insoluble in ionic form so bacteria have developed ways of **chelating** iron by excreting iron binding chelates into its surroundings. Once bound and drawn in, the iron is degraded (from Fe3 to Fe2 for example) before it is released and used by the cell.

Bacteria with flagella have a “sense” to swim towards concentrations of food in the environment and away from harmful substances and this process is called **chemotaxis**. More than 30 genes are known to be involved in the functioning of chemotaxis. This ability to move around could confer increased virulence in bacteria like anthrax that are not motile as well as increasing the prospects of it being contagious once they can migrate from the original location of infection to other sites.

Energy is the “currency” which the cell uses to do almost all its living functions. Like money which is used by everyone in society to buy and sell and moves things, all bacteria cells use energy for every transaction. This energy comes primarily from **ATP**. ATP is formed by fermentation reactions in the cytoplasm. Some of these fermentation reactions are very simple and do not have electron transport systems while other fermentation systems are more complex. Bacteria that cannot live in the presence of oxygen like Clostridium species will lack the more complex ATP producing systems. Lactic acid production is the simplest form of fermentation while bacteria can and will use many other types of sugar fermentation to build ATP.

Many bacteria and the cells of higher organisms can also make ATP through electron transport using oxygen in a process called **oxidative phosphorylation**. This transport system is found in the cytoplasmic membrane and produces much more energy for each molecule of food than fermentation will. Oxygen is used in these reactions as a final electron acceptor which has the effect of preventing substances from becoming toxic.

The best example of this is E-coli which produce lactic acid as a fermentation by product. If you grind and mix corn into a meal with water, the E-coli in the corn will begin to grow. If the corn mash is not stirred and is underwater, the E-coli grow and produce lactic acid which slowly lowers the pH in the mix. After about 24 hours, the corn is so acid that the bacteria die off from the toxic acid from fermentation. Bacteria growing on top of the medium are able to use oxygen in the air but they also die from the toxic acid. When growing in oxygen, E-coli can use the oxygen in the air for ATP energy and do not produce or accumulate toxins. They also grow much faster in oxygen and produce many more cells on the same amount of food when producing ATP this way. [Just think of fermentation to make ATP, like working for $1 an hour. By adding oxygen to the mix, you get a pay raise to about $10 an hour if you are a bacteria species.]

Anaerobic bacteria do not have oxidative phosphorylation to produce energy. They use other systems which can utilize organic compounds or nitrate instead of oxygen as receptors in the energy reactions. These can produce ATP for them instead. A few bacteria can derive energy from reactions with hydrogen or sulfur, while a handful can photosynthesize light (blue green algae).

The most primitive bacteria rely solely on fermentation for energy while each more advanced bacteria could use fumarate, nitrate, and finally molecular oxygen as the acceptor to produce energy (ATP). Bacteria however differ in their response to oxygen around them. These include –

Anaerobes or aerotolerant bacteria such as *Streptococcus* which form ATP from its substrate and cannot pass electrons to oxygen. Their growth is not affected by oxygen in the air around them. These organisms will also usually have soluble oxidases (enzymes) to deal with free oxygen that can act as an oxidizing agent to them.

Anaerobes like *Clostridium* can grow only in the absence of oxygen because the O2 in the air is toxic to them. There are two chemical reactions which use O2 as a final electron acceptor. Both of these reactions reduce O2 but also create a by product called an O2- radical called a superoxide. It is highly active and toxic in many ways. Almost all higher bacteria and animal cells produce a detoxifying enzyme called superoxide dismutase which reduces the superoxide to hydrogen peroxide (which can also be toxic and removed by cells producing catalase or other peroxidase) and O2. Clostridium lack superoxide dismutase and without it they cannot remove the toxic form of oxygen they produced.

[This led to one of the earliest ideas the author had for the category of planet killers. Clostridium is a primitive species on the evolutionary scale. By conferring on one of its members, Clostridium perfringens for example, the gene for producing superoxide dismutase, the primitive C. perfringens now leaps ahead on the evolutionary scale. If its toxin formation capabilities remain intact, it can now grow in oxygen and convert virtually all upper life forms into bacteria food on contact. (See volume 6-A for Clostridium species toxins and life cycles). Likewise, by converting C. botulinum by the same method, you may not infect everyone, but the organism would grow in the soil organic material yielding massive botulinum toxin and poisoning the planet with toxin production. Don't panic yet, the toxin is destroyed by oxygen so it would accumulate only in water and inside of packed soils. But you get the basic idea. This can be accomplished with all the Clostridium species and they can be further modified to do much more as we will see in later chapters.]

Facultative anaerobes are bacteria that can ferment to produce energy without oxygen, but also use oxygen to produce energy. Most bacteria can do both. E-coli and Staphylococcus are good examples.

Strict aerobes such as Mycobacteria tuberculosis or Pseudomonas will grow only in oxygen due to differences in the way they metabolize sugars.

In all animal cells, the reactions which use nitrogen are at the level of ammonia which is converted to glutamate and their is no oxidation or reduction of the nitrogen. Many bacteria and plant cells can reduce nitrate to obtain nitrogen which they turn into ammonia (NH4-by specialized soil bacteria). The process is called nitrification. Many bacteria can take atmospheric nitrogen and convert it to NH3 which is useful to plants in the soil via nitrogen fixation.

Some bacteria are so fastidious in the nutrients they require that even today it is difficult for scientists to create media on which they will grow. Some can only be grown inside of host cells (like viruses) to be studied. Some can grow on very simple nutrient mixes such as *Staphylococcus aureus* which can meet all its nutrient requirements from human sweat and other biological fluids.

Many bacterial species produce toxins that kill or injure hosts. When these cells kill a nearby animal cells (or the animal), the cells lyse and release their own cell parts as nutrients which the bacteria can now use as food. The diptheria toxin is so potent that a single molecule kills mammal cells upon entry through the cell wall (see volume 6-A for information on all major bacteria toxins).

Some bacteria produce a polysaccharide from sucrose which acts as an adhesive on teeth so that they can stay in a nutritious environment and ultimately cause tooth decay.

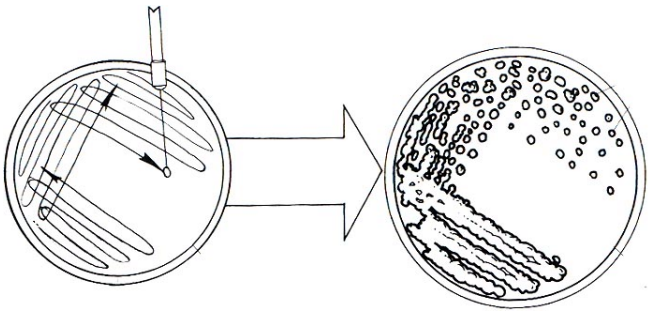
All these strategies can become useful in producing new types of weapons. Converting toxin producers that can meet all their basic requirements from human sweat is one strategy as is the reverse idea of converting the bacteria that live on sweat into toxin factories. Imagine producing anthrax that can live on and adhere to tooth enamel as opposed to blood and tissues. These forms of infection would be highly contagious and almost completely resistant to treatment with vaccines and antibiotics.

Growing Bacterial Cultures

The details of growing specific bacteria on nutrient gel or in liquid soups is covered in broad detail in Volume 6-A. A quick review is necessary for the reader to become familiar with the necessary concepts of growing bacteria.

Gel media are prepared by taking Jell-o, agar, or egg white, adding the desired nutrients to them and then sterilizing them by boiling in water or in the case of egg white, by frying. Agar and Jell-O are dissolved in liquid while boiling and then form a semi-solid mass while cooling. The result is a plate of nutrient gel.

Bacteria are “streaked” onto a plate of nutrient gel with a wire loop or swab or are spread in diluted form onto the surface with an eye dropper or pipette. This is called **inoculation**.



These plates are then incubated at 37 C for agar or egg, and below 78 F for Jell-O (so it stays below its melting point). If the nutrient needs of the bacteria are met, single cells form a pile of cells as they begin to grow into a **colony**. The repeated growth and cell division of all the cells causes enormous numbers to be produced (from doubling every 30 minutes or so) so that the colony often becomes visible in 12-24 hours. All the progeny of a single cell are clones of the original parent. A low number of the daughter cells will not be perfect clones due to **mutations**, or changes that occur in copying DNA. If two different cells are deposited next to each other during inoculation, both will grow adjacent or together with the genetic makeup of two parents.

A pure culture is maintained by repeated **subculture** of a colony. The colony is restreaked onto another plate or transferred from one liquid soup to another. Most bacterial work requires pure cultures that are known by desired species from the start. Liquid cultures are diluted and then a measured volume is spread evenly on the culture plate in a process called plating. This is done to produce a count of the living cells in the original volume and is called a **viable count**.

In virtually any sample of biological mass you will find a mix of many bacteria. In disease samples, many of the bacteria are normal "flora" which live on the host consuming waste materials and do not cause harm. The biological machinery that enables them to do this can be valuable when combined with toxin producing bacteria. The toxin or disease producing bacteria are usually recovered from the site of infection as part of the bacterial mix. These mixed samples are usually streaked onto a plate to dilute them and so that they form separate colonies which can be isolated from each other and studied. The different bacteria produce different sizes, shapes, textures, surface mucus, color and other properties described in pictures in Volume-6A.

Gel plates mixed with 5% blood (sheep is preferred) seem to support the growth of most disease and human related bacterial species. The use of blood and other complex nutrient substances make the media **enriched,** which supports the growth of finicky bacteria. Antibiotics and other ingredients can be added at different levels to inhibit some bacteria while allowing others to grow. Antibiotic resistant strains can be developed by

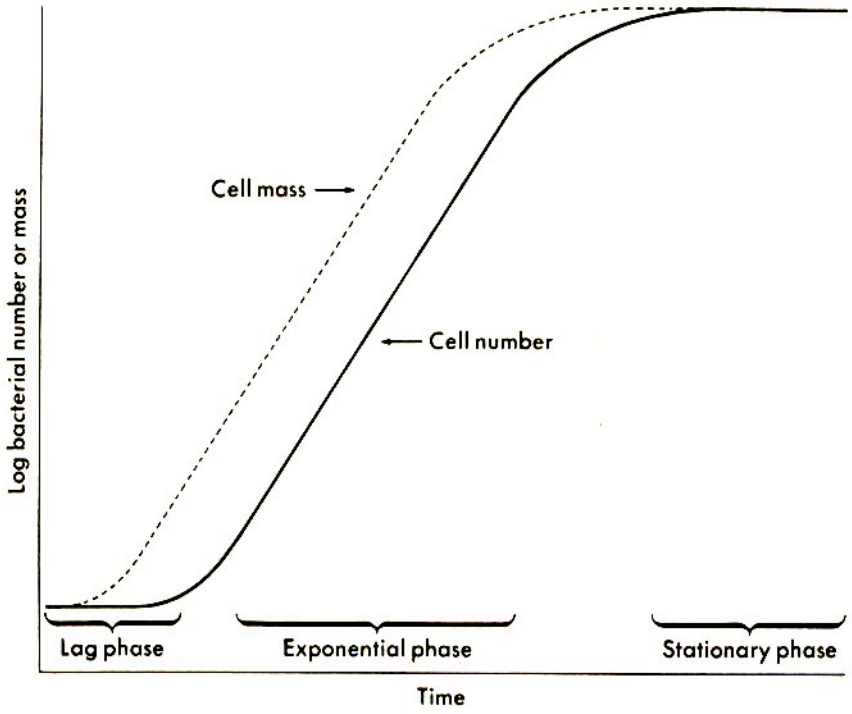
using very low levels of antibiotics and sub-culturing the best colonies onto progressively stronger plate dosages.

Most bacteria do not grow at pH of less than 5 while yeast strains will grow in this range. Some, like anthrax prefer a somewhat alkali environment for growth and spore formation.

Modifying bacteria depends on an ability to detect the rare mutants and genetic recombinants desired and specialized media are often used to selectively recover these strains. Mutations that are resistant to an antibiotic for example, are the only ones that grow with it in the medium with it added and this allows for the rapid screening of large numbers of cells. Only the mutations that can grow in its presence will grow on it.

Bacteria isolations are usually done on gel plates but most large scale production of very high numbers of bacteria is achieved by growing them in liquid soups. These soups are often rich broths of boiled yeasts and/or beef hearts which contain all the ingredients of cells rich in basic nutrients. All the necessary sugars, vitamins, minerals and amino acids necessary to meet the metabolic pathways of almost all bacteria species are found in these rich soups.

These liquid cultures are usually agitated or stirred mechanically to prevent the cells from settling and to provide oxygen in the liquid (aeration). Growth is measured by plate counts, turbidity (using a light meter to measure the cloudiness of the soup), cell counts on a microscope slide, or assaying for specific proteins or DNA. Most bacteria growth in liquid solution follows the chart below in which a lag phase occurs (while the machinery gears up to use the particular food in the soup) first. This is followed by exponential growth and ends in a stationary phase.



When cells are taken from the exponential phase and placed into an identical fresh medium, there is no lag phase. If the medium is different, they usually go through the lag phase first.

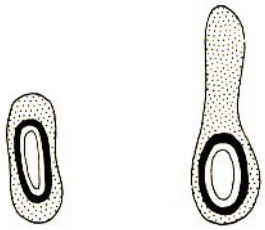
During exponential growth, the mass of cells essentially doubles for the average cell generation time (usually 30-45 minutes). As the cell density increases, nutrients become depleted and wastes become toxic to the cells, the cells change their metabolic pathways and their cellular composition. Fresh medium can be introduced while cells and waste products are removed or harvested in order to maintain the culture in exponential phase. This dilution maintains a constant environment for the bacteria.

It has been found that fast growing cells are usually physically larger than slower growing ones and they are richer in ribosomes and may contain two copies of the same chromosome.

When the nutrients become depleted or the medium acculmulates too much toxin or waste (usually acid), the growth stops. This usually occurs at about 10,000,000 cells per ml or about 1/1000 of the total liquid volume. The cells in this stationary phase in nature must either remain viable (such as anthrax producing spores) or die. Some use their neighboring cells or parents that die off as food, while others become dormant and go into chemical hibernation.

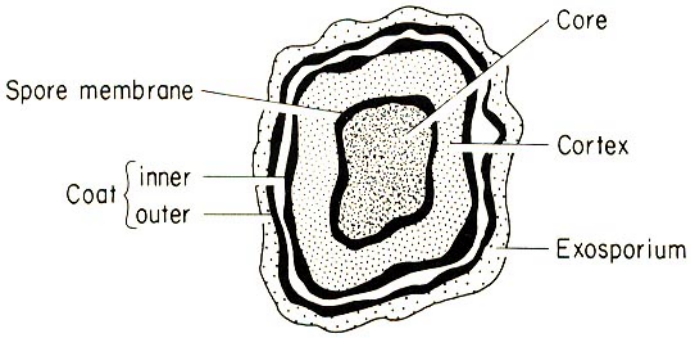
Usually, viable counts fall off fairly rapidly in the stationary phase. With disease organisms like plague or tularemia, the viable counts fall off rapidly and die off almost completely in a few hours or days without a fresh host or medium.

When the supply of food is limited or runs out, several gram positive bacteria form highly resistant, dehydrated outer shells and cell mass called **endospores** or **spores**. These have no metabolic activity and are adapted for long survival under intense heat, cold, drying, freezing, radiation and even in toxic surroundings. Clostridium, which cannot live in air and anthrax, are the best known of this type of bacteria. It takes about 6-8 hours to produce a mature spore. The most common shapes of spores are seen in the following diagram.



This form of protection is believed to be due mostly to drying since it is known that most bacteria that do not form spores can be preserved indefinitely by removing their water in the frozen state (freeze drying) and keeping them in a vacuum (lyophilization).

In spore formation, the nucleus of the cell and its cytoplasm become walled off with a series of new protective layers. The core become surrounded by a spore membrane which has an exterior cortex made up of peptidoglycan structure and finally an exterior coat which is usually made up of proteins rich in cysteine. These proteins cross link different chains by disulfide bridges (like keratins). This coat provides strong chemical resistance and this resistance is completely absent in mutants with a defective coat. It also protects the cell from ultraviolet and ionizing radiation. In some spores there is an outer lipoprotein layer called the exosporium.



The spore core contains only a fraction of the original cell material. Its DNA and a small amount of protein synthesizing machinery will let it produce all the parts needed for it to resume growth. There is no detectable messenger RNA, amino acids, and related enzymes in spores. Upon germination, messenger RNA (mRNA) is formed after hydrolysis of storage proteins.

When cells **germinate**, they form vegetative cells. Usually, the spores are activated by some external agent such as heat, low pH, or sulfhydryl compounds. Favorable medium can also cause **activation**. Many spores activate due to damage of the impermeable coat which is often seen in aging or older spores. It can also be induced by grinding the spores with glass powder which damages the exterior coat. [This is also an interesting form of preparing spores for immediate use in weapons because they begin their activation phase immediately].

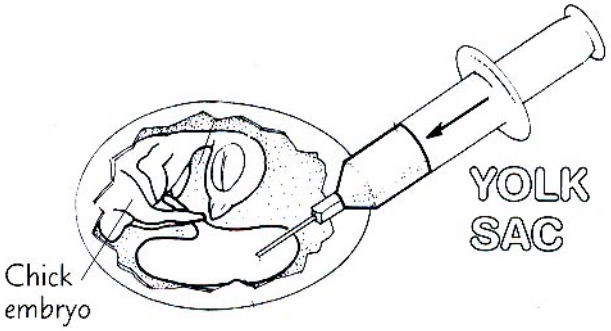
The next phase is **initiation** which requires water and a triggering germination agent. Germinating proteins or inorganic ions (Mn++) penetrate the damaged coat and cause the peptidoglycan to hydrolyze quickly. The cell quickly absorbs water and stored energy is converted to ATP. In minutes, amino acids and more energy are liberated.

The final phase is **outgrowth** as the cellular machinery is formed and the spore wall turns into a thicker vegetative cell wall. As the cell wall increases in volume, it bursts out of the spore coat.

[Spores can be destroyed by boiling the spores, and then cooling the liquid to prompt germination. This is followed by a second boiling to kill the unprotected vegetative cells.]

Spores can survive for very long periods in boiling water (up to two hours) and have been recovered from the intestines of Egyptian mummies and cultured (viable after 3,000 years).

Some bacteria will not grow on gel media or in broths. The Rickettsiae which causes typhus is usually grown in a fertilized egg or embryo so scientists culture it by injecting it into the yolk sac of a developing chick.



Chapter 2
Immunity, Virulence, & Disease

Disease is a state in animals and humans where normal function of tissues and cells is disrupted or adversely affected. It can be caused by poor nutrition, genetic defects, physical wounds, trauma, and structural disorders. When illness is induced by bacteria the result is infectious disease. Infectious disease bacteria are transmitted to people by –

1. **Droplets** which are expelled from the mouth and nose in tiny particles as an aerosol. These droplets usually contain the infectious bacteria or viruses.
2. **Insect** vectors whereby bacteria are carried inside blood sucking species and transmitted from one host to another via bites and fluid exchange. Mosquitoes, ticks and worms are examples of disease causing insects.
3. **Contaminated water** has been the cause of epidemics. Bacteria from ill humans and animals are passed out with solid and liquid waste in the stools and urine. These become washed into the water supplies and contaminate drinking and bathing water. Those individuals using this water become infected with the organisms.
4. **Wounds** caused by animal bites, broken glass, and any other puncturing device (pungi sticks in booby traps) can inject bacteria through the skin and into the tissues.
5. **Human mucosal contact** via kissing, sex and close personal physical association can spread bacteria from the mucosal surfaces of one person to another.

When the bacteria enter the body, they fight the body's defenses for survival and food. The body usually mounts a rapid and dynamic defense called the **inflammatory response**. The first response to invading bacteria that are detected by the body is for **phagocytes** to migrate to the infection site. Phagocytes engulf and destroy bacteria and associated particulate matter if they can reach the site and overcome bacterial defenses.

Bacteria may attack the tissues that connect the cells enabling further invasion into the body. Some may cause blood clots which stop phagocytes from reaching them and turn the clotted cells into bacteria food. Some bacteria produce enzymes or toxins which kill surrounding cells turning them into food. Some bacteria release **hemolysin** which ruptures the membranes of red corpuscles and lowers the oxygen carrying capacity in the tissues. This allows bacteria to overcome local tissue resistance. If the oxygen supply is reduced low enough, **anaerobic** bacteria can grow and infect. Clostridium spores are anaerobic and cause gas gangrene. One of the symptoms of gangrene is anemia (reduced red corpuscles).

Some bacteria produce poisons called **toxins** which damage host tissues and organs. There are two types of toxins. The first is **exotoxins** which are produced mainly by gram positive bacteria as part of their normal metabolism. They are usually proteins which are accumulated in the cytoplasm and then released with waste products into the

surrounding fluid and tissues (exo means out which is why they are called exotoxins). The toxins are usually dissolved into the blood and spread throughout the body by the circulatory system. Exotoxins usually cause direct damage to the organs and tissues and interfere with cell activities. If the exotoxin is absorbed into the intestinal tract linings they are called **enterotoxins**. These usually cause diarrhea, nausea, cramps and vomiting. If the toxin can gain access to the nervous system and cause harm it is called a **neurotoxin**.

The second type of toxin is **endotoxin,** which are produced mainly by gram negative bacteria. They are complexes of LPS that make up the cell wall (Chapter 1) and remain in the living bacteria, usually without causing harm. When the bacteria cell dies off, the cell wall disintegrates, releasing the LPS toxins into the tissues and fluids. These toxins are systemic which means that they damage a wide range of tissues and can disrupt the life of the host. Some endotoxins cause blood vessels to dilate which lowers blood pressure and can cause shock. Other endotoxins induce fever and muscle weakness. Serious conditions are so serious they are called endotoxin shock.

Toxins make bacteria more pathogenic by causing harm to the host tissues and often are the primary cause of the disease.

Disease Resistance and Human Immune Responses

The most important defense to infection is the skin barrier. It is impenetrable to most bacteria but can be damaged by burns and puncture wounds. Layers of dead skin cells constantly slough off carrying bacteria with them. The gastrointestinal tract has a similar lining of cells that resist invasion. The cells lining the stomach secrete hydrochloric acid which lowers the pH to about 2.0 which kills most bacteria and breaks down food. Some bacteria like Salmonella and the virus that causes hepatitis A are acid resistant and some survive the stomach acid when large numbers are present. When they reach the intestines, they can penetrate the lining cells and establish disease.

Bile is produced in the liver and stored in the gall bladder. Bile and enzymes from the pancreas are discharged into the intestinal tract and can disrupt the cell membranes of invading bacteria. Lysozyme is an enzyme produced in tears and saliva that affect gram positive bacteria by digesting peptidoglycan. If the contact is long enough, it kills the bacteria. If is also present in the openings of the skin such as pores, hair follicles and sweat glands providing some protection at these entryways.

Glandular secretions in the respiratory tract trap and kill bacteria in the mucus. Surface cilia sweep bacteria and debris in the mucus droplets from the cells lining the respiratory tract towards the mouth to be swallowed or expectorated.

The mucus also contain antibodies called secretory immunoglobulin A (sIgA) which binds to both bacteria and the mucin helping to carry the invaders away.

Bacteria that breach these outer defenses now encounter special cells which try to engulf and devour them, as well as other debris and foreign particles. This process is called **phagocytosis** and if specific antibodies are involved it is called **opsonization**.

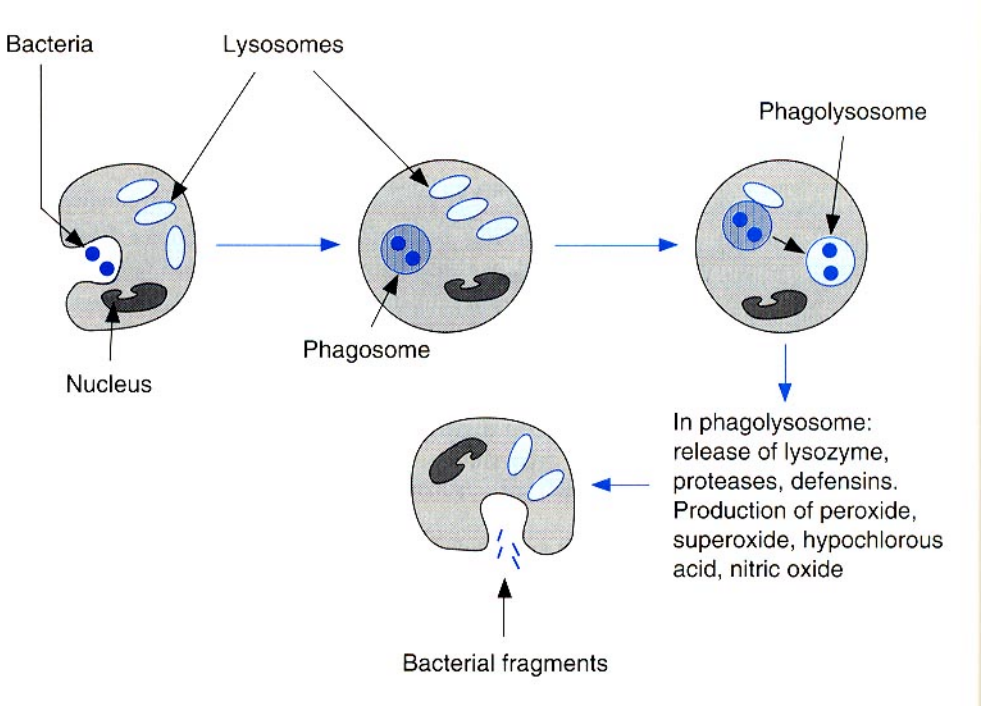
There are three kinds of phagocytes called **neutrophils, monocytes,** and **tissue macrophages.** Nuetrophils make up about 65% of the white blood cell population. They are found in the blood and connective tissues assuming countless shapes as they move through the blood vessels and between cells. They have a multilobed nucleus which makes them easy to identify. They migrate from the local capillaries into injured tissues where they swarm around bacteria and devour them.

Monocytes make up 4% of the white blood cells and work only in the blood. They are physically larger than the neutrophils and have a large kidney shaped nucleus filling almost the entire cell interior. Both neutrophils and monocytes surround bacteria and excrete digestive enzymes that dissolve ingested particles and bacteria. Some bacteria like virulent anthrax strains produce capsules around themselves which protect the bacteria from the digestive enzymes.

Macrophages are found in many tissues and most importantly in the brain, spleen, bone marrow, liver, kidney and connective tissues. They are not found in the blood. They may be fixed in place in cells lining the blood or lymph systems, or may float freely in the body tissue fluids.

Phagocytes are attracted to bacteria and debris by chemical signals (chemotaxis) and when they draw near to them, the cell membrane begins to wrap around the bacteria and completely encircles it. This newly formed pocket is called a vesicle. It is drawn from the cell membrane to the cytoplasm where it merges with a lysosome filled with digestive enzymes. The disruption and breakdown of the bacteria and debris begins as the enzymes are released into the vesicle. Normally, the bacteria are broken down into fragments which are then ejected by the phagocyte into the surrounding tissue and fluids where they are no longer harmful.

The digestive materials of lysosome include lysozyme, proteases, defensin, peroxide, superoxide, hypochlorous acid, and nitric oxide. It is a potent mix which destroys most invaders.



The body also has protein molecules that are soluble and circulate in the blood and are called **complement**. Circulating complement can bind with the polysaccharides in the cell membranes of some bacteria in a process called opsonization. The opsonized bacteria are more readily recognized by phagocytes which have special receptors for the bound complement and they engulf these bacteria much more rapidly than when the complement is absent.

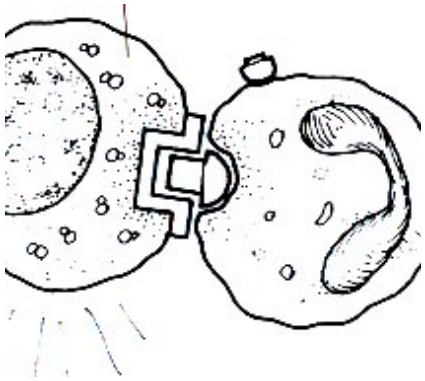
Inflammation is the bodies response to irritation that occurs near blood vessels. The blood vessels dilate turning the skin surface red (when it is a surface cut). Swelling is caused by the flooding of plasma or blood fluid into the intercellular spaces, and the warm blood heats the surrounding skin surfaces. Pain often accompanies the injury when pain receptors are affected. Blood clots are formed in torn blood vessels and new vessels are formed within a few hours around the infection site. While the bacteria and its toxins affect the local tissues, white blood cells, primarily neutrophils in skin infections, adhere to the margins of the blood vessels and then push out between lining cells and enter the connective tissues. Huge numbers congregate in these areas swarming into the injured site by chemotaxis and phagocytose the bacteria.

The neutrophils engulf all the debris including clotted blood, dead tissues and bacteria which is most often opsonized. After having digested large numbers of particles the neutrophils rupture their own cell membranes and die forming the pocket of **pus** at the sight of infection. Once phagocytosis is complete the local blood vessels diminish in number and fibroblasts secrete new fibers which is seen as the scar tissue.

As the area restabilizes the neutrophil populations return to a normal state and healing is completed.

Specific response to infection by bacteria also occurs whereby a structural part of an immune component fits a specific part of a bacteria. This interaction is called the **immune response**. The part of the immune component fits the bacteria part like a glove or like a specific size, unique wrench fits a bolt head. There is a great diversity of receptor structures and immunity components that fit together and these molecular pairs are often unique to each strain of bacteria. The body can remember over time what this strain of bacteria surfaces will have for receptors and produce the correct fitting component for it. It makes these components so that they only fit the bacteria surfaces and do not affect their own cells. In other words, the body can tell its own cells from those of an invader.

The part of the bacteria cell wall that causes the immune reaction is called an **antigen** and the surface of it (the keyhole) is called an **antigenic determinant**. A white blood cell called a lymphocyte produces the "key" to fit the exact "keyhole" on the bacteria surface. This key is called a **receptor**. It is impossible for receptors to fit any other bacteria surface antigen that does not fit its exact shape. It is a one of a kind fit for each antigen and receptor for each species of bacteria. There are millions of antigens and receptors to fit each one that are produced by the body over its lifetime. The drawing below shows how the receptor fits an antigen.



When a new antigen is encountered by a lymphocyte, the cell is activated. Once activated, it is capable of responding over and over again to the same antigen days later, and even years later and these later responses are greatly enhanced because of its immunological memory. This memory is transferred to the offspring or daughter cells of the activated lymphocytes and they can produce the identical receptors for each invading bacteria that their parent cells did.

Each new bacteria infection produces large numbers of antigen specific lymphocytes and after each infection, these cells with the specific antigen receptors are called memory cells. These memory cells are long lived and remember the previous encounter with the specific antigen.

Some phagocytes that ingest bacteria have a special ability to initiate an immune response. These phagocytes engulf the bacteria as they normally would but they also activate lymphocytes which then secrete substances called **antibodies** which attack the bacteria during phagocytosis. This is the immune response.

Antibodies on the surface of these lymphocytes, or floating free in the fluids can attach to the bacteria and assist in phagocytosis. Some of the lymphocytes secrete antibody toxins that specifically kill the bacteria and these are called "killer cells". Some lymphocytes called "lymphokines" secrete antibody that activates phagocytes to ingest the specific antigens of the bacteria.

The human body begins to form its **immune system** in the 3rd to 6th months of fetal development. This complex network of cells starts in the bone marrow where **stem cells** are formed and multiply into two major cell lines. One cell line is the "hematopoietic" which produces the red blood cells and most white blood cells. The second cell line produces lymphocytes. Cells called B-lymphocytes are produced in the bone marrow or fetal liver and migrate to lymphoid tissues and organs. About 20% of them remain in circulation and can transform into **plasma cells**. Both lymphocytes and plasma cells can secrete antibodies and those that do so in body fluids produce soluble antibodies.

The thymus is a two lobed organ overlying the upper part of the heart. It is large in children and it forms T-lymphocytes or T-cells. These make up about 75% of the blood lymphocytes. They have different receptor sites than the B-cells and do not produce antibodies. T-cells are responsible for **cell-mediated** immunity. The thymus begins to deteriorate at about the age of sixteen and declines in cell production with age.

The body has a system of lymph vessels which contain lymph fluid derived from intercellular tissues. The primary cells found in the lymph fluid are lymphocytes or lymph. Lymphoid tissue is made up of a net of fibers that support masses of T-cells and B-cells and various amounts of plasma cells and phagocytes. This matrix of cells make up most of the lymphoid organs such as the bone marrow, thymus, spleen and lymph nodes. The tonsils, appendix and adenoids are also lymph tissues. Some of the lining membranes also contain lymph pockets called mucosal associated lymphoid tissue or MALT.

B-cells and T-cells respond to infection like fire trucks to a fire and these encounter most of the antigens that enter the body fluids and tissues. Most of these antigens are proteins on the surface of invading cells. Sometimes, the bodies own tissues are mistaken for foreign proteins and the immune system can attack its own body.

Bacteria can have hundreds of antigens on its surface. The key part of every antigen is a molecule on its surface, the antigenic determinant. This molecule interacts with the receptors on lymphocytes creating the immune response.

The antigen can have several locations on its surface that act as antigenic determinants (several keyholes each having its own specific key). Each separate location can produce its own immune response as well as each of the hundreds of antigens on the surface of a bacteria.

It is possible to have over one thousand different protein antigenic determinants on a single bacteria surface that the body can have receptors for.

Antigens can also be proteins on animal skin cells (cat hair), pollen, and medications which all can produce immune responses (and occasionally allergies) when they contact body fluids or lymphocytes.

Body tissues are normally recognized as "self" and are not attacked by the immune response. In autoimmune disease of the thyroid, the lymphocytes do not recognize the thyroid tissues and produce antibodies to it. This results in inflammation of the thyroid and tissue damage.

As mentioned earlier, T-cells are involved with cell mediated immunity. T-cells only recognize protein antigens that are part of the surface of another cell and do not respond to anything else. T-cells will not recognize or bind to soluble proteins. The phagocytes first present an antigen to a T-cell called a helper T-cell. It can be any one of the molecular parts from the surface of a bacteria that acts as an antigenic determinant. Most of the time it is a part already broken down by digestive enzymes during phagocytosis. The digested protein fragment is expelled from the phagocyte in the presence of the T-cell.

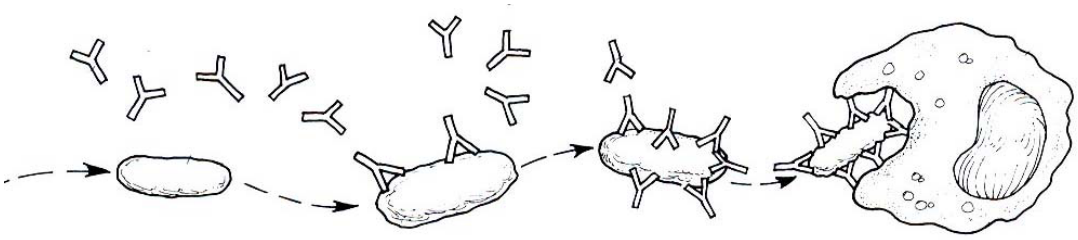
This phagocyte is called an APC (for antigen presenting cell). When the APC encounters a helper T-cell, the T-cell interacts with the specific antigenic determinant and this process "activates" the T-cell. These activated helper T-cells begin to proliferate rapidly and secrete **cytokines** which enhance the inflammatory response and activate neighboring B-lymphocytes and more phagocytes. Some of the helper T-cells turn into memory cells and "memorize" the original T-cell interaction. These cells live long lives and rapidly respond in a second encounter with this antigen. This allows the body to have long term immunity to the same infection in the future.

T-cells called cytolytic T-cells (**CTL's** or cytotoxic cells) also proliferate rapidly. These cells directly engage bacteria with certain antigens and lyse them (destroys their cell walls killing them). The CTL's are especially sensitive to cells containing viruses and to cancer cells. They will also form memory cells.

Plasma cells are formed from B-cells and are found in body fluids and blood. They produce antibodies on their outer membrane surfaces and these react with a variety of antigens, and not just the proteins like the T-cells. These will react with antigens that are not part of a cell and that are dissolved in the fluids (soluble). This solubility gives it the name of **humoral immunity** (humoral means fluid).

B-cells are activated when they come in contact with a bacteria antigen that they have a receptor for. Some B-cells differentiate into plasma cells. Once activated, the plasma cell enlarges and its cytoplasm fills up with ribosomes and protein synthesizing organelles. These produce thousands of antibodies and they release them into the surrounding fluids and blood. Each plasma cell (or B-cell) produces only one type of specific antibody and thousands of different antibodies are produced every second in the body during infections.

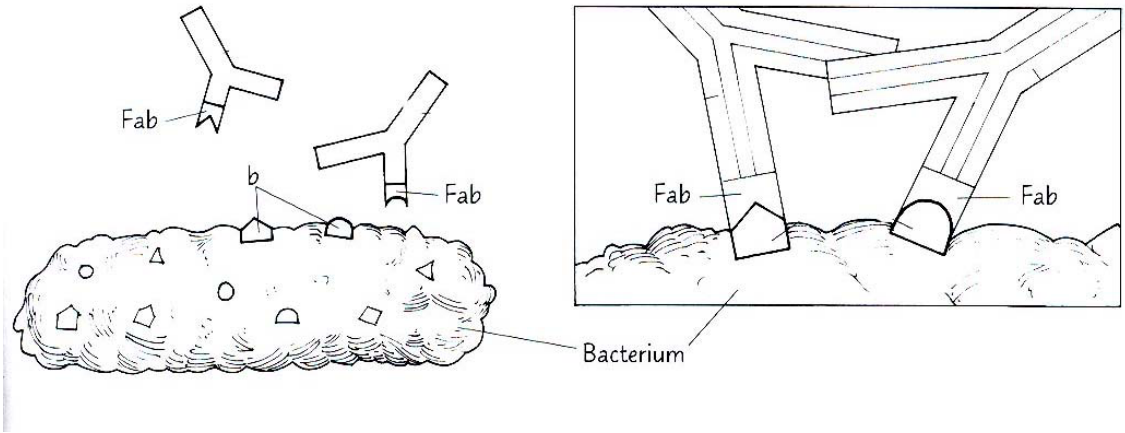
An antibody is a molecule that has a "Y" shape that allows it to "fit" onto the surface of invading bacteria. The phagocytes engulf the bacteria and are assisted by the antibodies in destroying the bacteria cell. This process is shown in the diagram.



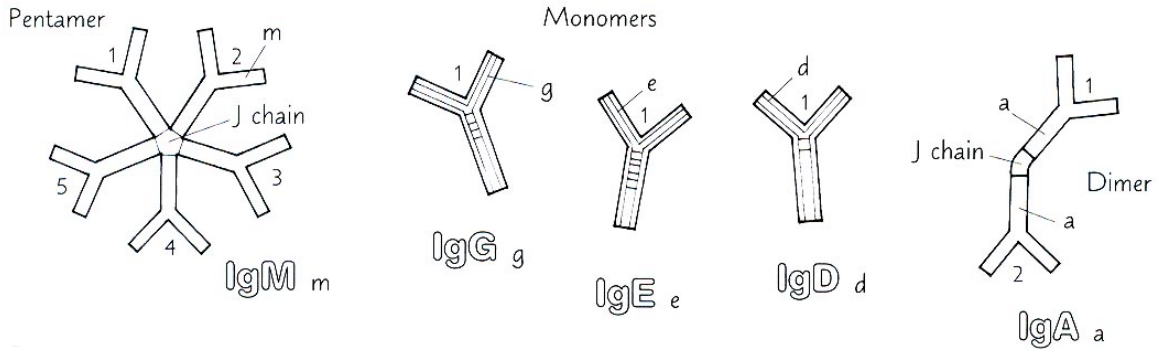
Antibodies pour into circulating body fluids by the millions once B-cells are stimulated by antigens. Usually, about 17% of all the protein floating in the blood of humans are antibody molecules for the thousands (or millions) of antigens fitting each type of infection we have encountered in our lifetime.

The Y-shaped antibodies have a hinge where the two arms divide which allows the antibody some flexibility in its shape. The stem of the antibody is called an Fc (fragment, crystalline) because it tends to crystallize when it is separated from the arms of the Y by enzymes. The end of the arms of the Y each contain the specific antibody

molecule that fits onto the specific bacteria surface. These are called Fab (for antigen binding Fragment). Only the exact fitting Fab will fit onto the corresponding site.



There are five different types of antibodies that exist in the human body and these are called **immunoglobulin** or **Ig**. They are all composed of proteins called globulins and each of these are involved in the immune response.



IgM is the largest of the antibodies and has five antibody subunits. Each subunit is called a monomer (mono means one) and since the IgM has five subunits it is a "pentamer" (penta means five). IgM is the first antibody to appear in circulation once the B-cells are stimulated by antigen. App. 5-10% of the circulating antibodies are IgM. The Y shapes are connected to each other via a central hinge connection called a J chain.

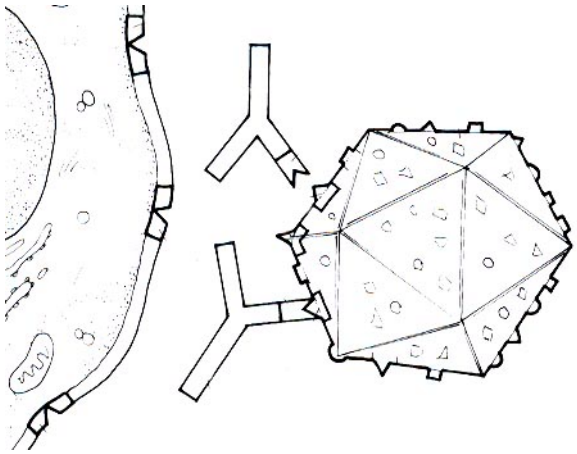
IgG makes up about 80% of the circulating antibodies and it is the main "antibody" that is referred to in the literature. It is a monomer since it has only a single Y. It is called gamma globulin and it assists in the destruction of antigen begun by IgM.

IgE and IgD are monomers and exist in very low concentrations. IgE is found in persons with allergic reactions. IgD is found on the surface of B-cells where the stimulation takes place. IgA is a dimer (di for two) with two Y shapes on a central J chain hinge. It is found mainly in the tears, saliva, GI tract, and respiratory tract as well as blood. It is also produced in mothers milk and passed on to nursing infants providing resistance to infection.

The differences in the IgG, IgE, and IgD are in the bonds holding the Y arms to the stem and in the "complement" molecules (binding sites) on the stem. The differences

in the Fab region can run into millions and possibly billions for each of the different antigens encountered in all human infections.

When a virus invades our bodies, they have antigens on the virus particle surface that match our human cells and these allow them to attach to our ells and inject them with their DNA. Soluble antibodies such as IgG in the blood may have the same antigen binding sites from a previous encounter with the virus. They also form them during the infections initial encounter with B-cells and the formation of plasma cells. These antibodies can bind to the virus on these same sites and prevent the virus from using the sites to bind to human cells and infect them. The virus continues to float in the fluid and are eventually engulfed by phagocytes. This process is illustrated below.

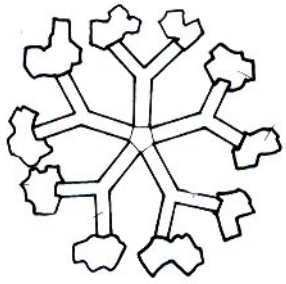


If the virus mutates, the surface proteins, or binding sites may be changed and then the previously encountered antigens are no longer effective. The specific sites are gone or have been changed and a new round of infection favoring the disease will begin. Mutation of this type is quite common with the aids virus and annual flu epidemics. Bacteria cell surfaces can also be altered by mutation, and other methods so that antigens are no longer effective.

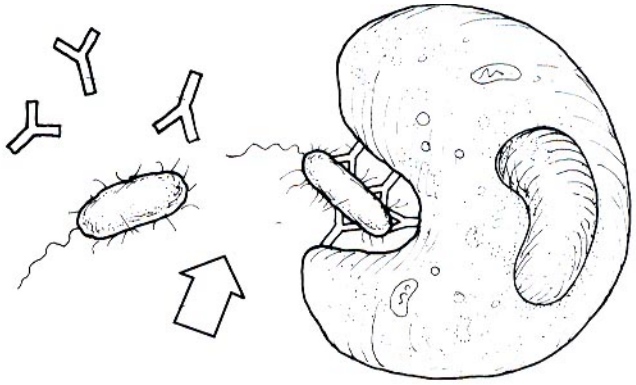
Deadly toxins can also be neutralized in a similar fashion by antibody and these are called antitoxins. When antibodies react with antigens on bacteria cell walls, the bacteria tend to accumulate in "clumps", which is called **agglutination**. These clumps are ideal for large scale phagocytosis since the phagocytes do not have to float around and find individual invading cells any longer. These large clumps attract huge numbers of phagocytes.

Agglutination tests are conducted to identify different bacteria infections in hospitals. To perform the test, a sample of unknown bacteria is mixed with antibody for a specific bacteria like E-coli. If clumps form they can be seen in a microscope, and it is the specific E-coli. If they do not, then it is another strain or species of bacteria.

When antigens are soluble (not attached to cell surfaces) they react with antibodies to form enlarged masses. Cross linking occurs when antigen and antibody molecules link up in multiple numbers at the same time. If there is a lot of IgM present, all five of the sites will link to antigen and the antibody becomes “heavy” and precipitates out of the solution of blood or fluid. These precipitated complexes are much more vulnerable to phagocytosis. Lab tests have also been devised to measure cross linking and precipitation.



In enhanced phagocytosis, the antibodies fit onto the bacteria surface antigens. The stem of the antibody contains complement that fits receptors on the phagocyte and this speeds phagocytosis (opsonization).



The **complement system** consists of proteins in the plasma of blood that disrupts the cell wall membrane of bacteria producing pores so that the insides leak out. It also induces the inflammatory response and enhances phagocytosis. In these ways the complement system “complements” the immune response.

The proteins begin their work when an antibody binds to the site of an antigen and then the “complement” protein (C1) binds to the antibody. Once the C1 is activated other proteins begin a cascade of reactions attacking the membrane molecules of the bacteria. This attack complex punches tiny holes in the bacteria wall and the essential fluids and small machinery escapes from the bacteria insides causing cell death. There are a variety

of small reactions taking place to ensure that these complexes do not attack host cells and the cascade is only activated in the presence of an antibody-antigen binding site.

Natural immunity to infection or toxin is acquired by

a) a microorganism induced illness
b) injection with a vaccine or toxoid
c) from a mother during pregnancy (lasts 3-6 months)
d) receiving antibodies from another person or animal for the specific disease

The first two acquired immunities usually last for years or even a lifetime with repeated exposure. The B-cells contact the bacteria, turn into plasma cells and produce antibodies with structures that match the antigen sites on the bacteria cell wall surfaces. Memory cells remain after the event to resist subsequent encounters.

The mothers antibodies run out quickly because the mothers B-cells are not passed on and memory cells are not formed. Receiving antibodies from another source is also short lived for the same reasons and the proteins may actually cause an allergic reaction. The protection usually only lasts for a few days or weeks but is often used when the disease is caused by a toxin or is serious and cannot be treated otherwise. The virus hepatitis A is often treated this way.

Monoclonal Antibodies

The medical profession long dreamed of having commercially available antibodies to react with disease causing antigens. The dream was realized in 1975 when Georges Kohler of West Germany and Cesar Milstein of Argentina developed a technique to hybridize cells by fusion and produce clones of antibody producing cells. [This technique is also useful in producing modified weapons and will be taught in a later chapter.] In this case, the scientists invented a new way to mass produce antibodies and these cell factories are called **monoclonal antibodies**. The scientists were awarded the Nobel prize in 1984 for their work.

To produce monoclonal antibodies, the target antigen is isolated and purified. This antigen is injected into a healthy mouse which stimulates the immune system of the mouse. The B-cells are activated and form plasma cells which produce antibody. Some of these plasma cells are extracted from the spleen of the mouse and placed in a culture dish. The plasma cells live only for a very short time and die off before they can reproduce themselves in sufficient numbers to be valuable. In order to save the plasma cells, they are “fused” with immortal cells whose progeny (daughter cells) survive indefinitely and produce the antibodies. The immortal cells used were cancer cells recovered from lymphoid tissue called “myelomas”. Myeloma cells are extracted from the spleens of mice with this type of cancer and mixed with the culture of plasma cells forming a mixed culture. In the mixed culture dish (with special food added to encourage fusion), many of the myeloma cells fuse with the plasma cells forming a hybrid called a hybridoma. These

new cells grow quickly at the expense of the unfused cells which die in the culture medium. The hybrids have inherited the immortality of the cancer and produce the antibodies of the previously cultured plasma cells.

Masses of these identical hybridomas are clones just like the single colonies of the bacteria cultures. As the clone grows in colony masses it produces only the antibody from the antigen injected in the mouse. Since they are derived from a single clone of cells, they are called monoclonal antibodies. After a time in culture, the hybridoma cells secrete a large amount of monoclonal antibodies more pure and uniform than antibodies found in the blood.

Monoclonal antibodies can be used to attach to specific sites on bacteria masses in culture and then these sites can be isolated for use in vaccines. They can also be used to test for the presence of disease organisms, and to directly fight cancer cells. They have also been used to cleanse bone marrow prior to transplant operations. Scientists hope to attach chemicals to monoclonal antibodies as a direct way of delivering medication to desired infection sites.

Testing Methods

A method for testing the presence of antibodies produced in large numbers in response to bacteria infections is the **Fluorescent Antibody Test**. This key component of this test comes from a lab animal that is injected with the human antigen for the bacteria disease in question. Lets use salmonella in this example.

The animals immune system detects the human antibodies to salmonella as antigens and it responds by making antibodies to these antigens. These antihuman antibodies that react with any human antibodies are harvested and have molecules of a fluorescent dye attached to them. These are called the fluorescent antibodies.

A sample of the patients blood is taken and allowed to clot for 10 minutes. The clotted blood is centrifuged with the fluid on top and the clotted cells pressed to the bottom. The yellow fluid that is poured off is the serum. If the patient has salmonella, large numbers of antibodies will be present in the serum. The fluorescent antibodies are added to the serum on a microscope slide and the slide is washed after a few seconds. Antibodies to salmonella attach to the fluorescent antibody and remain on the slide. All unattached antibodies are swept away. The slide is examined under fluorescent light and if the dye has accumulated on the surface from reaction with the antibodies, the slide will glow an orange or bright green color. If the slide is black, the patient did not have salmonella antibodies in sufficient quantities to show up in the test.

A similar method to the above is called the **Radioimmunosorbent Assay (RIA)**. In this test, the antihuman antibody is combined with a radioactive material instead of a fluorescent dye. These antigens from the microorganism are bound to and coat microscopic polystyrene beads. Blood serum is added to the beads and if the antibody is present, it reacts with and attaches to the coating. The sample is washed and then the radioactivity is measured. If there is radioactivity, it means the antibodies were present

and reacted with the radioactive antibodies and they remain attached to the beads. If they were not present, the radioactive antibodies are washed away. The stronger the radioactive measurements, the more recent the infection.

Poison Ivy Immunity

The cell mediated immune response can cause problems with certain types of exposure. When a person comes in contact with poison ivy, the chemical **urushiol** enters the pores of the skin, usually on the hand. leg or arm that brushed against the leaves. The urushiol combines with a protein in the skin which marks it as an invader. This combined molecule is then phagocytosed and taken to T-cells in nearby lymph tissues. The T-cells are sensitized and begin to proliferate. T-memory cells also leave the lymphoid tissues and are drawn to the site of first exposure. Over the following weeks and months, they accumulate to high levels. When the person encounters the poison ivy the second time, the urushiol once again combines with the protein and are taken up by phagocytes. This time the phagocytes not only destroy the combined molecule, they also send out digestive enzymes to fight off the "urushiol infection". These enzymes fill fluid spaces and damage local cells. They cause blisters that itch profusely and become red and granular. As the phagocytes remove the urushiol and damaged tissues, the blister heals and disappears in a few days.

The sensitized T-cells remain in the skin indefinitely and will initiate repeated reactions in the individual on each subsequent exposure. [The author wonders about combining urushiol molecules with other immune response magnifiers that produce lethal self inflicted attacks-more on this later in the book].

Infectious Disease Processes

Upper respiratory infection begins when organisms enter the nose or mouth. Most become trapped in droplets of mucus and saliva. The tissue lining of the upper respiratory tract is called **mucosa** and it is only one cell layer thick and covered with tiny hairs (cilia). The cilia move the tiny droplets and trapped particles from the nasal cavities, throat and upper lungs to the mouth to be swallowed or spit out. When conditions are right or when very large numbers of bacteria are present, they penetrate the mucosa infecting the local tissues.

One of the best known bacteria in these types of infections is *Corynebacterium diphtheria*, a club shaped gram positive rod which causes diphtheria. It produces a potent toxin that can kill a cell with a single molecule by interfering with protein synthesis. As the C. diphtheria cells die and accumulate in the inflamed tissues, a thick leathery membrane is formed. If can block the respiratory passages suffocating the host. When the infection reaches the bloodstream, it is carried to other tissues and organs and cause systemic infection. Diphtheria is rare in the US because most people are immunized against it with toxoid in the DPT (diphtheria, tetanus, pertussis) vaccine.

Whooping cough (pertussis) usually affects infants under six months of age. It is caused by *Bordetella pertussis*, a small gram positive rod with a capsule. B. pertussis often populates the nasal and pharyngeal mucosa and may be carried into the lower airways causing inflammation (bronchitis). It causes massive mucus production by irritating the tissues. The patient coughs to clear the mucus. The bacteria is sensitive to many antibiotics and killed B. pertussis cells are used in the vaccine.

Neisseria meningitidis is a small gram negative diplococcus which can infect upper respiratory tissues. It migrates from this first infection site to the bloodstream and then into the meninges (membranes that envelope the brain and spinal cord) and causes an inflammation called meningitis. *Haemophilus influenza* can also cause meningitis via the same route and is seen most often in infants where it causes various neurological symptoms.

Streptococcus pyogenes is a gram positive coccus that grows in chains and multiplies rapidly in the mucosa causing "strep throat". It causes a local inflammation when phagocytes attack the bacteria and local lymph nodes swell as the immune system responds. The mucosa surface becomes red and the patient experiences high fever. When large numbers of bacteria penetrate the mucosa and enter the bloodstream it often causes septicemia while infecting other organs. Some strains of streptococci may be infected with a virus (bacteriophage or phage) and release toxins in the blood that damage vessels and cause the characteristic rash of "scarlet fever".

Lower Respiratory Tract Infections

The primary tissues of the lower respiratory tract are the bronchi, bronchioles and alveoli (tiny air sacs). Some bacteria can move past the upper respiratory defenses, or if they are tiny single cells can be breathed directly into the air sacs. The alveoli are the tissues which allow blood to release CO2 and take in oxygen (gas exchange).

The lower tract is lined with mucosa and smooth muscle and can easily become obstructed by infection. The alveoli has a thin single cell layer next to the bloodstream capillaries. When these sites are infected, the infection easily spreads to the blood and other tissues and quickly becomes life threatening.

Mycobacterium tuberculosis is a rod shaped bacteria that causes the lung disease tuberculosis. It is transmitted in tiny droplets of sputum that are coughed or sneezed out. It is often transmitted by a carrier who does not have symptoms of the disease which allows for its rapid spread in dense populations. Once the bacteria reach the lungs they localize and form hard nodules called tubercles. They tend to affect tissues in apex of the upper lung. M. tuberculosis can spread into the blood and infect many organs without causing symptoms for months. The respiratory sputum becomes bloody as the lungs deteriorate. Antibiotics can prevent the spread of the bacteria but the tissue damage remains.

Streptococcus pneumoniae causes the condition called pneumonia. It is an inflammation of the lungs where the air sacs fill with debris and particles called "exudate". Many organisms can cause pneumonia but S. pneumoniae is the cause about 90% of the time. Young and old age groups are the most vulnerable and the infection often follows trauma, surgery or severe flu. Resistance is lowered and the bacteria invades the tissues. A protective capsule surrounding the bacteria resists phagocytosis and aids significantly in infection. The disease causes chills, fever, muscle pain, weakness and chest pain.

Bacillus anthracis is the cause of inhalation anthrax. It is an extremely rare infection in the United States but is a favorite biological weapon because it produces spores that are long lived. When single cells are breathed in and reach the alveoli, they infect the air sacs, spread to other organs and produce a potent toxin that often kills the host. The anthrax cells also have an exterior capsule that resists phagocytosis and those anthrax strains that lack the capsule cannot resist phagocytosis and cause infection.

Mycoplasma pneumonia cause a mild form of pneumonia called "walking pneumonia" because the symptoms rarely force the patients into hospitals. The bacteria has no cell wall and is not affected by penicillin but other antibiotics are effective.

Legionnaires disease was first encountered in 1976 at an American Legion convention in Philadelphia. The bacteria are spread by wind current and cause the classic pneumonia. In the first epidemic in 1976, 34 out of 221 patients died.

Ingestion Infections

Bacteria in the food, water and sometimes air may enter the body from contaminated sources and initiate infection via the digestive tract. The mouth, pharynx and esophagus have multi-layered linings that are highly resistant to bacteria invasion. Food and water regularly "wash" down bacteria into the stomach so there is little chance of colonizing these parts. The stomach and upper small intestine have a single layer of cells that excrete acid and kill most bacteria.

Most of the small and large intestines are also lined by a single layer of cells and have a less hostile environment for bacteria. The contents also move slowly through these parts allowing time for colonization and infection of the tissues.

There are specialized cells that line the digestive tract that excrete a variety of enzymes that are harmful to most bacteria. The list is long and complex but only those bacteria that have resistance to these enzymes are successful in initiating infection here.

Food poisoning is caused by many organisms. *Staphylococcus aureus* is the most common cause. This species is a normal colonizer of human skin and feed off dead and decaying skin cells. They often contaminate foods producing toxins. When they enter the GI tract and survive the acids, they can produce toxins and enzymes that cause injury and help initiate infection of the tissue linings. They induce vomiting, cramps, diarrhea and when the infection spreads it can cause shock and septicemia. Since the toxins are

produced at room temperature, refrigerating foods below forty five degrees usually prevents this source of poisoning. *Clostridium botulinum* causes the deadliest form of food poisoning producing toxins in unsterilized canned foods.

Salmonella typhi is ingested with contaminated water or food and causes typhoid fever. Sewage plant workers are the most frequently afflicted and it is often transmitted by food handlers in third world countries. S. typhi enters the small intestine and quickly penetrates into the bloodstream causing bacteremia about one week after ingestion. It produces high fever, sweating, weakness, vomiting, anorexia and diarrhea. It can spread to other organs as well.

Salmonella enteriditis is the cause of classic salmonellosis which is spread via contaminated food and water. The skin of poorly cooked poultry is the most common source. The bacteria produces ulcers in the intestinal lining cells which often produces blood in the stool. In about forty eight hours the classic symptoms of cramps, diarrhea, nausea, vomiting and fever occur. The disease generally passes in a few days.

Vibrio cholerae causes severe fluid loss from diarrhea following infection. It is a gram negative, comma shaped bacillus that is endemic (permanent and found nearly everywhere) in India. It produces a potent toxin that causes water and electrolytes to leak form cells in the intestinal mucosa. They do not invade the mucosa and generally grow on its surface.

Wound Disease

Anthrax as mentioned earlier is a rare infection but is common in livestock throughout the world. Most often, it infects the skin via scratches, cuts and punctures. This form is less fatal in humans (about 20%). When the spores contact the tissues under the protective skin layer, they colonize the site and induce reddish vesicles which form craters or ulcers. They may invade under the skin causing cellulitis and if they reach the bloodstream they can develop in the spleen, liver and kidneys.

Clostridium tetani is a gram positive spore forming rod that is anaerobic. They germinate in pus filled necrotic wounds where the blood and oxygen have been cut off. They produce one of the most lethal toxins known that diffuses into the surrounding tissues and reaches the spinal cord where it blocks neurotransmitters. The result is “lockjaw” or tetanus. It is prevented by vaccination with tetanus toxoid.

Clostridium perfringens is a gram positive anaerobic rod that infects in wounds where oxygen is cut off like C. tetani. It produces gas bubbles and destructive toxins at the wound site causing the condition known as gas gangrene. Without antibiotics or amputation, the disease is 100% fatal.

All of the described infections are covered in much greater depth in Volume 6-A. We provided a brief review here so that the general mechanisms of the disease processes are understood.

General Principles of Virulence

A number of virulence factors are important to understand in designing infectious or toxic biological agents, The list of potential factors can run into the thousands and be used to create millions of possible weapons types but a few highly illustrative examples will be described here.

One of the most important parts of a bacteria that permits it too infect is its ability to form a protective capsule. There are hundreds of strains of anthrax and all of them produce the deadly toxin that can kill a host and all produce spores.

The strains that do not produce capsules are not a threat as a weapon or infectious agent because the body's phagocytes engulf and digest the unprotected bacteria on contact before they can multiply in large numbers and produce toxin.

The capsule of successful infecting organisms resist all the enzymes and acids produced in phagocytes and the bacteria is still alive when the phagocyte expels it instead of being digested. In some instances, invading bacteria also excrete their own toxin that kills the phagocytes during the devouring process. The anthrax toxin has a component that appears to help it do that.

Also of importance in virulence with anthrax and other spore producers is the ability to germinate when desired in living host tissues. The spores germinate in three stages. The first may involve physical damage or aging. This is accomplished in the laboratory by grinding with ground powdered glass. If the glass remains as part of the weapon, it can cause tiny microscopic wounds that can cause bleeding and provide a local food supply for the germinating spore. The second part requires the presence of germinating proteins. In the case of anthrax, these can be mixed into the scratches and cracks produced by the ground glass which prepares the spores for reproduction. The third part of the germinating process is too add water. This is provided on contact with mucosal and skin surfaces. The cell wakes up and begins to grow immediately.

During the normal infectious process, it takes 5,000 to 8,000 spores to infect primates and produce 98% fatality rates. Lower numbers of spores produce lower fatality rates. This may be due to the spores producing separate infection sites and toxin. The body may defeat a handful of germinating infection sites but as the numbers increase, so much toxin is produced that a lethal dose is achieved even if the body successfully fights off the invasion over a period of days. By drastically increasing the germinating rate by preparing the spores with cuts, germinating proteins and an injury producing glass or chemical carrier, the number of spores needed to initiate infection can be reduced. Only tens or hundreds of cells may be needed for fatal infection which is consistent with the numbers found in other major disease weapons like tularemia and plague.

When people are treated for cancer using radiation therapy, PMN's (polymorphonuclear leukocytes) that are produced in stem cells in the bone marrow are

killed. Immunosuppressive therapy can also kill PMN's. When PMN counts get low enough, widely found bacteria that live in soil and water can now cause serious infection and disease. There are thousands of these species that produce harmful enzymes and toxins that never cause human infections because the bodies defenses routinely devour them. The cells that mount these defenses are killed in immuno-therapy and radiation and leave the body open to a new type of invasion.

Bacteria that can infect the body with reduced defenders include *Serratia* and *Enterobacter* which are common soil species and normally harmless *E-coli* and *Klebsiella* strains that colonize the skin, mucosal and intestinal tracts. So many of these species can now infect that it becomes almost impossible to protect these patients in hospital wards. Antibiotics are often not very helpful because they usually do not kill the bacteria. They inhibit their growth and damage them so the immune cells can catch up with the infection and finish them off.

These species of non-infective bacteria that can infect these types of patients number in the thousands and each can be modified to be able to resist these defensive cells. An obvious approach using modification methods is to provide them with shielding capsules like that possessed by virulent anthrax strains. Others strategies can include the addition of excreted toxins that kill immune cells or combining pairs of organisms that each do one of these actions and will grow together in infection (synergistically). Some cells can live in and infect monocytes and macrophages. Mycobacteria are an example. Virus particles also have this ability and many bacteria infections follow viral exposure. [Virus particles are preserved by freeze drying and some can be incorporated into bacteria weapons-this is another book for the future].

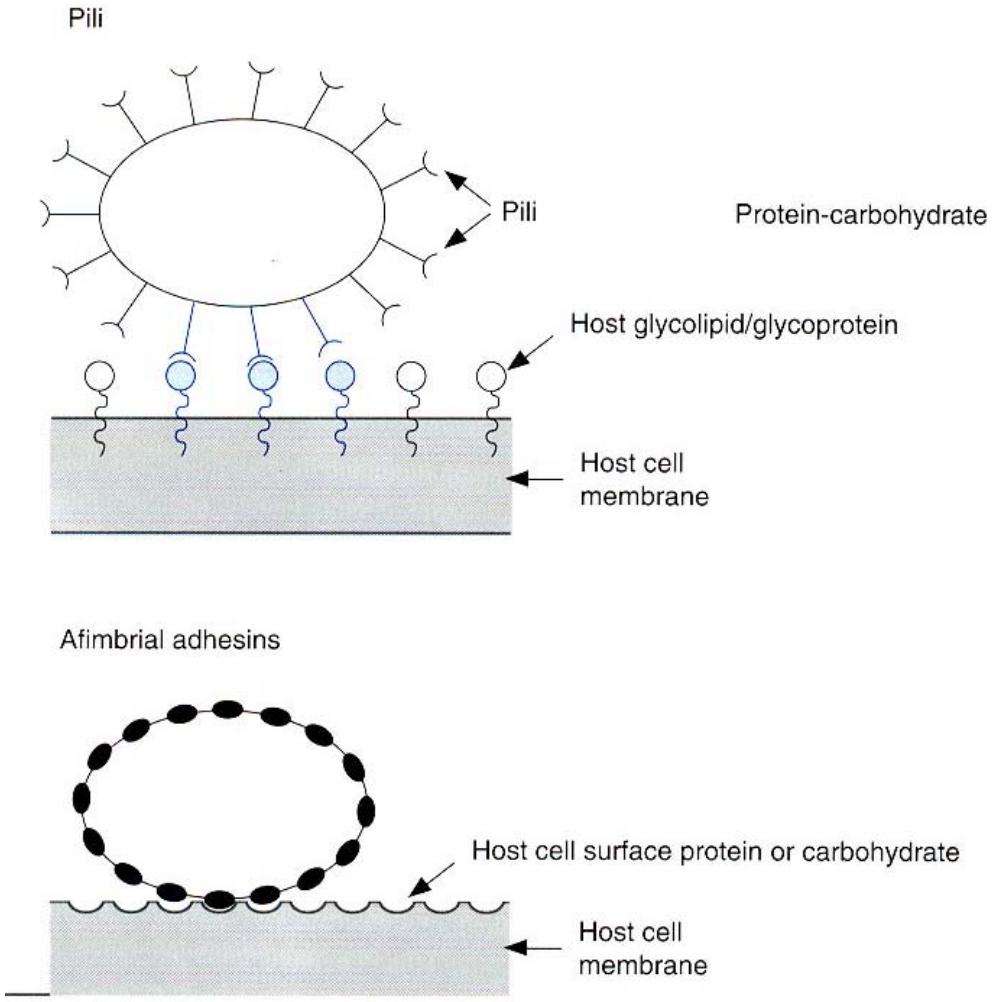
Some bacteria produce carbohydrate and lipid antigens on their cell surfaces rather than protein antigens. These are processed by the bodies defenders differently. Bacteria and capsules with a carbohydrate coating cannot be engulfed by the phagocytes unless they are opsonized by antibodies that are bound to the coating. This allows for very effective first time infections since antibodies are not present from a previous infection. This is also a common type of infection in infants since the T-cell part of the antibody response does not develop until about the age of two.

The addition of nutritive factors like the geminating proteins can also provide starter food for bacteria weapons (see volume 6-D on enhanced weapons). The addition of iron ions aids many bacteria in establishing infection and high local concentrations of iron interferes with phagocytosis. Bacteria that have superior methods of holding and processing iron also show superior abilities to infect.

There are a number of common factors which allow many bacteria to have superior ability to infect. These are found in virulent strains of bacteria species but are lacking in avirulent strains of the same species. It is possible to introduce the cloned genes responsible for these factors into avirulent strains of the same and other species. The most frequent virulence factors encountered are listed in the chart –

Virulence factor	Function
Pili	Adherence to mucosal surfaces
Nonfimbrial adhesins	Tight binding to host cells
Bacterial triggering of actin rearrangement in host cells	Forced phagocytosis of bacteria by normally nonphagocytic host cells; movement of bacteria within host cells or from one host cell to another
Binding to and entry of M cells	M cells used as natural port of entry into underlying tissue
Motility and chemotaxis	Reaching mucosal surfaces (especially areas with fast flow)
sIgA proteases	Prevent trapping of bacteria in mucin
Siderophores, surface proteins that bind transferrin, lactoferrin, ferritin, or hemin	Iron acquisition
Capsules (usually polysaccharide)	Prevent phagocytic uptake; reduce complement activation
Altered LPS O antigen	MAC not formed; serum resistance
C5a peptidase	Interferes with signaling function of complement
Toxic proteins	Kill phagocytes; reduce strength of oxidative burst
Variation in surface antigens	Evade antibody response

Adherence is necessary for bacteria to stay in the same spot to colonize a surface. This is most important in the mouth and digestive tract which is washed continuously by fluids. Practically all known bacterial pathogens have some way of attaching themselves firmly to host cells. The two most common methods are using pili to bind to host cell surface molecules such as carbohydrates and using surface proteins called afimbrial adhesins (illustrated below) which bind tightly to host tissues.



Bacteria have produced different pili which each have specific receptors that bind to the molecules on the specific type of host cell. These pili and receptors determine what body site the bacteria can infect. Bacteria with pili that bind at the tips with intestinal lining cells will infect the intestines but will not infect lung lining tissues because the molecules at the tip of the pili do not fit receptors on the different types of cells. The opposite is true of bacteria which colonize and infect the respiratory tract. These will not colonize the intestines because their pili tips do not fit the receptors there. By changing the genetic machinery to produce different types of pili tip proteins, it is possible to produce bacteria strains capable of infecting new body sites that they could not adhere to previously.

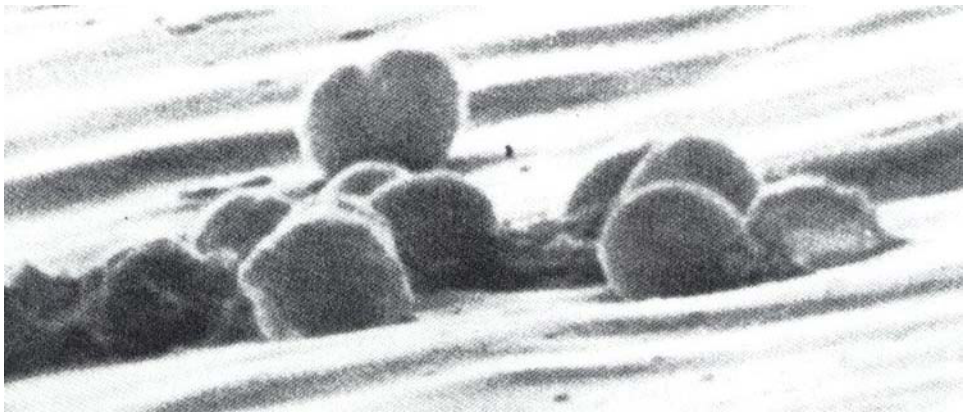
Bacteria growing in the body are constantly losing and reforming pili which helps them evade some of the immune response. The antibodies which bind to the tips of pili will prevent their attaching to the host cells. By breaking off and forming new pili with new and different binding proteins, the antibodies are no longer effective. Some bacteria

like *gonorrhea* change pili tip proteins so frequently that it is impossible for the body to mount an effective antibody response and prevent colonization.

One of the reasons that pili are necessary for adhesion is that the surfaces of both bacteria and host cells are negatively charged and there is an electrostatic repulsion that takes place at the cellular level. The pili allow the bacteria to come close to the cells without touching and being pushed away. After a loose connection is made with the pili, other bacterial proteins are used to make a tighter attachment and bring the cell surfaces together. These connections are called afimbrial adhesins.

Most bacteria that use pili are gram negative bacteria. Gram positive bacteria use afimbrial adhesins which attach to proteins or carbohydrates. Some bacteria attach to the host cells in a layer and the cells grow on top of them and are bound to them by a polysaccharide matrix (like a dense spiderweb) forming a **biofilm**. The normal bacteria populations of the mouth and intestines form biofilms to stay in one spot. These dense mats act as barriers which prevent other harmful bacteria from finding a spot to colonize. Dental plaque is an example of a biofilm that causes disease. *Psuedomonas auruginosa* forms films in the lungs of cystic fibrosis patients. Biofilms also commonly form on medical plastic implants when they are not completely sterilized at the time of surgery. Biofilms are much more resistant to antibiotics than free living bacteria and they also partially resist phagocytosis. The problem with biofilms on plastic implants is so great in hospitals that it is practically considered to be a virulence factor all by itself.

By providing the machinery to excrete a biofilm matrix, the ability to colonize or resist host defenses in human tissues can be conferred on many candidate bacteria species. (Staphylococcus binding to a PVC catheter-photo)



Invasion of host cells is accomplished by some bacteria which attach to the host cells. They do this with bacterial surface proteins that act on the normal host cell and instruct it to form structures like phagocytes that envelope the bacteria. Since these normal cells are not phagocytes, they do not have machinery to harm the bacteria and act as a protective coat to the bacteria while being consumed from the inside. The proteins produced by the bacteria that initiate this engulfing action are called **invasins** or invasion factors.

Some bacteria that are engulfed by host phagocytes use similar proteins to damage the phagocyte membrane and permit it to escape its vesicle and live on inside the cytoplasm of the phagocyte. Contact with the bactericidal chemical pocket is avoided. While inside the phagocyte, they are protected from host antibodies and complement, have abundant food in the cytoplasm and partial protection from antibiotics. *Shigella* and *Listeria* species that are virulent produce these proteins and invade phagocytes in this manner. These bacteria can hitch a ride inside the phagocytes and reach other parts of the lymph system and body organs which spreads the infection.

Many harmless bacteria and some infectious species without protective capsules would make very effective bio-weapons with the ability to produce invasins.

Motility allows bacteria to move in a direction towards food and away from harmful substances. This is especially helpful if the environment is the mouth or intestinal tract where fluids are constantly moving everything downstream That is why most successful colonizers of the GI tract have flagella and are motile. They move in the desired direction because they have complex biological detection machinery that comes with flagella to detect the environment and swim in the desired direction (like sperm). It permits bacteria to move through viscous mucin in the respiratory tract as well. It also helps bacteria survive in lakes and rivers. There is also non-flageller motility that aids some organisms in spreading from cell to cell and through organs which are found in myxobacteria and spirochetes.

Secretory Immunoglobulin A Proteases is an enzyme produced by some bacteria which attacks the stickiness in the mucin layer. This stickiness is caused in part by sIgA molecules that simultaneously bind to bacterial antigens. This enzyme is used by some bacteria to cleave IgA in the hinge region separating the part that binds to the bacteria from the part that interacts with the mucin. This helps them resist being trapped by the mucin and being moved with it up the throat and expelled making them more effective in initiating respiratory infections.

Iron acquisition mechanisms are necessary for most bacteria to successfully invade and colonize the human body. Iron concentrations in nature are low and in the human body, free iron is especially low. Almost all human iron is bound to proteins such as hemin and ferritin. Bacteria have developed mechanisms for acquiring iron from the human body. The most well known is **sidephores** which are small compounds that chelate (bind into a special form) iron with a very high affinity.

These sidephores are excreted by bacteria into the surroundings and they form tight complexes which are then drawn into specific receptors on the bacteria surface. Once inside, the sidephore is cleaved releasing the iron for use by the bacteria cell. Some bacteria also have receptors for sidephores produced by other organisms which is their way of freeloading iron.

Some pathogenic bacteria can bind the iron proteins to their surfaces directly and then break them down inside. Many infectious bacteria produce exotoxins only when they are low on iron. These kill neighboring cells which release their iron stores on cell death and lysis.

This provides bound iron which still must be cleaved from the protein, as well as the basic cell nutrients for food.

Capsules are loose unstructured networks of polymers that cover the surfaces of some bacteria. Most are made up of polysaccharides but some are made up of proteins or mixtures. The capsules in infectious bacteria prevent complement activation and phagocytic killing. Capsules that are rich in **sialic acid** are usually the most effective at protecting the bacteria from host defenses. This acid has a high affinity for a blood serum protein (**H**) which interferes with the complement action. Some bacteria produce this acid on their cell surface without a capsule and are also more resistant to phagocytosis.

Complement that does bind to the bacteria surfaces under the capsule are prevented from making contact with phagocyte receptors by the thick capsule network. This capsular material also mostly shields the bacteria membrane from the membrane attack complex (MAC). Some MAC proteins can usually gets through the loose network of the capsule by diffusion and so some of the invading bacteria are killed while others are not. The more effective the capsule, the more virulent the strain of bacteria.

Bacterial meningitis is caused primarily by three species (*Neisseria meningitidis, Haemophilus influenzae and Streptococcus pneumoniae*). All three produce polysaccharide capsules and the most dangerous strains are also serum resistant. Some strains become serum resistant by attaching sialic acid residues to their LPS molecules and mdifying their LPS O antigen side chains. S. pneumonia are gram positive and naturally serum resistant. Once these organisms reach the bloodstream, they are protected from the phagocytes by effective capsules. Some E-coli cause meningitis in newborns because they produce a capsule of sialic acid residues that do not activate complement. It prevents MAC formation in this way and this renders all the non-specific blood defenses ineffective.

People who can make antibody's against capsular antigens are fully protected from the disease. The immune system of infants have not done this so there is no protection to the earliest infections except maternal antibodies. A vaccine made up of capsular polysaccharide (attached to a protein to make it a better antigen) of H. influenae type b is used in young children.

Capsules that are composed of sialic residues do not produce antibody responses and effective vaccines for these types of infective strains have not been developed. E-coli (K1 strain) and many N. meningitidis strains have this type of capsule.

During outbreaks of N. meningitidis, up to 80% of the population may have their throats and noses colonized while only a small percentage actually come down with the disease. This is because most of the adult population has had some previous level of exposure and immunity. Outbreaks in Africa are almost always associated with the dry season when mucus membrane surfaces thicken and dry up. Influenza also predisposes populations to outbreaks due to damage caused to cells lining the membranes.

Meningitis causes death within 24 hours in many cases because the bacteria release the toxins in the cell wall (LPS) as they die and these toxins are already inside the spinal fluid and blood where they can cause shock and death. Administering antibiotics that kill and lyse large numbers of bacteria can release all the toxin at once and cause more short term harm than good. Corticosteroids are often given with antibiotics to reduce the inflammatory response of the toxins that are released on cell death.

Circumventing complement and phagocytes is also accomplished by several other methods. Gram negative bacteria with LPS antigens can form sialic acid on its O antigen. This prevents complement formation on tips of these antigens. Some bacteria also produce longer chains of the LPS antigen which seems to hold the MAC proteins too far away from the bacteria to effectively destroy the membrane. (Like a man using a spear to keep a man with a sword from getting too close to harm him). Bacteria that are not killed by the MAC are called **serum resistant**.

Some bacteria produce enzymes that directly degrade parts of the complement that acts as attractants to phagocytes (S. pyogenes), while others produce proteins that kill phagocytes, inhibit their migration, or reduce the strength of their oxidative burst. These substances not only prevent phagocyte recruitment to the site, they also protect the bacteria from phagocytes that do reach the site.

As mentioned earlier, some bacteria have developed methods of escaping the phagosome inside the phagocytes before it can merge with the lysosome. Some bacteria can prevent the fusion of the lysosome with the phagosome. Others have acquired some resistance to the chemical complex in the fusion and survive. These usually involve production of enzymes like catalase and superoxide dismutase that detoxify the superoxide. Some have cell walls that are resistant to lysozyme. These bacteria are among the most deadly infective strains known. The only defense left after survival inside phagocytes is the activated macrophages and the cytotoxic T-cells.

Evading the host antibodies has already been mentioned where the pili can fall off and new proteins form on the new tips. Some bacteria can also vary their surface proteins as well to make the first antibody response obsolete. Bacteria can also produce surface structures that resemble the host cells carbohydrates (hyaluronic acid and sialic acid). Bacteria can also coat themselves with host proteins like fibronectin. Some produce proteins that bind the wrong end of the antibody and prevents opsonization.

This antibody coat may prevent recognition of the bacteria by the immune system. Bacterial binding of iron proteins may also produce an effective exterior coat.

Recent studies of bacteria show that they produce many proteins and stop making others as they colonize a new site of body tissue. The number of genes necessary to produce the entire sequence of processes that enable a Salmonella to survive in macrophages is greater than 200. As the bacteria move from a mucosal surface, to the underlying tissue, to the bloodstream, and into the liver, they go through several stages of adaptation. Each new environment causes them to shift production of one enzyme or protein system to another to adapt to the new food sources and dangerous surroundings.

It has also been found that a single mutation to eliminate a virulence trait has often had no effect by itself on virulence. It has often taken several mutations to have much effect on degrading virulence. Likewise, in the efforts to use mutation alone as a method for producing disease causing weapons, scientists found that single mutations that improved disease were rare and generally ineffective by themselves. It often requires many sequential changes in genes to produce superior virulence by mutation alone. Modern methods of using several techniques in combination has changed this science considerably.

The author has previously published the concepts of using food particles that surround bacteria as inhalation weapons. The concept is that the bacteria, growing from the inside out form a very large infective mass that is protected from antibodies and phagocytes. This occurs in nature as well.

In bacterial endocarditis, the harmless streptococci in the mouth can enter the bloodstream during oral surgery. Normally, the phagocytes will digest them all in a few hours to a few days with no problem. In individuals with endocarditis, the irregular and turbulent blood flow around the heart valves causes clots to form on the valve surface. The streptococci reach these surfaces and colonize them. The phagocytes cannot reach some of the colonizing bacteria due to biofilm, turbulence and more clots forming on top of the mass. The immune response can cause inflammation around the heart without attacking the bacteria. Phagocytes can be attracted to the area but be unable to reach the colonies. The bacteria can also produce enzymes that turn some of the surrounding tissue into food. All of these cause injury and disease but the bacteria is a normal healthy strain possessing no virulence factors at all.

Many virulence factors lie in the genes of the bacteria. The ribosomes in a bacteria produce the instructions necessary for adapting to a new tissue the bacteria has attached to. If the directions it provides for producing new enzymes are wrong, the bacteria dies. If they are correct, it survives and infects (grows). This machinery has been aided by billions of years of mutations and natural selection. It can be speeded up and significantly enhanced by man.

Virulence Factors Used to Harm the Host

Bacteria produce a variety of substances to fight back against host defenses and turn surrounding matter into food. The most important of these have been briefly mentioned, the exotoxins and endotoxins.

Exotoxins are excreted from the bacteria, hence the term exo. Endotoxins are embedded in the cell wall (LPS) of gram negative microorganisms and are released on bacteria cell death. A few bacteria produce toxins that are held within the cytoplasm and also released on cell death. All of these toxins are proteins.

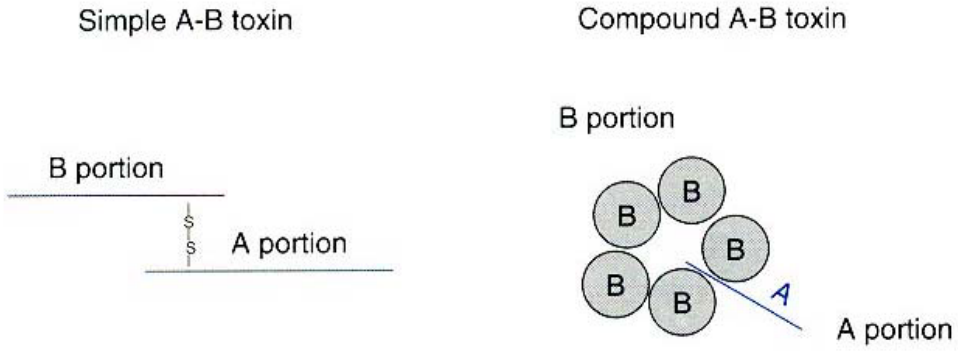
In some species most or all strains of the bacteria will produce the same toxin. Some gram negative bacteria also produce exotoxins as well as LPS toxins. Exotoxins vary considerably in the types of cells they attack.

Exotoxins that attack a variety of cell types are called cytotoxins while those that attack a specific type of cell are named according to the cell that they affect. Neuerotoxin, leukotoxin, and cardiotoxin attack nerve cells, leukocytes, and cardiac tissue.

Exotoxins fall into three categories-

1. The A-B toxins where part A controls the enzyme action that attacks the host cell and part B attaches the toxin molecule to the host cell receptor.
2. Toxins that do not have A-B portions and act by disrupting the cell membranes. Hemolysins and Pospholipases are examples.
3. Superantigens which stimulate T-cells to release cytokines.

The simplest kind of A-B toxin is that which has a single binding portion and a single enzyme part. As the A part of this toxin enters the host cell cytoplasm it separates from the B part. The "compound" A-B toxin has the same kind of binding unit but its B portion has many parts that also separate form the A part when it enters the host cell.



Both kinds of the B part of the toxin binds to and enters the host cells. The B portion determines the host cell specificity for the toxin. If the B portion binds to a glycoprotein that is found only on the surface of neurons, then it will be a neuron specific toxin even though the A portion could kill many other types of cells if it could gain entry into their cytoplasm.

Once the A portion enters the host cell, it begins to attack the cell enzymatically. Most of the A part toxins follow the same series of chemical reactions. They attack proteins which are essential for cell functioning.

The cholera A part attacks a protein that regulates ion flow in the cell. This causes water to leak from the host cell which is seen as diarrhea. Diptheria toxin part A attacks proteins involved in cell protein synthesis which kills the cell directly. Shigella A toxin cleaves host cell rRNA molecules which also shuts down protein synthesis.

Membrane disrupting toxins attack the plasma membranes of host cells. There are two kinds of these toxins. One is a protein that inserts itself into a host cell membrane and forms a channel or pore that lets the insides of the cytoplasm leak out and free water or plasma to enter. The osmotic strength inside a cell is much higher than the outside so the new holes in the cell wall produce an inrush of water which causes the cell to swell and rupture. This type of toxin has no enzymatic activity but kills cells simply by attaching to a host cell surface and inserting into membranes. The host cell molecule that is usually used as a receptor is cholesterol rather than a carbohydrate.

The second kind of membrane disrupting toxin is the enzyme phospholipase. These lipases remove part of the lipid portion of surface phospholipids which stabilizes the phospholipid layer in human cells. When this part is removed, the membrane destabilizes and breaks apart and the cell lyses.

Membrane destabilizing toxins attack many cell types and their action is seen on culture plates of bacteria that produce them. They attack the cells in the surrounding medium causing cell lysis and the is visibly seen as a halo around the bacteria in which the surrounding cells have lysed from the toxins effect and the cell contents have leaked out and started to break down. The outer edge of the halo is the outside edge of the toxins diffusion into the medium. All human cells have phospholipids so these toxins attack all types of cells and are called cytotoxins.

Superantigens are an unusual bacterial toxin. They are proteins that forma bridge between antigen presenting cells (APC's) and T-cells. The APC's normally process antigens by cleaving them into peptides and presenting them to T-cells. The superantigen binds to the presentation sites causing many T-cells to link to them rather than the few that normally do. The result is stimulation of the production of cytokines by the T-cells. The net effect is that APC's that normally stimulate 1 in 10,000 T-cells will now stimulate 1 in 5. The results can be nausea, vomiting, malaise, fever and shock. This a common effect of staphylococcal food poisoning.

Many exotoxins have parts that make them antigenic. Antibodies can be formed that attach to the toxin molecule and prevent it from attaching to the cell surface. Vaccines have been produced by manufacturing the toxin in quantity, and then using genetic mutation, chemical attack or heat to render the toxin inactive but still leave its surface parts intact. These parts can then be injected into a patient which produces antibodies to the toxin parts. This type of vaccine is called a toxoid.

[The anthrax vaccine has made headlines in recent years as a possible contributor to gulf war syndrome. This author has previously described his views on this in Volume 6-A but will revisit the controversy here. The anthrax toxin has three parts. The part that causes direct harm to cells is the one that kills humans during infection. Another part of the vaccine is an invasive substance that opens doorways on the cell surfaces for toxin and other substances to enter. The toxin used in the vaccine was tested so that its toxic activity was eliminated in the lab and this made the vaccine seem safe. The portion of the toxin that allowed entry to the cell was not tested for (according to published accounts).

Even though the toxin itself could do no harm to those receiving the vaccine, the vaccine parts could still effect cell surfaces allowing exterior substances to enter that could be toxic. There are billions of different molecules floating around in the blood plasma. The cells do not normally permit entry of any of these without being a proper fit or chemical reaction for the cells biology. When the cell surfaces are artificially opened to permit other floating substances, the cell can now be effected by whatever enters it that is floating around. There is essentially no biological guard at the gate. The new vaccine should be tested for these invasive substances for FDA approval.]

Exotoxins cause disease in a variety of ways. When bacteria produce a toxin in foods and these foods are ingested, they cause food poisoning. This illness is caused by the injurious effects of the consumed toxin and not by an infection by the bacteria. Antibiotics are useless but the disease is limited to the amount of toxin absorbed. No more toxin is being produced by bacteria so the illness ends when the toxins and their injurious effects run out (or the patient dies).

Bacteria that colonize a wound or mucosal surface do not enter the bloodstream but grow and produce exotoxins that act locally or diffuse into the bloodstream and attack susceptible organs and tissues. Cholera bacteria adhere to mucosal surfaces in the intestine and produce the cholera toxin that causes mucosal cells to discharge water. The diphtheria toxin enters the bloodstream and attacks the heart and other organs. As the body detects these toxins, it produces antibodies to the toxin but these are often too little too late.

Bacteria will also produce exotoxins that attack local cells turning them into food and kill phagocytes entering the area. The toxin is used to aid bacterial growth and spread in the immediate tissues. Gas gangrene is this type of disease. The toxins produced by C. perfringens kill local cells and cut off the blood supply. The bodies phagocytes, antibodies and other defenses cannot reach the site to put up a defense.

More damage is caused to surrounding tissues in the same manner and the disease spreads. Before the advent of antibiotics, amputation was the only way to stop the spread of the disease. If the tissue damage is great enough, antibiotics are ineffective and amputation is the only remedy.

Hydrolytic Enzymes

Many bacteria produce enzymes like **hyaluronidase** and **proteases** which are also produced inside human cells to break down food particles. These enzymes attack extracellular matrix that binds the cells together and this disrupts the host tissues and may turn these parts into bacteria food.

Some produce **DNAases** break down the tissues further forming pus at the infection site. Phagocytes produce some of these same enzymes to clean up infection sites and so the damage in these areas is often mixed. These types of enzymes will not normally kill the host cells (except the phospholipases).

Autoimmune Response

Some bacteria produce antigens that are similar or identical to host cell proteins. When T-cells bind to the surface of bacteria during infection, a surface protein I also bound. This protein acts as a signal to prevent the T-cells from attaching to host cells. This prevents the bodies immune system from attacking itself. Some bacteria can produce this protein with the effect of causing antibodies and cytotoxic T cells to be produced which can attack the bodies own cells. In **Rheumatic heart disease**, antibodies and T-cells stimulated by antigens of *Streptococcus pyogenes* cross react with heart tissues and provoke an inflammatory response that damages the heart.

Bacteria also produce heat shock proteins that are released when the bacteria encounters various types of stress. Damaging pH, heat, and oxidative compounds set these in motion. These proteins normally help the bacteria synthesize proteins. When the bacteria is consumed by phagocytes (serious stress) it releases these proteins which resemble human cell proteins. This causes the immune system to be tricked into not recognizing the bacteria or attacking its own cells with the heat shock proteins.

Some bacteria elicit a huge antibody response which causes aggregates of antigen-antibody complexes to form. These aggregates can accumulate in filtering organs like the kidneys and activate the complement cascade. S. pyogenes can also cause this response.

Endotoxins and similar bacterial cell wall structures

Endotoxin is the lipo-polysaccharide (LPS) that is formed on the outer wall of gram negative bacteria. The lipid portion (lipid A) is embedded in the outer membrane with the core and O antigens extending outward from the bacterial surface. Lipid A is the toxic part of the molecule and because it is imbedded inside the cell wall, it cannot cause harm until the cell dies, and the cell wall begins to break apart (lyse).

Lysis occurs during the membrane attack complex (MAC) action of complement and during digestion by phagocytes. Some antibiotics also damage the cell membranes releasing the LPS toxins.

The lipid A is toxic mainly because of its ability to activate complement and stimulate the release of host proteins like cytokines (which signal and mediate the immune response). When they are produced in too high of concentrations, they become toxic to the host. Local inflammation in tissue is caused by LPS activation of complement. Cytokine release can lead to septic shock. Gram positive bacteria can also produce this same series of reactions if they are killed in the bloodstream in large numbers and large amounts of complement and cytokine release is activated all at once.

When bacteria infect the bloodstream, they usually cause fever, increased respiration and heart rate and increased PMN levels in the blood. If the infection is not overcome in the early stages, the bacterial populations become very large and the blood is flooded with bacteria parts from phagocyte digestion. These massive levels of bacteria fragments provoke an equivalent, massive inflammatory response in organs, circulatory system and other tissues. This leads to organ failure, circulatory collapse and death from septicemia (mortality rates over 70%).

The weapons concept behind this is that a bacteria does not need to successfully attack and destroy body parts or produce toxins that do. It can be superior in colonizing in very large numbers so that when they are consumed, their own body parts cause the septicemia reaction described above. Any gram negative bacteria with LPS can do this. Some of the LPS is more effective than others at producing this response. This is why some E-coli are harmless to people and kill mice while others are harmless to mice and kill people, Different strains produce different structures. They also produce different volumes of these structures in their cell walls.

Different types of LPS provoke different types of cytokine release that are often common for a species of bacteria. Some of these cytokines cause direct and indirect effects of fever, anorexia, wasting, hemorrhage in organs, migration of PMN’s, vascular damage, and activation of monocytes and macrophages which compound the damage. Some LPS will cause only one of these via the cytokines while most cause a combination. A few can cause them all.

The most effective species that cause this type of infection are E-coli, Klebsiella, Pseudomonas, Serratia and Proteus that are antibiotic resistant. Light antibiotic therapy in resistant bacteria leads to heavy antibiotic therapy after the bacteria have reached high levels in the blood and this can worsen the patients condition. Massive amounts of resistant bacteria are now finally all dying at once producing massive amounts of inflammatory LPS.

Bacterial regulation of Virulence Genes

Bacteria can float in a pond of cool water at 20 C and scavenge particles of food around it to survive, grow and divide. The pH is neutral, iron is abundant, and some oxygen is dissolved in the water to support metabolism. All of the cells internal machinery is finely tuned to operate and feed itself in this environment.

This same bacteria cell is now suddenly transported and ingested in a glass of contaminated water. It is inside of a human body at 37C, a much warmer environment. It experiences very low pH in the stomach, and when it survives that, it encounters a rise in pH in the intestines as well as high concentrations of membrane disrupting bile salts. This environment and that in the colon is mostly anaerobic so its oxygen is almost gone. The iron is bound to proteins and not found floating free. Finally, without the ability to adhere to cells, it will be washed down the GI tract and away from the host. The adhesins it used to stick to rocks or aquatic plants do not work here. All this happens in the course of only a few hours.

The bacteria encounters the signals that tell it that the pH has changed. It can no longer find free iron. Its food is different and the solid mass around it will not let it stick to it with its current adhesin. The surroundings are now hot. In this new setting, the genes inside the bacteria now order it to produce a new form of adhesin that lets it bind to the cell surfaces of the intestinal tract. It may even be able to form a biofilm with many daughter cells. It adjusts its machinery to operate in the higher heat and tells it to start scavenging for iron by manufacturing and using iron chelates.

Now the bacteria face the phagocytes so it begins to produce proteins that prevent it from being detected or consumed. If it is consumed, it excretes substances that let it evade the toxic chemical pouch of the phagocyte. It may even have a capsule that protects it.

This ability of the bacteria to turn off important cellular machinery and turn on the needed factors only when they are needed is one of the most important abilities that a disease causing bacteria can have. This ability resides in the genes of the bacteria and is controlled in a number of ways.

The bacteria can change their DNA sequence to amplify and rearrange the genes that direct the cellular machinery. They can change the number of transcripts using activators and repressors and they can also change the amount of active gene product. Later chapters will explain what all this means. The fact that bacteria can do this is seen when you grow bacteria in culture plates. Many bacteria do not produce toxin at 25C but start producing it at 37C. Some produce adhesins and invasins only at this higher temperature. The chart below describes some of the more common virulence factors that can be observed by changing the growth cultures.

Organism	Environmental signal(s)
Bordetella pertussis	Temperature, SO_4, nicotinic acid
Corynebacterium diphtheriae	Iron
Escherichia coli	Iron, temperature, carbon source
Listeria monocytogenes	Temperature
Pseudomonas aeruginosa	Iron, osmolarity
Salmonella typhimurium	Osmolarity, starvation, stress, pH, growth phase
Shigella spp.	Temperature
Staphylococcus aureus	Growth phase
Vibrio cholerae	Osmolarity, pH, temperature, amino acids, CO_2, iron
Yersinia spp.	Temperature, Ca^{2+}

The ability to change these genes by modifying methods varies. Mutation alone may change a tiny sequence and effect something small like bacterial resistance due to a single amino acid change in a surface protein. Mutation alone cannot produce the thousands of different directions needed in the gene to regulate the way the bacteria adjusts to its living conditions. Millions of years of gradual evolution and selection has evolved all these different types of machinery, each using a complex set of instructions.

Other tools allow for the exchange of blocks of genetic machinery. Plasmids are loops of DNA that bacteria exchange. A plasmid loop can contain complete sets of LPS instructions changing a non-toxic bacteria (in humans) to a toxic one. Cell fusion can permit virtually all the machinery from two pair of bacteria to coexist side by side in a new “hybrid”, and many hybrids may express the new directions differently. Viruses (phages) can also be used to insert new DNA instructions into bacteria. Finally, the specific desired DNA machinery genes can be snipped from one bacteria and inserted into the desired location in another bacteria using genetic engineering. This involves using enzymes to cut DNA fragments at the end of each complete set of instructions and then transferring them to the target bacteria.

Some of these techniques can be used in interesting combinations to produce desired results. Plasmids can be used to transfer new toxic LPS cell wall structures from a potent E-coli into plague. Mutation can then be used to make it antibiotic resistant. A gene coding an instruction for producing blood clotting substance can be added to protect the bacteria from the bodies defenses and provide a large food source for huge colony formation. When the cells finally die, they evoke a massive inflammation that causes septic shock and death. A new and deadlier plague has emerged.

This organism could then be fused with the healthy bacteria that cause dental plaque so that it can live in the mouth, use human food and be communicable in this fashion.

[These are just the possibilities with one dangerous bacteria, modified in single ways using each of the techniques described. There are tens of thousands of potential combinations with each bacteria, and there are hundreds of thousands of bacteria. The potential for weapons is in the millions. This does not even count the possible fusion combinations with human and animal cells (so the body could not recognize it as an infection), splicing in plant toxins, and using new virus proteins in combination. A targets own DNA could be used to splice in a sequence making a disease selective only for him. This is the future of warfare. Scientist warriors in their own home laboratories can now make millions of designer weapons and arm entire populations with them in a fashion similar to that of computer viruses on the internet. A person in the US can make a designer bug at home, mail it to China and have his underground contact their extinguish the local government or population on demand. The only limit is the imagination of the author.]

Previous volumes describe all the major bacteria, plant and mold infectious species and toxin producers. A good working knowledge of these (and a good technical library) is essential for producing modified bacteria weapons.

Chapter 3
Fundamentals of Bacteria
Genetics and Mutations

Genetics is the study of how hereditary traits are passed on from one generation to the next. It was known in ancient times in both plants and animals that the offspring of a pair of parents would show a highly variable blending of the traits of the parents. Often, the traits of one parent were much more obvious than those of the other parent.

By the mid 1850's, the process of plant hybridization was in common use and scientists had carried out a number of experiments to find if these blendings followed any solid rules. In 1865, Gregor Mendel published his now famous work on the heredity of traits like color and height.

In one of the most important experiments, Mendel selected pea flowers that were always purple or white. There is no blending of this trait to produce whitish-purple colors. He knew from experience that some colors would be passed on from parents that did not show the same color.

In his experiments, he would cross-pollinate (breed) purple flowered plants with white flowered plants. It did not matter which parent provided the pollen to the other plant, the results were the same. In his first generation, all the offspring produced only purple flowers. He then allowed these plants to self pollinate and in this next generation he yielded 705 offspring with purple flowers and 224 producing white flowers (out of 929 Plants). This ratio was almost exactly 3:1 and was observed in all other traits like height, seed color and texture, and pod inflation.

What he had discovered is that the gene for purple is **dominant** and the gene for white is **recessive**. He also realized that each trait is passed on in discreet units (which we now call **genes**) rather than blending and producing shades. The rules he discovered is that

1. The trait that is inherited is a discreet unit
2. Each plant must have a pair of these characteristics (one from each parent)
3. When an individual has two conflicting genes, only one will show up and is dominant over the other one.

Only one fourth of the flowers would be white due to the dominance in the purple gene in ¾ of the offspring. $1/4^{th}$ would inherit a pair of purple genes and would have only purple flowers. ½ would inherit both genes the purple dominant and showing this color. $1/4^{th}$ would inherit both white genes and these would be seen with white flowers.

This type of reasoning was tested over and over again for all traits and proved to be true for all distinct physical characteristics. They knew the rules governing genes, but they did not know the mechanisms.

In 1882, microscopes improved enough so that the insides of cells could be seen and studied. Cells that were dividing would produce a coiled up bundle that would stain brightly and this was called chromatin (later we would call it chromosomes). These early scientists saw the threadlike appearance of the chromatin appear from inside the cell during cell division.

When single bacteria cells divide and produce offspring, the process is called mitosis. The chromatin coils up and condenses into compact structures called chromosomes. They become shorter and thicker as mitosis continues and scientists could see that they were made up of two parts that they called chromatids. Pairs of chromosomes were found in all species and the size, shape and number of chromosomes is constant for any given species. Humans were found to have 23 pairs of chromosomes while some species have only one pair. Some species have more than 200 pairs of chromosomes.

As mitosis continues, the chromatids of each chromosome separate to form two daughter chromosomes. These move to opposite sides of the inside of the cell so that each daughter cell will now have an identical set of genes. The chromosome uncoils, and the cytoplasm divides to form two separate cells, each with the same chromosomes and cellular machinery as the parent had. Each new cell has identical cell membranes, nucleus, and other parts.

In sexual reproduction in humans and in pea plants, only ½ of each parents chromosomes are passed on so ½ of each of the chromosomes is found in the daughter cells. The single cells that divide produce identical offspring while sex cells that are paired produce a mix of the genes of the parents which are not identical to the parents.

A scientist in 1910 began a study of fruit flies. His name was Thomas Hunt Morgan and over the next decade he would produce many important discoveries in his laboratory by breeding and studying the fruit flies. He had hundreds of bottles of fruit flies under study at any given time and his lab was known as the "fly room" to his students. The flies would produce over 100 offspring each mating and could be raised in a pint bottle on mashed bananas or a mixture of molasses, cornmeal and a little yeast sprinkled on top. A new generation was produced every two weeks.

Morgan and his co-workers discovered that Mendels laws of inherited characteristics held true with practically 100% accuracy. They also discovered that many characteristics tended to be inherited together and this tendency cam to be called **linkage**. Two traits that were linked in the fruit fly were the black body and dumpy (short) wings. Both of these are recessive to the tan body and long wings of the "wild" type of fruit fly. Morgan crossed both types of fruit flies after obtaining pure strains of each.

As expected all offspring yielded only the dominant pair of traits, the tan body and long wings. When these hybrid flies were interbred, 3/4ths showed the dominant pair of traits and $1/4^{th}$ produced the recessive black body and short wings. Only a few (16%) would yield a mix of these paired traits.

More tests were conducted with similar results in succeeding generations. To explain these results, Morgan hypothesized that the genes for each of these paired characteristics must be on the same chromosome. The dominant parent must have each of the dominant genes on the same chromosome while the recessive parents must have the recessive genes on their same chromosomes.

In the few flies that did not inherit the paired traits, Morgan reasoned that there must be an exchange of genes between chromosomes. In other words, the chromosome is not passed on completely intact. It must be separated at some places and parts of the chromosome must be "traded" between each other in order for these genes to be mixed. In some of these trades he observed, the chromosome trades both of the dominant or recessive genes. In other trades, **crossing over** occurred in which 8% receive a mix of dominant wings and recessive body, while 8% received a mix of recessive wings and dominant bodies.

Different combinations of characteristics produced different percentages of crossing over and so one of the students of Morgans, A. H. Sturtevant developed the first crude system of mapping chromosomes using the hypothesis that genes that are further apart on the chromosomes should cross over more often than genes closer together. This method is still used today in conjunction with DNA mapping.

Some traits do not dominate one another and the result can be seen as a blending. The snapdragon is a good example. If red flowers are crossed with white flowers, all of the first generation (F1) flowers are pink. In the second generation (F2), self pollinated snapdragons will be ¼ red, ½ pink, and ¼ white. In this case neither trait dominates the other so a blend occurs. When the pairs of white or red come back together, they produce only the original color. The blending is sometimes called co-dominance or lack of dominance.

This also appears in humans with different blood types. Blood types A and B are not dominant over each other so if a person has both genes, they have type AB. If They have A or B in pairs by themselves then they are these types. Both A and B are dominant over O so a person can have one O with either A or B and will be A or B. Only if the person has both O genes, will they have the O blood type which is uncommon. People with type O are called universal blood donors. AB blood types are universal recipients because O blood types do not produce antibodies for the AB blood and AB blood recognizes AB types and do not produce antibodies to it or to O types.

In 1908, an English physician published a book describing patients who lacked the ability to produce specific enzymes in their bodies. These caused a variety of disease and he studied the families of these patients to find out if others had similar disease. He soon found that in every case, the disease was inherited and that they followed the rules that identified them as recessive Mendelian traits.

The effect of the defect for a missing enzyme is like having an assembly line of robots processing food. The robots do not know that one of their robots further down the line is not working so they continue to function as normal, processing the food in front of them. In the cellular machinery of humans, the robots are the pathways for processing nutrients for the cells. When one of the enzymes was missing, the process for the pathway continues until the missing enzyme robot is reached. Since he doesn't work, the food cannot be processed any further. People with this disease have defects that show up in their body chemistry. Their cellular robots process food for the cell only to the point of the missing enzyme instruction (robot). If the defect is a minor one, they find a way to live with it. If it is a major defect, they usually die from the effects.

Garrod pointed out in 1908 in his book, that all genes must control the characteristics of organisms by controlling the enzymes and proteins they produce. He could see the action of recessive genes in his patients so he concluded it must hold true for all our body and life processes.

In 1914, a German chemist, Robert Feulgen developed a method for staining DNA bright red. It would not produce this color in any other substance in the cell. This red color only appeared in the chromosomes and he believed that if the genes were only found in the chromosomes, then any chemicals substance found only in the chromosome must be stuff that genes are made of. This would provide a tool for separating the chemicals in the chromosomes from all other substances.

Transformation and DNA

By 1928, another discovery would aid in uncovering the chemistry of the gene. An English Bacteriologist Frederick Griffith would grow a strain of disease causing pneumococci (see volume 6-A) called the S strain on a culture dish. It was called the S strain because the bacteria had a capsule and this capsule produced smooth colonies, which is why they were called S (for smooth). He also had a mutant strain that could not produce a capsule that yielded rough looking colonies, which he called the R strain (for rough).

When Griffith injected his S strain of bacteria into mice the living S bacteria would kill the mice in a few days. The living R bacteria were harmless without the capsule and generally would not harm the mice. He then injected killed S strain bacteria into the mice to see if the dead cells had a toxin that would kill them. The mice were unaffected. He then injected both killed S strain bacteria into the mice with living R strain to see what would happen. All the mice died and when he did the autopsy, he found living S strain bacteria in the tissues of the dead mice.

Over the next three years many scientists studied this **transformation** as it was now called. They found that they could mix killed S strain bacteria in a culture dish with R strain and the living R strain would transform into S strain pneumococci. In 1933, James Alloway, an American scientist reported that he had not only killed the S strain, but he had extracted the DNA and mixed it into the living R strain and produced S strain

cells. Once these bacteria were transformed into S strains, they would permanently produce only S strain cells that had a capsule and caused pneumonia.

They deduced that the transforming material that yielded a capsule on the bacteria must be a gene and the search was on to discover the chemical nature of the gene and how it worked. By now they knew that the chromosomes were made up of proteins and some thought that they might be repeating **nucleic acids**. They doubted that the four simple **nucleotides** that made up these nucleic acids were too simple to account for the incredible variety of millions of genes that they knew must exist to produce all the vast differences in life on the planet.

In 1944, after 10 years of painstaking work, Owen Avery, Colin McCarty, and Maclym Mcleod announced that they had isolated and purified the molecules in the S strain pneumococci and tested each one for its ability to transform it into a capsule and disease producer. These three American biologists found that the only substance that would transform S strain into R strain cells was a particular kind of nucleic acid called **deoxyribonucleic acid** or **DNA.**

In 1941, American scientists George Beadle and Edward Tatum reported a series of experiments that had conducted on a common bread mold called Neurospora. They could grow it in a test tube with sugar (glucose), mineral salts and only one vitamin (biotin). This means that the Neurospora must have enzyme systems in its body to synthesize all the proteins (and specific amino acids-proteins are made up of amino acids) and vitamins except for biotin. If does not need to get them already preformed in its diet like humans and many other organisms do.

Beadle and Tatum irradiated many Neurospora with X rays which they knew would produce mutations (changes in the genes). These mutations occur at random and some of the mutations would kill the spores, while some would make them unable to grow in the test tube on the minimal food. These mutants that are different from their parent strains are called nutritional mutants. Each of these mutated spores were placed in a separate tube of enriched medium that had all the basic parts needed for growth. Each single amino acid, vitamin and mineral were provided.

Any mutant spore that lost the ability to grow on the minimal food could still grow on this enriched medium. In a few days, each mutant spore would produce a colony in its tube with hundreds more identical daughter spores with the same mutation. A few spores from these test tubes were then placed in three more test tubes. The tubes contained –

1. Minimal food plus all the Amino Acids
2. Minimal food plus all the Vitamins
3. Minimal food plus a variety of Sugars

The spores would then grow in the medium that supplied what it needed. If it could not grow without a needed amino acid, it must be a mutation that lost the ability to synthesize its own amino acids. The same was true of a needed vitamin or sugar. Once

they knew what category was needed, they would then grow the spores in more test tubes containing each specific amino acid (or vitamin or sugar) until they found the one the mutant could not grow without. The mutant would grow only in the tube with the amino acid that it specifically needed.

If the mold grew in the tubes with minimal medium plus all the amino acids, they would then place the daughter spores in test tubes, each containing a different amino acid plus the minimal medium. In this way, they discovered which amino acid was needed. If the needed amino acid was arginine (for example), then they knew that the mutant mold had a mutation in its gene for making arginine from the basic food. The mutant could no longer take its basic food and make arginine and so the mutation had damaged the gene that instructed the mold cell to produce arginine in a specific pathway. It now had to be supplied in the diet or the mold would no longer grow.

Beadle and Tatum discovered many nutritional mutants and they recognized that genes were responsible for the ability of the enzymes to catalyze steps in the pathways that the mold used to make its own amino acids like arginine. Each different type of mutant had to have a different defective step in the pathway for each of the defective enzymes. Since these defects were inherited by each daughter cell, their had to be something wrong with the genes so they determined that each gene must control the production of a specific enzyme.

This means that a defect in gene A will produce a defect in synthesizing enzyme A. Defects in genes B & C produce defects in enzymes B& C and so on. This became known as the one gene, one enzyme hypothesis. As science has progressed in the last 60 years, it has shifted to being known as one gene, one polypeptide. Genes also control the production of proteins as well as polypeptides.

Bacteriophage and DNA

The next breakthrough came in 1952 with the publication of experiments involving **Bacteriophage**, a virus that infects bacteria. Virus particles are so small that they cannot be seen with a light microscope. Pictures of them could be taken with an electron microscope however. The bacteriophage enter living cells and take over the machinery of the cell, usually forcing it to make copies of itself.

Bacteriophage (phage, for short) have a head, stalk and arms. The arms attach the phage to the target bacteria cell. Then the phage injects its core into the cell leaving its outside coat as a ghost. A hundred or more new bacteriophage particles would be produced inside the cell in less than an hour and then the bacteria cell would disintegrate releasing the phages to infect new cells. Scientists knew that the core the phages were injecting must have genetic material for making new phage. Their question was “Is it DNA or protein?

Alfred Hershey and Margaret Chase began their experiment by growing E-coli bacteria on a culture plate containing radioactive phosphorus. When the bacteria consumed phosphorus, they would incorporate the radioactive form into their cells. They also grew more E-coli on nutrients containing radioactive sulfur which the bacteria consumed and incorporated into their own molecules. The bacteria became radioactive.

In the next step, each of the bacteria were inoculated with phage culture. The phage infected and consumed the bacteria in the production of more phage and now the radioactive ions were part of the virus particles. Two different strains of radioactive (hot) phage were formed. One set of phages with the hot sulfur atoms and one with the hot phosphorus atoms.

Hershey and Chase knew that protein contained sulfur, but no phosphorus. They also knew that DNA contained phosphorus and no sulfur. They decided they would follow the radioactive ions to see whether it was protein or DNA that the phage was injecting into the bacteria. They now used the radioactive phage to infect more bacteria. One set was infected with the hot phosphorus phage while another set of bacteria was infected with the hot sulfur phage. Enough time was allowed for the phage to inject their cores into the bacteria but not enough for more phage to be produced. Next, they mixed the cells in a kitchen blender just long enough to knock the empty phage coats off of the bacteria cell walls but not long enough to harm the cells themselves.

The cultures were then spun in a centrifuge. The outer empty coats of the bacteriophages remained suspended while the phage core inside the cells settled to the bottom. In lab tests, the scientists found that almost all the radioactive sulfur was found in the liquid suspension containing the empty phage coats which meant that they were made up of protein. In the cell with the bacteria containing the core, they found all the radioactive phosphorus which meant that the core was DNA and this must be what the phage was injecting into the bacteria. This also showed that protein was not injected with the core. This supported the idea that genes were made up of DNA.

By 1950, scientists knew what that most of the DNA molecule was composed of four different nitrogenous bases. By studying the composition of these bases in a number of lab animals, a researcher, Erwin Chargoff discovered **Chargoffs rule**. This rule states that in any sample of DNA, the amount of **adenine** is equal to the amount of **thymidine** and the amount of **cytosine** is equal to the amount of **guanine**.

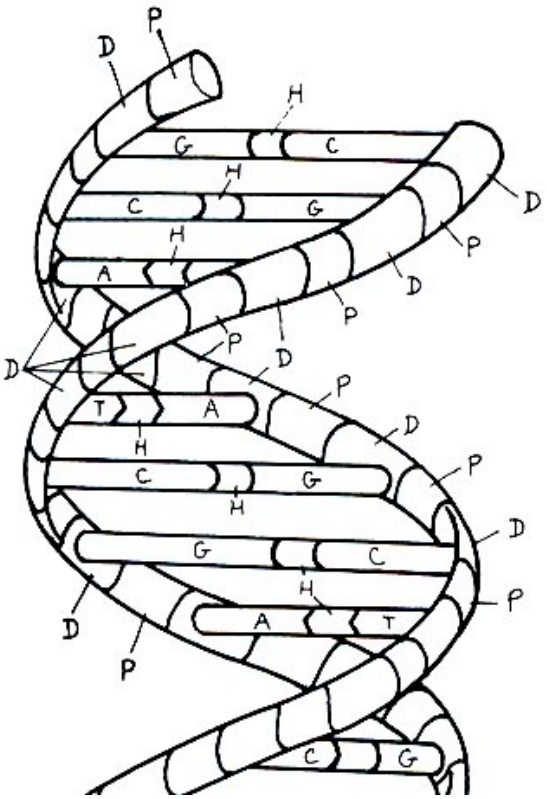
These four substances made up virtually all DNA. He also discovered that the ratio of adenine and thymine compared to cytosine and guanine is constant for all cells of a given species but these ratios vary from species to species. These ratios provided key clues to the structure of DNA and how only these four molecules could carry all the instructions of life.

The Double helix

More clues came from scientists at Cambridge University in England who purified crystals of DNA and did X-ray diffraction studies of them. By beaming X-rays at the crystals and exposing the rays to X-ray film the scientists could study the arrangements and spacing between the atoms in the crystal.

Another team of scientists at this time, were also working on the structure of DNA and this group included a visiting American with a recently earned Ph.D. degree, James D. Watson. He led the scientists in a theoretical and intellectual approach of examining all the evidence including the new X-ray results. They used Chargoffs rule to deduce that adenine always paired with thymine and cytosine always paired with guanine. The chemistry of the bonding was also consistent with this idea. They used the X-ray data to recognize that the form of the molecule is most likely in a **helix** shape.

The final structure then must be a double helix in which the DNA molecule appears like a ladder that has been twisted.



The base pairs of **Guanine (G)**
Adenine (A)
Thymine (T)
Cytosine (C) are shown as the rungs of the ladder.
Hydrogen bonds (H) hold the base pairs together

The deoxyribose (D) and phosphate (P) groups (in the diagram) alternate continuously for the entire length of the molecule and form the uprights or **backbone** of the ladder. (This is the phosphorus detected in its radioactive form described in the earlier tests.)

The sequence of these four different bases constitutes a code (the bases are a four letter alphabet) and this code specifies all the information for almost every form of life on earth. The information in a single human chromosome is about 140 million base pairs and humans have 23 chromosomes. The chromosomes would be about two inches long if laid out in a straight line. In order to fit inside a nucleus of a cell, the chromosomes coil tightly around each other and the rungs of the ladder are actually stacked close together.

The order of the pairs in the helix are important because a single change in the order can yield a **mutation** that can cause the loss of ability to produce proteins and other important substances. The wrong single molecule or base pair out of sequence can cause the death of a cell or even the death of the entire organism.

In 1958, Watson and two other scientists on this team were awarded the Nobel prize in physiology and medicine for their discovery of the double helix. It was important because the double helix carried all the genetic information in its varying sequences of the base pairs, and it was also able to duplicate itself and pass on this same genetic information to every daughter cell.

DNA Replication

Using radioactive tracers, scientists soon learned how the DNA copies itself. The DNA unzips itself like a "zipper". The two halves of the zipper are separated at the center of the base pairs.

As the DNA unzips, an enzyme called **DNA polymerase** adds new base pairs to each side of the zipper to form two new copies of the DNA. Only adenine "fits" thymine so that these two and only these two go together during this assembly. This is also true of the other pair, guanine and cytosine. This assures that each daughter strand is a perfect copy of the parent strand of DNA. When the process if finished, two complete and perfect copies of the DNA molecule are the result.

The DNA polymerase actually assembles the base pairs with the needed base + sugar + three phosphates in what is called a **nucleotide**. The nucleotide is assembled in the nucleoplasm surrounding the DNA and it provides the energy for the production of the new DNA. The DNA polymerase moves along the parent strand drawing in

nucleotides from the surrounding nucleoplasm and assembles the new daughter strands as they go. Segments of DNA are connected by using a separate enzyme called DNA ligase which also acts to repair DNA when it is damaged.

Once the DNA replication is complete, the cell undergoes cell division in which one set of DNA goes to one end of the cell while the other goes to the other end. The cell divides with each new cell containing its own identical copy of DNA.

It has been discovered in recent years that the DNA polymerase has an extra enzyme that acts as an **exonuclease** (nucleotide cutting out enzyme). In about one per 10,000 to 100,000 the polymerase makes a mistake and adds the wrong base to the DNA strand. This exonuclease recognizes nearly all such mistakes and removes the wrongly added base pair as fast as it is added so that another attempt to add the correct one can be made. The result is that about one in one billion base pairs has a single error during copying.

In bacteria, the chromosome forms a closed loop. It unzips as hydrolases break the hydrogen bonds. The strands are assembled as two separate strands in the cytoplasm of the bacteria by the DNA polymerase (and other polymerases). The two DNA strands are completed as cell fission occurs and each new bacteria cell has its own DNA strand constructed from the parent.

Transcription and Making RNA

The major activity of bacteria cells (and almost all other living cells) is the production of proteins. These proteins are used to make cell walls and membranes, carry large molecules through the cell membrane and form enzymes that catalyze chemical reactions in the cell. They can also be toxins. In larger organisms, proteins are used to make antibodies and all other cells.

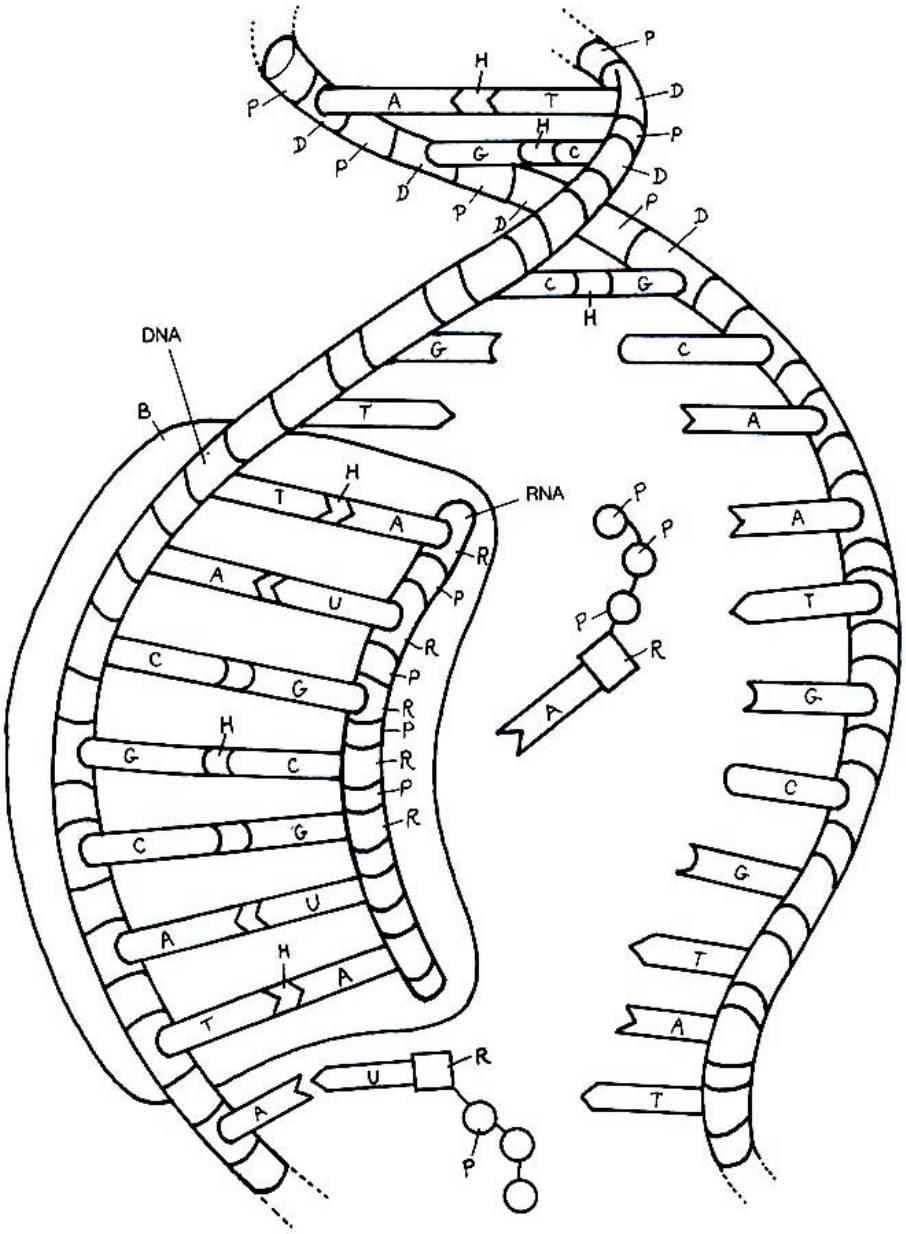
Cells need a process to make proteins and live. This process begins with DNA. In a process called **transcription,** a substance called **RNA** is formed from the DNA code. The RNA molecule then carries this code to the ribosome's where the protein is put together in a process called **translation**.

In the cytoplasm of bacteria, free nucleotides have already been assembled and float around freely. Some of these are DNA nucleotides used to make DNA. Others have been made for RNA and fit to form RNA strands. RNA is made from part of the DNA strand.

A section of the DNA molecule that is needed to make the specific RNA unzips itself just like it does for making a DNA strand. The hydrogen bonds come apart at the center of the base pairs as they unzip. Now instead of forming a new strand of DNA, it forms one strand of RNA using RNA nucleotides. The strands are made in an identical fashion as DNA except that RNA does not use the base thymine. Instead, it uses a

different base called uracil in its place. All RNA nucleotides have uracil instead of thymine to form a base pair with adenine. RNA also uses ribose sugars attached to their phosphate groups.

The RNA is thus assembled from a short segment of DNA and is identical in the instructions it is made up of (except for uracil). When it is finished it looks like ½ of a ladder cut down the middle.

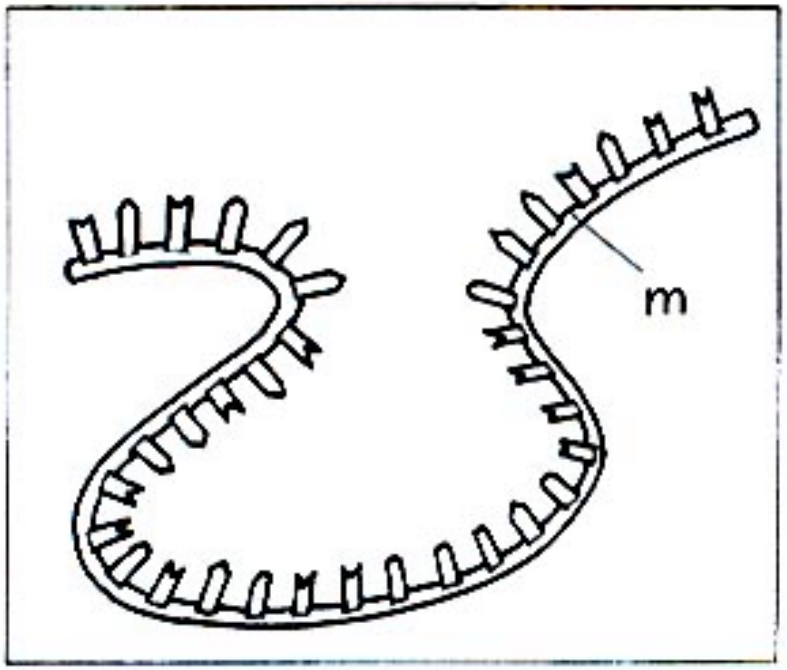


Three kinds of RNA are produced from the DNA of bacteria-

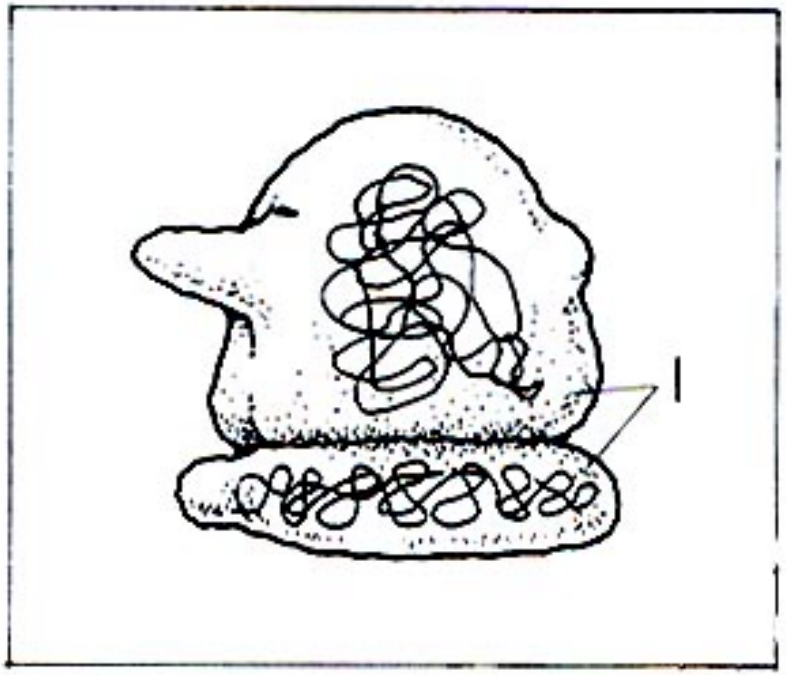
Messenger RNA (mRNA) which carries the genetic code to the ribosomes. Each mRNA provides the base sequence to synthesize and produce one specific protein, one amino acid at a time in the correct order.

Ribosomal RNA (rRNA) is the substance that makes up the ribosomes. The ribosomes are assembled from rRNA and are composed of them. The ribosomes provide a framework or scaffolding or factory for the mRNA to work in.

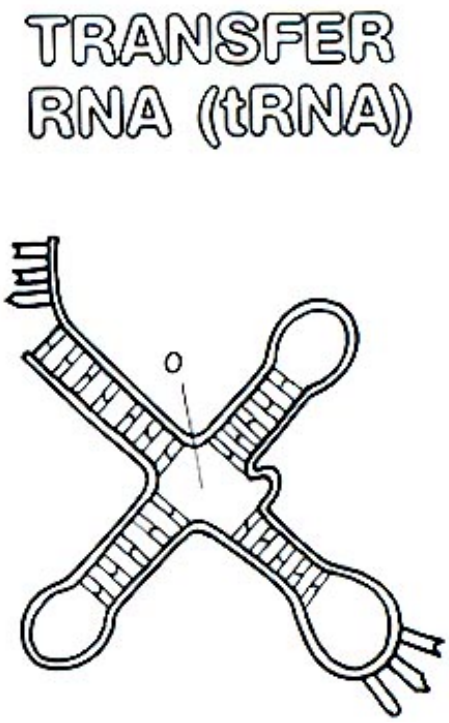
MESSENGER RNA (mRNA) m



RIBOSOMAL RNA (rRNA) l



Transfer RNA (tRNA) captures amino acids floating inside the cell and brings them to the mRNA at the ribosome.



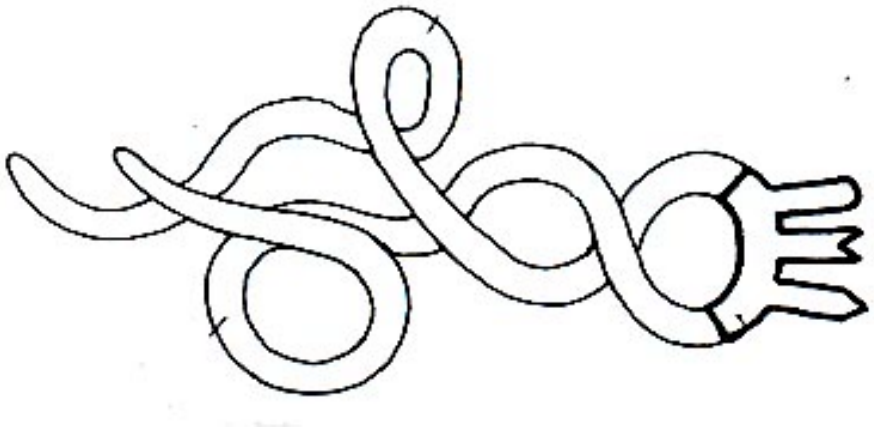
The sugar in RNA is made of ribose rather than deoxyribose. When the DNA unzips to produce RNA, a new enzyme called RNA polymerase attaches and produces the RNA in place of the DNA polymerase. The RNA polymerase only works off of one side of the DNA strand to transcribe the DNA. This polymerase knows where to unzip the DNA and start work on the correct sequence because part of the DNA code is made up of sequences called promoters. These sequences of bases (the alphabet of the genetic code) form the words of a road sign that say "RNA Polymerase-start here". Promoters are the road signs that tell all the different locations on the DNA where the RNA polymerase should unzip and begin work.

The RNA daughter strand does not remain attached to the DNA. It separates from the DNA and the DNA zips itself back together. The RNA then migrates out of the nucleus and into the cytoplasm. DNA can be millions of base pairs in length because of all the instructions it needs to carry. The rRNA strands in bacteria are typically 120, 1500 or 3000 nucleotides long. The mRNA runs from 900-1500 nucleotides in length.

Transfer RNA is special because it must be able to attach to every kind of amino acid in order to obtain and bring to an assembly point, all these protein parts for the cell. All tRNA are about 80 nucleotides long and all of them end in the sequence CCA

(cytosine, cytosine, adenine). This end always serves as the attachment point for the amino acid to be transferred.

The tRNA forms three loops of unpaired bases. The center loop has a recognition code called an "anticodon" that makes sure that this particular tRNA is attracted only to a complementary set of bases on the mRNA called a "codon". One of the loops have a set of bases on its end that allows it to attach to the ribosome's while another has a set of bases that allow it to attach only to a specific amino acid. Each of these codes must be correct in order for RNA to produce the correct protein.



[The anticodon of tRNA with three unpaired bases in the center loop.]

Translation: Making Proteins

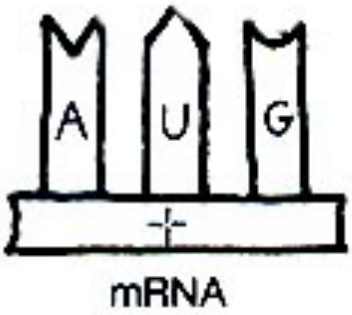
There are 20 amino acids that are necessary for most life. In the bacteria cytoplasm, all 20 kinds of amino acids can be found in abundance. When these amino acids are assembled together they form **polypeptides**. When two amino acids are brought together, they are bonded by a **peptide** bond and a **dipeptide** is formed. When a third amino acid is attached to the first two a **tripeptide** is formed and so on. This is repeated continuously and as many (poly) amino acids are brought together, they form polypeptides.

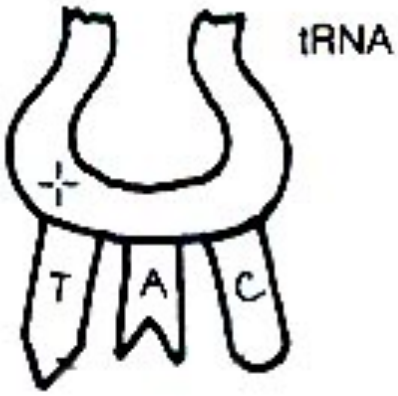
Proteins are made up of amino acids. Some are small peptide chains while others are larger with tens of thousands of amino acids in the molecule and these form enzymes and toxins. Each protein is unique (different from all other proteins) and so each sequence of the amino acids that is used to produce it is unique.

To produce the proteins needed by the cell, the RNA is produced and put to work. The ribosome is made up of rRNA and some protein. There are more than 30,000 ribosomes in most bacteria. Each ribosome has two parts, which attach to mRNA molecules as well as two more parts which allow tRNA to attach their amino acids to it.

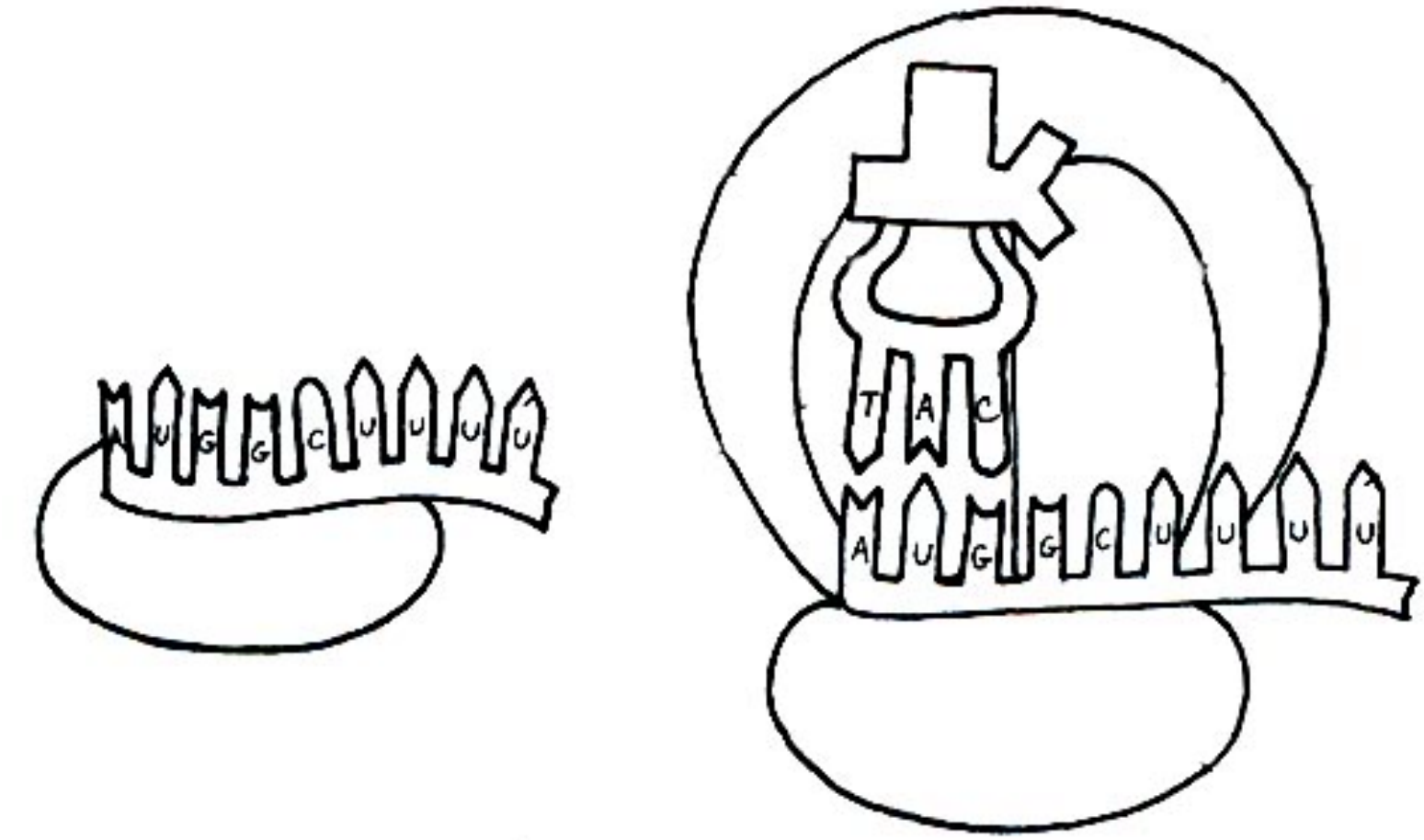
The tRNA will have 75-85 nucleotides in a typical bacteria. These roam the cytoplasm attaching to amino acids and then carrying them to the ribosomes. The ribosomes bind together the bases of the tRNA with the bases of the mRNA. This binding causes the tRNA with the correct amino acid to be placed in line first. This binding is guided by an enzyme called **synthetase**. The anticodon of tRNA is lined up with the codon of mRNA so that they match perfectly. This makes sure the correct amino acid is the one selected for that spot in line. For example, tRNA with the sequence AAA (for 3 adenine bases) lines up with UUU (for 3 uracil bases). This particular lineup always carries the amino acid phenylalanine.

Below is the drawing for a codon of mRNA and the anitcodon for tRNA. The tips of each base is illustrated with a different form so that they can only link up with the complementary forms.



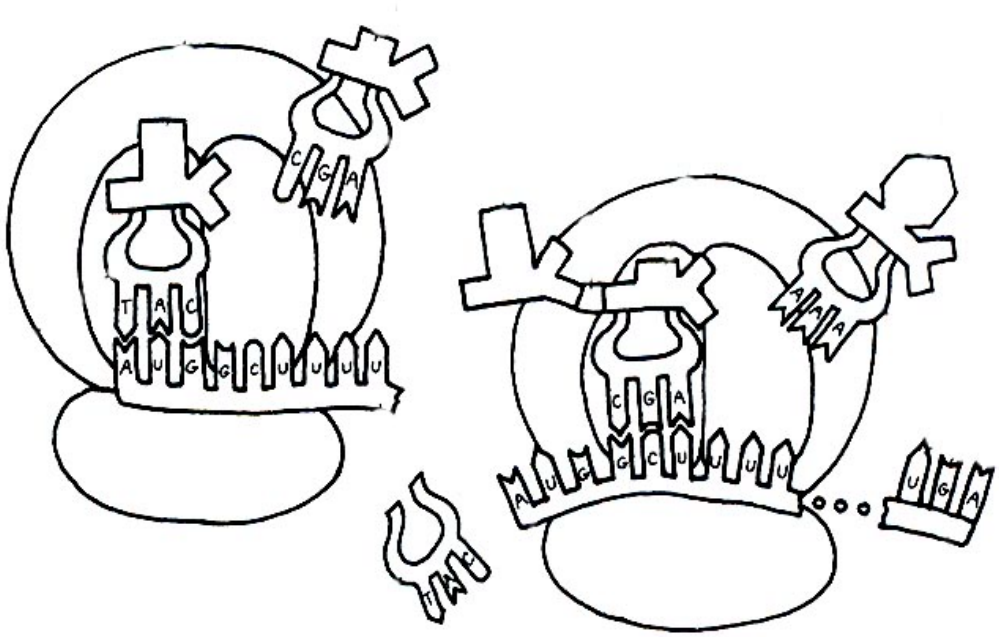


These are brought together as shown below –

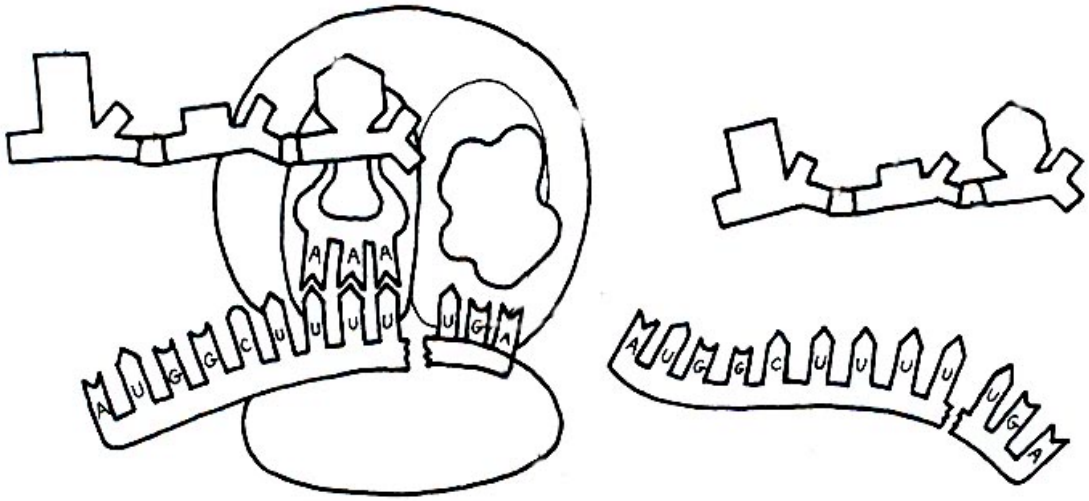


The large circles in the drawing represent the large and small parts of the ribosome which hold everything in place for the RNA to work. They function like a workbench.

More tRNA brings the next sequences needed to form the molecule as shown –



Once the entire polypeptide (protein) is finished, the mRNA has a code of sequences that stops the process. This sequence is called a stop codon. It signals a release factor protein to arrive and react with the molecule and cause the finishing reaction and release of the completed protein –



The ribosome then separates into its two subunits and the mRNA may bind to more ribosomes to make more copies of the polypeptide. After a while, the mRNA is broken down by an enzyme called ribonuclease. This prevents it from making the same copies forever. This allows the DNA to form new RNA for production of other proteins from the available amino acids.

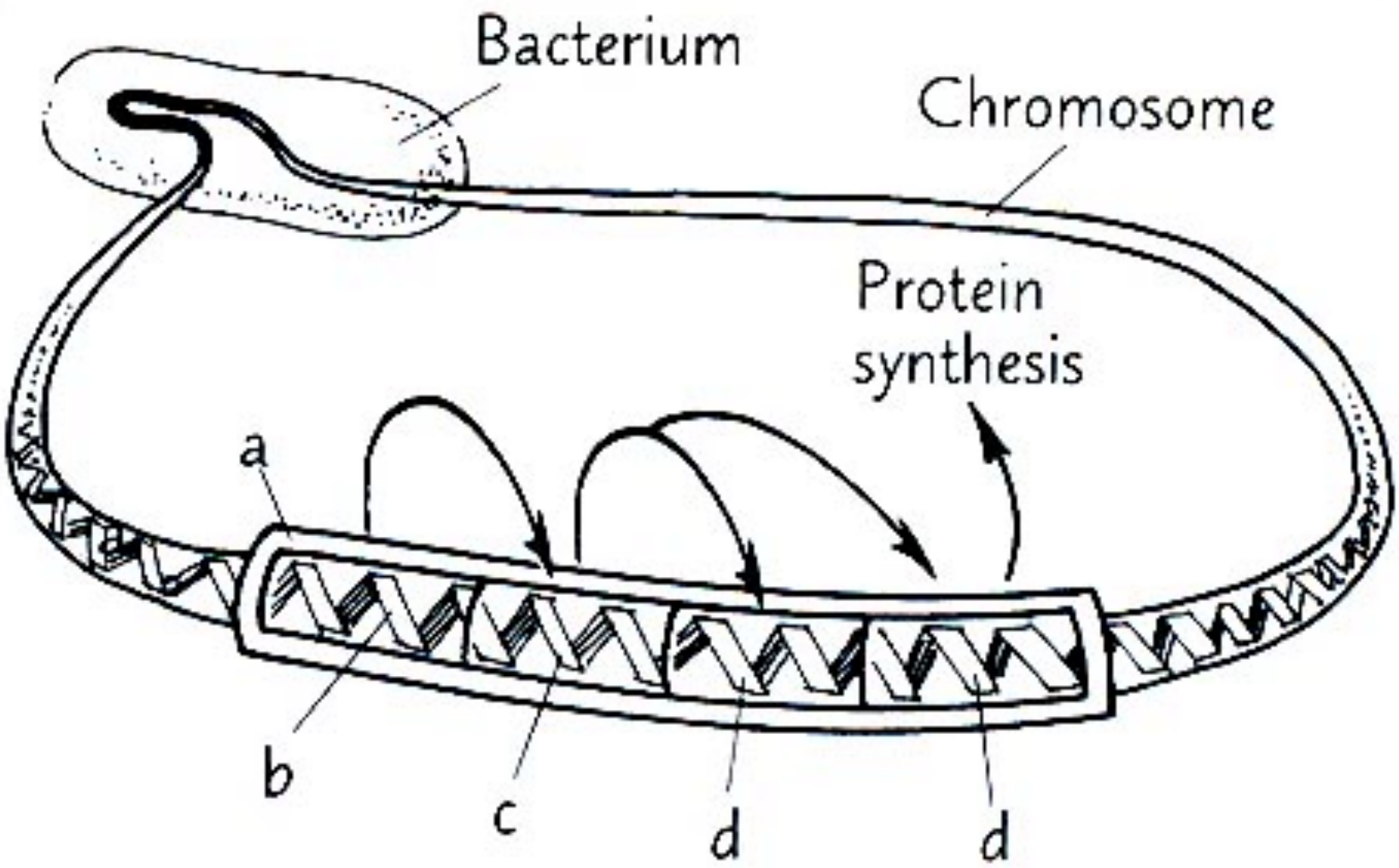
Regulation of Protein Production

The cell "knows" which proteins it must make to use the chemicals in its environment, make more cell mass for cell division and also produce toxin to make food from its surroundings. All the proteins are not produced at the same time. The bacteria have mechanisms which tell it to activate DNA to make RNA and then tell it when to stop (inactivate it).

The DNA contains a group of genes called an **operon**. The operon has three different functioning parts or genes. These are –

1. Operator genes which turn the structural genes (for RNA) on and off
2. Structural genes which are the sequences used for RNA and protein production
3. Regulator genes which produce a protein called a "repressor" protein. This repressor protein binds with operator genes that force the operator gene to turn off. When it is turned off, the structural genes will no longer initiate the synthesis of enzymes.

In the drawing below, a gene group called the **operon** from the chromosome highlighted as "a". At one end of the operon is a regulator gene (b). Next comes the operator gene (c) and finally the structural genes (d).


Bacterium
Chromosome
Protein
synthesis
a
b
c
d
d

The DNA dictionary uses base pairs as its alphabet. The base pairs form the words of the dictionary and these words are the 20 amino acids and the stop code that are produced by the sequence of the code. The three sequence codes for each of these amino acids are called triplets and each possible combination is listed in the chart below –

THE TRIPLET CODE FOR DNA

FIRST POSITION	SECOND POSITION			THIRD POSITION
	T	C	A	G	
T	PHE	SER	TYR	CYS	T
	PHE	SER	TYR	CYS	C
	LEU	SER	STOP	STOP	A
	LEU	SER	STOP	TRP	G
C	LEU	PRO	HIS	ARG	T
	LEU	PRO	HIS	ARG	C
	LEU	PRO	GLN	ARG	A
	LEU	PRO	GLN	ARG	G
A	ILE	THR	ASN	SER	T
	ILE	THR	ASN	SER	C
	ILE	THR	LYS	ARG	A
	MET	THR	LYS	ARG	G
G	VAL	ALA	ASP	GLY	T
	VAL	ALA	ASP	GLY	C
	VAL	ALA	GLU	GLY	A
	VAL	ALA	GLU	GLY	G

The first letter in the combination is in the up and down row on the left. The second is listed at the top and the third is on the right. If the combination is TTT, this is the first combination in the upper left. It codes for Phe or phenylalanine. TTC also produces the same amino acid phenylalanine. Some of the sequences like TGA are **stop** sequences that tell the RNA to end here.

This DNA dictionary has redundancy in the code so that there is less chance of errors and damage. There are 64 possible codons of which 61 code for amino acids and 3 are stop codes. This same genetic code applies to every human cell, every bacteria, every virus, and every known plant and animal. This code enables the production of all life on earth.

You can see from the code that if a single error occurs, or if the DNA is damaged, and a repair enzymes repairs it incorrectly, then there will be incorrect bases produced. This error is then passed on to all daughter cells for every generation afterwards. Because there are more than one set of base pairs to produce the same amino acid, there is a chance that the same amino acid will be produced anyway, even if there is an error. This kind of change is called a **silent mutation**.

If a new codon codes for a different amino acid, then a different amino acid is inserted into the protein at that particular point. If this substitution does not affect the proteins function, then the mutation is harmless. If it falls into a spot like the active site of an enzyme, it could inactivate its function, or cause it to become a different type of enzyme.

An example is sickle cell anemia in which there is a single substitution of the amino acid valine for glutamic acid in the sixth position of two beta chains in the hemoglobin molecule. Only 2 amino acids out of 574 are wrong, but the person with the same error on both of the genes coding for hemoglobin will have this disease.

Point Mutation

The DNA of bacteria can be altered by several methods. The most common in nature (and produced deliberately in laboratories) is **point mutation** which results in a substituted protein.

Some substances can cause mutations to occur. Nitrous acid (HNO_2) is an example. It is a simple compound that occurs in nature and can be found in the soil where many bacteria live. The nitrous acid can enter the cell and float in the cytoplasm. When it comes into contact with the DNA, it reacts with some of the adenine to produce "hypoxanthine" molecules. These hypoxanthine molecules mimic guanine in the DNA and this ends up being substituted in the RNA as well.

The result is mutated DNA and a point mutation has occurred at every point where this change has occurred. This change is permanent and irreversible. This single

change can influence enormous alterations in the structure and function of the proteins and the end function and behavior of the bacteria. The abnormal DNA is not a problem until the RNA picks up the change during transcription and pairs cytosine with the new guanine mimicking base. It would normally have placed thymine in the sequence with adenine.

When chemicals like nitrous acid are absorbed in large amounts by bacteria, enormous numbers of mutations can occur. Most of these changes kill the bacteria. Some do not. A few rare ones may actually improve its life and those that are beneficial will give the bacteria an advantage over others that do not have the same mutation.

Mutant Strains

Replicating DNA is an amazingly accurate process. Millions of nucleotides are perfectly matched and the few occasional errors are corrected. Occasionally, the errors are not repaired and the mistakes are permanently perpetuated in all descendant cells and these are generally called mutations. They are all stable and heritable changes in the nucleotide sequences and any bacterial culture will **always** contain a low number of spontaneous mutants of various kinds.

The mathematical probability of base pair change during replication of the chromosome at a particular position will vary from 10(-7) to 10(-11). The exact rate depends on whether the base is "methylated" and can be easily repaired. This rate is generally specific and constant for each organism. In addition to this rate, the effects of **mutagens** (substances which cause mutations) can be added when they are present. Radiation from X-rays and ultraviolet light will cause mutations as well as many classes of chemicals.

Many mutations are non-lethal and have no obvious effect on the organism. For example, a bacteria cell that has lost the ability to degrade lactose and use it, would not be affected unless lactose was the only energy source in the food around it. Mutations that interfere with essential functions like making RNA polymerase are called **lethal mutations**.

Wild type strains of bacteria are generally those strains that are found in the wild. It can also mean those strains that are used in the laboratory as seed stock for producing mutant strains. Mutants can be differentiated from wild type strains by chemical sequencing of their DNA.

Some mutations change proteins so they are only stable at low temperatures. The E-coli wild types grow at 20-45C while a mutant RNA polymerase strain may only grow at 30C or less. These strains are called **conditional** mutants.

Some strains have mutations that are not observed without special conditions. These would include **antibiotic resistant** strains. They may produce a protein or enzyme that inactivates an antibiotic and its effect is only seen in the presence of the antibiotic

and is seen by successful growth (compared to no growth with same strain colonies without the resistance mutation.

The wild type E-coli can use lactose as a carbon source (in the example above) because it has working *lacY* and *lacZ* genes. Mutation in either of these genes can prevent the strain from growing on lactose. The spontaneous frequency of these mutations occurring naturally in culture medium is 10(-5). To find the mutant strain that occurs in 10(5) [or one in one million-ten followed by five zeroes] wild type cells, you would directly screen the cells by growing 1,000 colonies on a 1,000 plates with lactose indicators to find the 1 colony that cannot use lactose.

To test for mutant strains in weapons production, the mutation for the desired trait such as capsule production or a specific toxic protein is screened for. This screening usually uses a selection medium that weeds out all other cells without the desired trait. If you want an antibiotic resistant mutant, you add the antibiotic to the plate and only those cells that have the resistance mutation will grow.

To find a toxic protein producer by indicators, a chemical indicator can be added to the medium that is affected by the toxin. Blood is added to a medium to detect toxins that "lyse" blood cells. These destroy the cells and form a halo around the colony. Other cell types or toxin sensitive food particles and/or bacteria can be used as well to detect the toxin producing mutants.

If you have a mutation rate of 10(-5) for a particular trait like not being able to use lactose, then you can grow a colony or plate to 10(8), which will yield 10(3) mutant colonies. This is because the mutant will produce daughter cells with the same mutation at the ratio of 1 per 10(5). [Cells in liquid culture often reach levels of 10(8) per milliliter of medium and individual colonies on agar or Jell-O usually greatly exceed this number] If the liquid medium is aerated or shaken to mix oxygen with the solution, cells will reach 10(9) per ml.

With such high mutation rates, it becomes apparent that DNA damage can be significant. This is observed in humans with skin cancer from years of sun tan and sun burn without protection. The rates would be much higher if not for genes that repair damaged DNA.

Studies in E-coli have identified more than 30 separate genes involved in different processes to repair damaged DNA. Altering any of these 30 genes will affect the mutation frequency of any group of base pairs. With very few exceptions, all of the known damages to DNA can be corrected with special enzymes that use the information on the undamaged ½ of the DNA strand as a blueprint for making a repair. These can usually repair all the damage effects of chemical mutagen and ionizing radiation if they are not damaged themselves.

Sunlight is so destructive to DNA that no known bacteria on the surface of the earth live without mechanisms to repair radiation damage. E-coli have four separate

systems that participate in ultraviolet light (UV) damage repair. The large range of different systems to cope with damaged DNA will not be covered here but can be found in most molecular microbiology textbooks in your local college library.

Gram negative bacteria like E-coli and Salmonella have 4.5 x 10(6) base pairs of DNA. The mutation rate per base pair is known to be 2 x 10(-10). This yields a total mutation rate of .9 x 10(-3) and this is consistent among most bacteria species. This rate does not take into account instances of reversion mutation. When a mutation occurs that changes the DNA, it is called a **forward mutation**. Mutations that occur afterwards can revert the mutation back to its original form and these are called **backward mutations** or **reversions**. When colonies are left to grow for long periods of time without changes in food or environment, they tend to revert towards equilibrium as the number of forward mutation events begins to equal the number of backwards events. At this point, the number of mutants in the culture becomes constant.

Mutations usually occur as single base pair changes as mentioned. The base pairs are called **purines** if they are adenine-guanine and **pyrimidines** if they are cytosine-thymine pairs. [These are the names of the classes of chemicals that they belong to when paired together.]

If a purine is replaced by a purine (or pyrimidine by a pyrimidine), it is called a **transition**. If they are replaced by the other chemical group, a pyrimidine replaces a purine for example, then it is called a **transversion**.

If the mutation changes the amino acid that is being produced from one kind to another, it is called a **missense mutation**. Some amino acids can be replaced in a protein by a similar amino acid (threonine and alanine for example) and the protein is often unaffected. Its structure and operation remains almost identical. Some other amino acid substitutions can destroy the protein that is being assembled and this is lethal to the bacteria in many cases.

If a terminating sequence is produced by a mutation (ATC, ATT, ACT) then the RNA is instructed to stop here and the protein cannot be finished. This is called a **nonsense mutation**. It often produces fatal or extreme changes in the organism.

If one or more base pairs is removed from the DNA, it is called a **deletion mutation**. If one or more base pairs are produced by a mutation, it is called an **insertion mutation**. In practice, these types of mutations often involve entire genes and for every three base pairs added or subtracted, a new amino acid is produced or lost.

Bacterial Conjugation

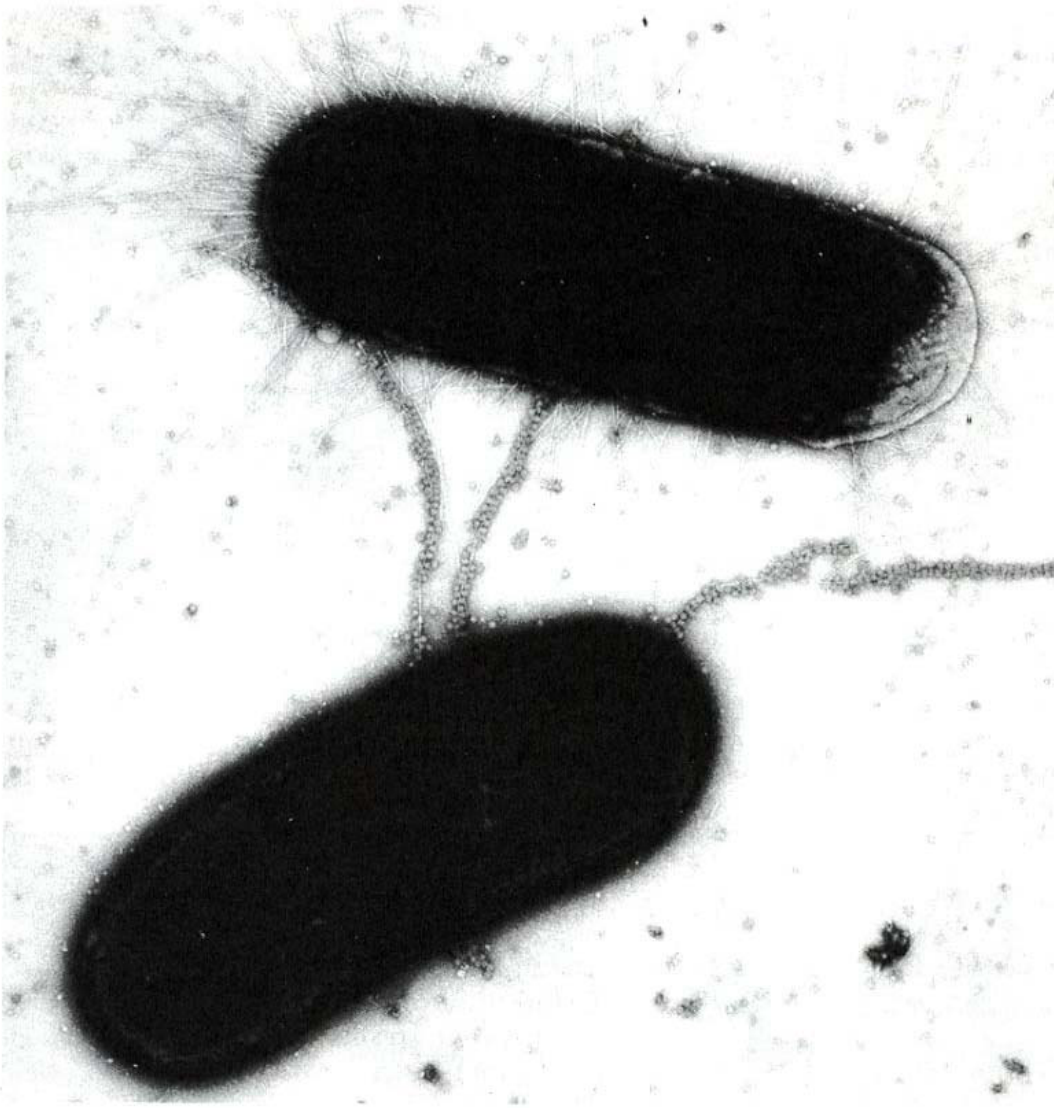
In the early 1950's, scientists discovered that some bacteria had a packet of genetic information separate from their DNA. It was in the form of a loop and usually contained about 20 genes. This loop was called a plasmid or is sometimes called a fertility factor. The scientists observed that some bacteria with the loop could give it to other bacteria and these became known as fertility bacteria. They could donate this strand of DNA to other bacteria without a plasmid

For plasmid transfer to occur, the bacteria must come into close contact with one another, with their cell walls nearly touching. To get many transfers to take place in a culture the cell density must be large with 10,000,000 cells per cubic millimeter of medium. As a plasmid donor approaches a cell without a plasmid that can receive one, the donor extends a tubular appendage known as a sex pilus towards it. They make contact and the pilus acts as a conjugation bridge (like a refueling line between two flying aircraft). The plasmid in the donor cell duplicates itself and then migrates across the pilus bridge into the cytoplasm of the receiving bacteria. The cells then separate and the receiving cell now becomes a possible donor. It can now make copies of itself and produce a sex pilus with which to transfer it.

Plasmid transfer between bacteria is one of the primary ways that antibiotic resistance is conferred from one bacteria to another in nature. Quite often, one or many of the genes in the plasmid will code for an enzyme that destroys a particular antibiotic and this ability to produce the enzymes is passed around between the bacteria by plasmid transfer.

Another form of conjugation is chromosome transfer. A donating bacteria can transfer its chromosomal DNA directly to another bacteria and this ability to do this designates the bacteria as an Hfr cell (for high frequency of recombination). These particular cells use the sex pilus to make contact with other cells. The donating bacteria Hfr cell produces an almost entire copy of its DNA and plasmid and then donates both to the receiving bacteria. The receiving bacteria then use enzymes to remove its own DNA and replace it with the Hfr DNA and plasmid chromosome.

The photo shows two E-coli connected by F pili. The "male" on the bottom has three pili of which two are connected to the "female" on top.



Conjugation of both these types have been observed among various genera of bacteria, especially gram negative species. The amount of DNA transferred by conjugation can be from a few thousand base pairs to the entire chromosome. This appears to be a significant way that antibiotic resistance is spread and how new disease organisms are born.

The chromosome of E-coli will take about a minute to transfer a stretch of DNA from a host to a recipient during conjugation. The segment of DNA that is transferred is usually about $1/100^{th}$ of the total chromosome.

Bacterial Transduction-Using phage to transfer genes

A virus penetrates a bacteria cell wall by attaching to a receptor on the wall and injects its core of DNA inside. It leaves its virus coating on the wall outside. This DNA takes over the bacteria cellular machinery and tells it to make copies of itself as described earlier. The cell then releases hundreds of new copies of the phage so they can go off and infect more cells.

Once inside the bacterial cytoplasm, the virus DNA orders the production of bacterial enzymes that fragment its own bacteria DNA. Certain bacterial DNA segments are used as the starting materials for the replicating molecules of the phage DNA and its protein coats. This is helpful if the virus protein coats are made up of the same proteins that the bacteria use in their cell wall. That way the body does not attack it as a foreign substance.

In some of the phage particles, the entire DNA of the bacteria is substituted and you have a situation where some of the phage virus have phage DNA and some now have only bacteria DNA. When these phage are released and they find other cells to infect, the virus with phage DNA repeats this process. The virus with bacteria DNA injects itself into another bacteria and this cell now has new bacteria DNA being injected into it instead of viral DNA. This injected bacteria DNA is now incorporated into the host bacterial chromosome and so the host will now have new genetic traits produced by the new DNA.

This method of transfer of genes between bacteria is called transduction. Usually the phage only infects one particular species but in some cases, they can infect many species. These viruses can be used to transfer genetic DNA from a toxin producer like anthrax to a non toxin producer like the Staph or Strep that colonizes the mouth and nasal passages. If the DNA for toxin production is incorporated into the recipient bacteria successfully without disrupting other parts, a new weapon and disease is produced. A likely contagious one in this case.

This method is used in laboratories in which a virus is used to infect target bacteria with the desired DNA. Once the cells lyse and their viruses are released, they are transferred to a medium with the target bacteria. These become infected. Only those bacteria that survive this infection will grow on a culture plate and these include cells that may have DNA from the original bacteria in them. If the transfer of genes is accomplished, and they grow on culture medium after infection, they are called **transductants**. The amount of DNA transferred is limited to the size of the virus DNA but can approach 200,000 bases in length.

Transductants may receive any part of the genetic material from the original bacteria so each transfer is random. It takes a large number of transfers to yield a desired new genetic property in the recipient bacteria. In the case of anthrax, the DNA for its toxin is so large that it is unlikely that the complete toxin could be transferred by this

method (although not impossible). Other methods for larger DNA segment transfer are available.

Phase Variation

Some bacteria have a control mechanism that allows them to "choose" between two different types of DNA to use to produce a particular protein or cell part. The best example is certain strains of *Salmonella* which can change their flagellar antigens. These strains can usually produce either of two different flagellar antigens called H1 and H2, but only one at a time. These bacteria shift from one to the other at rates far higher than that of ordinary mutation rates.

When Salmonella infect a host and it is the H1 type, the bodies defenses produce H1 specific immunoglobulin to help fight the infection. An enzyme then directs a significant portion of the Salmonella (from the original infectious cells) to produce H2 flagellar proteins to evade the immune response and continue the infection. They can also switch back to H1 as they may invade other hosts by passing out of the intestinal tract and into water and food supplies or soil. This ability to shift from one type of antigen production to another is called phase variation.

Trypanosomes have developed a far more elaborate system of phase variation in which they produce many different surface antigens. They change these constantly during infections, often "outwitting" the antibody system of the host to exhaustion. (this type of protective changing by switching the DNA instructions for antigens regularly is a very effective strategy).

Cell Fusion

Bacteria cells have been combined by a process called protoplast fusion. The bacteria cells are turned into **protoplasts** by stripping them of their bacterial cell wall. This is usually done by treatment with antibiotics or enzymes. Polyethylene glycol is added to the medium mixture of the two cell types. Diploid cells (bacteria with two chromosomes, one from each donor type) are produced and these yield haploid cells (normal bacteria with one chromosome) of which many show the genetic traits of both parents. Stable diploid bacteria cells have also been reported.

Animal and plant cells have also been fused with bacteria. Using this method, anthrax could be fused with human blood cells (for antigens that resemble blood and are not attacked by the immune system). It can be fused with diphtheria bacteria for cells that can consume and grow in mucus membranes making it communicable, or fused with the fungus that causes athletes foot for a species that can turn skin into food directly.

Genetic Engineering

Experiments in bacterial transformation, plasmid exchange and transduction led to more research on techniques to insert and delete genes from DNA. They wanted to know if bacteria could have non-bacteria DNA inserted into them that would alter bacteria to mass produce specific proteins. In agricultural science this was done to mass produce Lysine and other amino acids for livestock feed. In bio-weapons research, it was studied for mass production of Botulinum toxin and other potent enzyme systems.

In the 1960's certain enzymes were discovered that would cleave bacterial DNA at designated points. These would become known as **restriction enzymes**. At the points of cleavage, foreign DNA could be inserted. The researchers then discovered that **ligase enzymes** would attach these foreign enzymes to the strands of the bacterial chromosome.

From experience it was found that using the entire chromosome to transfer DNA was difficult but that using plasmid DNA was much easier. Using these small ringlets of DNA, they learned to maneuver genes and "engineer" new forms of bacteria that could produce specific proteins on demand. If these proteins happened to be toxins inside of harmless bacteria, then they made good weapons. If they happened to be lysine producers, they made good commercial products.

Genetic engineering methods begin by breaking open a bacteria with a plasmid inside. The "donor" plasmid is isolated from the bacteria cell and then it is cleaved at a desired point by a restriction enzyme. The cleaved part is removed and broken down and the plasmid is ready for its new DNA to be inserted in this same section. The foreign donor DNA can be from human cells, animal cells, plant cells or insects as well as other bacteria. The foreign DNA is cleaved into fragments and the selected fragment is combined with the plasmid DNA at the restriction point using the ligase enzyme.

This combined plasma is called a chimera because it has the DNA from two unrelated species (usually). Chimeras are inserted into bacteria by putting the target bacteria colonies and the chimeras into a calcium chloride ($CaCl_2$) solution and heating the water quickly. On heating, the cell walls open up and the membranes permit the plasmids to pass through and enter the bacterial cytoplasm. On cooling and placing in culture medium, the bacteria soon reproduce with each colony producing cells with chimera plasmids. The bacterial protein DNA that was inserted into the plasmid is soon being used by the cells and the desired protein is soon in production. This protein can then be harvested.

This last method occurs in nature with some frequency. A bacteria that is living (or a bacteria that has died and lysed) releases some genetic material into its surroundings. The DNA can obviously degrade, but until then, it can be taken up in contact with any other living bacteria which may take up the DNA, transport it inside its own cell wall and membrane and allow it to recombine with its own chromosome. This new recombinant cell is called a **transformant**.

In theory, any piece of genetic material can be transferred by this method which is greatly enhanced using the calcium chloride solution and rapid heating described above. In actual practice, DNA of around 10,000 bases in length is usually transferred per event.

This means that you may take a toxic bacteria like anthrax, kill it and add an enzyme to lyse its cell wall (lysozyme) or a mild chemical (caustic soda). Billions of these bacteria with degraded cell walls can be placed into a calcium chloride solution with a desired recipient bacteria and heated. The cell walls of the billions of the recipients open on the heating and permit various strands of DNA from the decomposing anthrax to be taken up. Some may take up the ability to form a capsule. Others may take up the ability to produce the anthrax toxin while others might even be capable of producing spores. The greater the numbers of mixed cells and genes, the better the chance of getting a desired transformant.

By the 1980's, a huge range of medical uses were found for genetic engineering. Insulin, human growth hormone, vaccines, and blood clot dissolving enzymes could all be mass produced inside of bacteria with chimera DNA.

Theory of Evolution

In 1859, Charles Darwin published his origin of species in which he described how species change with time. Mankind had used "artificial selection" for centuries to develop useful varieties of domestic animals and plants.

One of the earliest examples occurred in Germany over several hundred years. The German farmers were hounded by badgers that were constantly stealing chickens so they would use dogs to hunt them. They decided centuries ago, that they needed a dog that could chase the badger into its hole and have strong claws to dig with and large enough jaws and teeth to be a match for the badger once it was cornered. No such dog existed at that time. Dog breeders were given the job of producing dogs from the shortest legged dogs that they had. They would continue to select those with shorter and shorter legs.

Over time, mutation, constant recombining of desired genes, and the "selective" pressure of the breeders produced the new breed. Using only the dogs with the best badger hunting traits as breeding stock for the next generations, they eventually developed a new breed that was half a dog high, two dogs long, with short legs, stout paws and claws and strong jaws. We know the breed today as the dachshund. They would also do the same thing with Great Danes to hunt wild boar and bears.

One of the most extreme examples of "natural selection" is seen in the giraffe. The developed long necks and legs that are longer in front than behind. This allowed them to reach the leaves and twigs on trees that other competitors could not, The giraffe with shorter legs and necks could not reach these undisturbed portions. They would go hungry while taller giraffes could continue to find food. Over time, natural selection

favored the taller animals with more food and these would produce more offspring that could succeed in a limited food environment.

Speciation means the formation of a new species. A biologist named John Moore did mating experiments between leopard frogs from southern Canada to northern Mexico. Those that were close to each other in range, say a couple hundred miles apart, they produced many good eggs. The farther apart that the leopard frogs originated from, the fewer eggs that they could produce and would hatch. The leopard frogs from the ends of each range combined to produce eggs that all died. The frogs from the two ends of the ranges had clearly become distinct and separate species. This was because they could no longer successfully mate with each other. Their DNA sequences no longer permitted them to reproduce all the correct proteins in tandem with each other so the offspring could live.

The genes of the frogs appeared to have adapted over time to differences in temperature. It was found that the eggs of northern leopard frogs could develop and hatch in water too cold for the southern frogs and the reverse was true for the southern frogs. They could produce and hatch in water too warm for the northern frogs. By a series of mutation and selection the similar leopard frogs eventually became two species.

In bacteria, we can use **artificial selection** to find and retain (feed) those bacteria which produce the traits we desire. If we want them to produce lysine, we can grow billions of new cells each day and by random mutations, find the better lysine producers (by using dyes that react with lysine production in the medium) and continually raise these. With each succeeding millions of generations, eventually, superior lysine producers will be found up to some upper limit (which is usually the point where the lysine production is so great it is toxic to the bacteria).

The most often used selective agents to inhibit growth of parental cells in artificial selection include antibiotics, bacteriophages and required growth factors. Finding the one in a billion cell with the trait that allows it to grow when all the others are inhibited is easy using this method because one billion cells to be screened can fit on the end of a toothpick and be streaked on this selective culture plate.

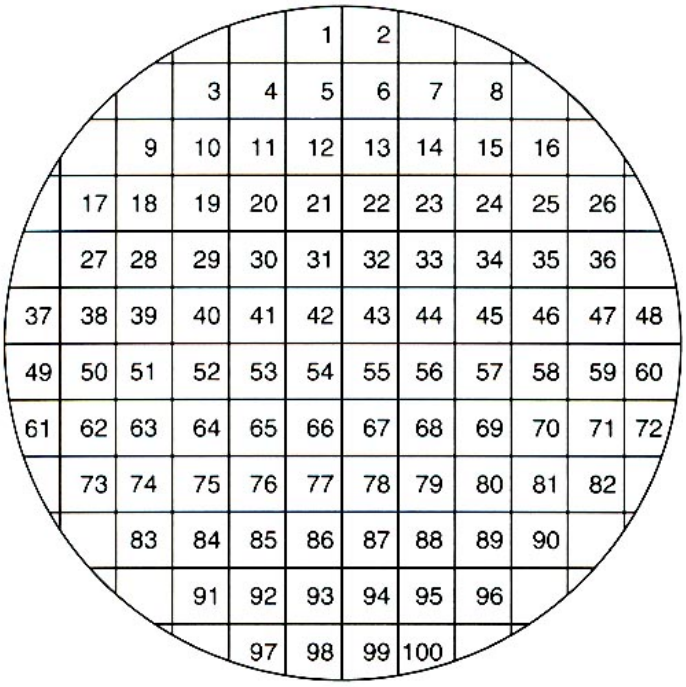
Colony Plating Techniques

Volume 6-A teaches techniques for streaking bacteria onto culture plates and transferring them to subcultures (other plates). In order to find specific strains from a general bacteria population that covers an entire plate and is mixed, a number of other methods are useful.

Replica plating is a method that was developed using a velvet cloth. The cloth has raised spots on it. Wire tips or even spaghetti spoons can be used in this technique. The idea is to be able to touch the agar or Jell-O with the tips or raised spots to form an “imprint”. This imprint from the bacteria culture has cells at each of the raised spots. This imprint is then pressed on a fresh agar or Jell-O plate to produce separate colony growths

at each imprint spot. This aids in separating the mixed bacteria samples. Velvet is preferred because only a few cells transfer through the velvets opposite side for imprinting as illustrated in the drawing below.

Numbered plates are used in professional labs and these are called colony grids.



Grid for 100 colonies

Bacteria can be stored by packing the culture in dry ice in a sealed container with a vacuum pump. The air is pumped out and the bacteria freeze dry. These are usually stored in a small glass ampule under vacuum for storage over many years. They can also be stored in an ordinary freezer for months at a time, with fair to good viability depending on the organism.

Cultures also store well when mixed into 10-16% glycerol solution (or 8% DMSO-dimethylsulfoxide) and frozen at –70C. To recover viable cells, you simply chip off a piece of the frozen stock, add to medium and grow overnight. This method of storage is usually good for five years or more. For storage in a home freezer at –15 to –20C, 40% glycerol is used in the liquid. Storage with this much glycerol can last for several years with good viability but is poor with some species. The glycerol should be mixed with water to dilute it and heated to sterilize it before adding it to a culture.

Cultures can be stored at room temperature by stabbing the colony cells into enriched agar or Jell-O (usually in test tubes) and refrigerating at 4C. This can last for weeks to years depending on the species.

Storing cultures in liquid usually maintains viability for a week or so and is not recommended for long term recovery.

Summary

We can speed nature along by using chemicals and radiation to induce increased mutation rates far beyond what nature would produce. From these we can have a far greater variety of selection and can find the most extreme and useful new mutations.

We can also use plasmids to obtain DNA from one bacteria and place it in another. We can use virus (phage) to do this as well. We can use genetic engineering to take any DNA from any living thing and place it inside of bacteria using genetic engineering.

This last sentence demonstrates that there is probably no limit to the possible new weapons that can be conceived and produced. Genetic engineering techniques are being taught in high schools today. These same methods can be used to arm millions of people overnight, almost anywhere on earth. This is the future of warfare and will almost certainly change the face of human existence in the next generation

Chapter 4
Producing Chemical & Radiation Mutations

It is possible to produce a wide range of mutations using chemicals and radiation. The ability of bacteria to reproduce themselves by the billions every day allows for large numbers to be treated and screened. These types of mutations usually only change the nature of the proteins produced. In other words, some new amino acids yield new proteins that make a bacteria resistant to an antibiotic. It is also useful for producing bacteria with new protein antigens in its cell wall, capsule and in the toxin it produces. Changes in these antigens will often make vaccines and body defenses less effective or ineffective. This occurs because the immune system no longer "recognizes" the new protein structure and has to produce new antibodies to fight it.

Mutations cannot be used to produce large changes in bacterial structures. A bacteria that cannot grow in oxygen lacks the long genetic sequence to produce enzymes that detoxify the superoxides formed while processing oxygen. This long genetic sequence cannot be produced by mutations because a long genetic stretch of correct instructions has to be added to make these enzymes. Mutations do not produce new stretches of DNA but they can produce new base pairs (usually one at a time). If the organism already has this long sequence of base pairs (the entire gene) then mutation can alter it or make it inactive.

The best example is Clostridium species. These lack the cellular machinery to grow in oxygen. Billions of years of mutation and evolution has not produced the machinery to utilize oxygen but it has produced some of the deadliest toxins the world has ever seen. Botulinum toxin of various types have been produced as well as tetanus and the various gas gangrene enzymes. These toxins cannot be produced in non-clostridium species by mutation because these other species do not have the genetic sequences to produce any part of these enzymes and proteins.

The toxins themselves can be altered by mutation so they are no longer antigenic. The proteins on the surface that act as antigens can be changed so that antitoxin is longer effective when produced by this new strain.

Mutagens are anything that increases the mutation rate of organisms. The most commonly used mutagens in laboratory use today are –

Radiation which provides energy to effect the DNA. X-rays are very high energy and they often break the phosphodiester backbone of the DNA strands. Ultraviolet rays are much lower energy providers that catalyze a reaction in adjacent pyrimidine bases to produce **dimers**. These dimers prevent the polymerases from functioning until they are removed so various repair systems go to work to repair the damaged segment. The UV mutations occur during these repair interactions.

Chemical Modifiers which act in a variety of ways. These include

Nitrous Oxide (nitrous acid) which induces transitions from G-C to A-T. It oxidatively deaminates adenine, guanine and cytosine.The result is that free amino groups are lost and replaced by hydroxyl groups which converts adenine to hypoxanthine, guanine to xanthine and cytosine to uracil. Nitrous acid also reacts with these bases in nucleosides, nucleotides and nucleic acids.

Hydrazine and Hydroxylamine (and its derivatives) which act mainly on cytosine but can attack uracil or adenine. It usually replaces a cytosine with a thymine residue. At a low pH (app. 6.0) and in high concentration, hydroxylamine reacts exclusively with cytosine. At lower concentrations, it reacts with all four bases. At low concentrations it is highly toxic and weakly mutagenic. The main effect of these mutations is C to T transition.

Alkylating agents attach ethyl or methyl groups on a purine ring which results in a mispairing of the base. The most well known of these include EMS (ethyl methane sulfonate), MMS (methyl methane sulfonate), and MNNG (*N*-methyl-*N*-nitro-*N*-nitrosoguanidine). MNNG has a great effect on the replication fork and is an extremely potent mutagen. It often mutates as many as 15% of the total cells for a specific trait such as maltose utilization. It produces so many multiple mutations that it becomes hard to yield a single desired result in an individual cell.

Base Analogs (chemical equivalents) are chemicals that have a ring structure similar to that of one of the nucleic acids (base pairs). They do not have the same chemical properties and this induces errors during replication. Mutations occur during repair. They also increase the effects of radiation mutations. Some base analogs like 5-bromouracil (5-BU) and 2-aminopurine (2-AP) have the same structure as thymidine and adenine respectively and can be substituted directly into the DNA. These substitutions induce errors during replication. Other aminopurines such as 2,6 diaminopurine are also mutagenic.

5-Bromodeoxyuridine (BudR) can replace virtually all the thymidine in DNA during synthesis when free thymidine is lacking. Organisms containing BudR substituted DNA are much more sensitive to the effects of other mutagenic chemicals and radiation including visible light. The effects of these mutagens in this case is nearly always lethal.

Some base analogs have ring structures similar to the base pairs but do not have deoxyribose phosphate which forms the backbone. This allows these **intercalating agents** to slip in between the base pairs without being linked into the DNA. It interferes with the base stocking during replication and can cause a gap in the new DNA strand. These points of intercalation cause extra base pairs to be produced and these gaps may result in mutations during DNA repair. The most widely used intercalating agents include acridine orange (and related dyes), proflavin and the poison gas group of nitrogen

mustards. [This last group, the mustards, easily pass into and through human skin causing genetic changes that kill most of the cells. That is why it is effective as a weapon. Sulfur mustards (WW1 war gas) also cause mutations, though at a lesser rate than the nitrogen mustards.]

Most of the acridine family of dyes provide mutagenic activity only during recombination and do not act on the DNA at any other time.

Cross-Linking Agents are chemicals that produce interstrand cross links in the DNA that must be repaired before the DNA can replicate. These induce mutations during repair. Cross-linkers include the antibiotics mitomycin C, porfiromycin and the compound trimethyl psoralen, which must be activated by exposure to 360 nm light. This last substance allows an experimenter to cause cross-linking at specific times during bacteria growth and reproduction by simply turning on the light. This allows only certain parts of the DNA strand to be affected during replication if you time it correctly.

The nitrogen mustard HN2 causes crosslinking and a single interstrand cross-link caused by HN2 has been sufficient to inactivate an entire 3,000 base pair DNA molecule (by blocking strand separation). This accounts for its great effect as a war gas.

[HN2 and HN3 mustards are prepared by reacting diethanolamine or tris(hydroxyethyl)amine with thionyl chloride respectively.]

Transposons are units of DNA that move from one DNA molecule to another and inert themselves nearly at random. They can act as catalysts to cause deletions and inversions. These are found in nature and are introduced into bacteria by some bacteriophage.

Mutator Mutations are segments of DNA that have been mutated and then cause other mutations to occur. The new **mutator genes** produce transitions, transversions deletions and frameshifts (base pairs in multiples other than three produce gibberish so the mRNA shifts the frame it is reading to produce more gibberish and this must be repaired). The genes called *mut*S, *mut*H and *mut*U have been recovered and produced for use as laboratory agents. These genes produce mainly transition mutations. A gene called *mut*D effects only DNA polymerase III. These induce transversions of which 95% are the G-C to A-T type or A-T to T-A. Some mutator genes are **antimutators** which reduce the mutation rates significantly.

The following charts lists the mutator genes found in E-coli

Locus	Map position (min)	Specificity	Strength	Defect (if known)
mutT	2	A:T → C:G transversions	Moderate	Prevents incorporation of A:8-oxodG mispairs by hydrolyzing 8-oxodGTP
mutH	61	G:C → A:T and A:T → G:C transitions; frameshifts	Strong	Lacks methyl-directed mismatch repair system
mutL	95	G:C → A:T and A:T → G:C transitions; frameshifts	Strong	Lacks methyl-directed mismatch repair system
mutS	59	G:C → A:T and A:T → G:C transitions; frameshifts	Strong	Lacks methyl-directed mismatch repair system
uvrD (mutU)	86	G:C → A:T and A:T → G:C transitions; frameshifts	Strong	Lacks helicase II and the methyl-directed mismatch repair system
mutD	5	All base substitutions; frameshifts	Very strong	Altered ϵ subunit of DNA polymerase III
mutY	64	G:C → T:A transversions	Moderate	Lacks glycosylase that corrects G:A and 8-oxodG: mispairs
mutM	82	G:C → T:A transversions	Moderate	Fapy glycosylase (8-oxodG glycosylase)
mutA	95	A:T → T:A, G:C → T:A, and A:T → C:G transversions	Moderate	
mutC	42	A:T → T:A, G:C → T:A, and A:T → C:G transversions	Weak/ moderate	
dam	74	G:C → A:T and A:T → G:C transitions; frameshifts	Moderate	Lacks DNA adenine methylase
ung	56	G:C → A:T transitions	Weak/ moderate	Lacks uracil-DNA glycosylase
sodA	88		Weak	Lacks superoxide dismutase, manganese
oxyR	89		Weak	Lacks positive regulator of oxidative damage genes
polA	87	Frameshifts; deletions	Weak/ moderate	Lacks DNA polymerase I

Another class of mutations is the terminator class in which a mutation causes a terminator to be formed at a codon. This causes the protein to be terminated at this new spot and results in a shortened polypeptide. If caused specifically with a toxic protein with multiple properties, like the anthrax protein, part of the protein can be removed. This may include the part that is toxic, or it may remove the part that produces the invasive effect.

This allows for the production of special weapons with new properties that limit the harm they cause. An example is a weapon that infects and makes people ill and unable to fight temporarily but does not kill. It can also include complete inactivation of the toxin while leaving all the other proteins and antigens intact. This allows you to make inactive toxin that is identical anti-genically to active toxin. This makes it a possible direct toxoid that can be used in vaccines.

Site directed mutagenesis is possible using DNA cloning methods. A single strand of DNA containing the region of interest is separated and an artificial primer DNA sequence is produced by changing the base sequence at one or more points. This method extends to genetic engineering and will be covered later.

The mutation rates for all the methods listed are generally known. They are usually calculated from direct plate counts of the affected vs unaffected colonies.

The following chart lists the main mutagens commonly used in laboratories

Mutagen	Specificity	Mechanism	Additional advantages	Disadvantages
MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine)	Principally G:C → A:T transitions	Generates O^6-methylguanine	Very powerful mutagen	Dangerous to handle; frequent secondary mutations
EMS (ethylmethane sulfonate)	Principally G:C → A:T transitions	Generates O^6-ethylguanine	Powerful mutagen	Dangerous to handle; some secondary mutations
UV (ultraviolet) irradiation	All base substitutions, although favors G:C → A:T transitions; frequent hot spots; also induces frameshifts, deletions, and rearrangements	Generates photoproducts that require SOS bypass		High amount of killing required (relative to EMS) for mutagenesis; not a powerful mutagen; certain strains too sensitive
BPDE (benzo[a]pyrene diolepoxide)	Principally G:C → T:A transversions; frameshifts	Generates adducts that require SOS bypass; may stimulate depurination		Extremely dangerous to hand and difficult to obtain
2AP (2-aminopurine)	A:T → G:C and G:C → A:T transitions	Acts as a base analog	Safe and easy to use; works well on *recA* strains	Relatively weak mutagen
ICR 191	Frameshifts, mainly additions and deletions at monotonous runs of G (or C)	Probably stabilizes looped out bases by stacking between them	Causes only frameshifts, which are usually nonleaky	Some strains too sensitive
5AZ (5-azacytidine)	G:C → C:G transversions			Weak mutagen
NH_2OH (hydroxylamine)	G:C → A:T transitions when used in vitro	Reacts with cytosine to generate N^4-hydroxycytosine	Useful for treatment of phage or plasmid DNA in vitro; can be powerful mutagen under these conditions	Causes only one type of base change; more laborious to use than many mutagens

Nitrous acid	Principally transitions, deletions			High amount of killing required for good mutagenesis
Sodium bisulfite	G:C → A:T transitions		Can be used in vitro	Weak mutagen
NQO (4-nitroquinoline-1-oxide)	G:C → A:T transitions, and to a lesser extent G:C → T:A transversions; some frameshifts	Makes adducts that require SOS bypass		Extremely dangerous to handle
Mutator genes				
Nonspecific				
mutD	All base substitutions, frameshifts	Lacks editing function for DNA replication	No treatment required; convenient for phage and plasmids	Genetic construction required for chromosomal mutations; must move mutator out after use or move phage or plasmid
Specific				
mutT	A:T → C:G transversions	Lack different repair systems (see Table 4.3)	No treatment required	Not as strong as *mutD*; requires strain construction
mutY, mutM	G:C → T:A transversions			
mutH, mutL, mutS, uvrD (mutU)	A:T → G:C and G:C → A:T transitions; frameshifts			
mutY mutM (double)	G:C → T:A transversions	Inability to repair 8-oxodG lesions and mispairs	Very powerful (as strong as *mutD*)	Requires strain construction
Transposable elements	Insertions; can be used for deletions and other rearrangements		Generate nonleaky mutations; mutations are often associated with antibiotic resistance markers to facilitate mapping and cloning	Will not result in missense changes; some inserts are lethal; requires some genetic expertise
Spontaneous (no mutagen)	All base substitutions, frameshifts, deletions, insertions		Wide spectrum of mutations; ease of application; no secondary mutations	Low levels of mutants; many siblings in each culture

Compounds that are also mutagenic and have been reported and studied in the literature include the antibiotics *Actinomycin B* which turns off RNA synthesis without affecting DNA activity (at low concentrations), porfiromycin, and streptonigrin. Aflatoxin B1 (described in detail in Volume 6-C Mold based weapons) combines with DNA to form complexes (base pair intercalations). It is also a powerful inhibitor of RNA synthesis. It drastically reduces the amount of nuclear RNA in liver tissue which makes it particularly carcinogenic to that organ.

Caffeine is mutagenic in bacteria during cell division and antimutagenic in non-dividing bacteria cells. Other common substances that cause mutations (and cancer in humans and lab animals) include cyclamates, ethylene and propylene oxides.

Repair Mechanisms

It is obvious by now that most mutations are actually the result of the repair mechanism failing to correct for damage. These mechanisms can operate independent of or subsequent to DNA replication.

In higher organisms, cells are more resistant to alkylation and other mutation processes than bacteria cells. This is partly because of DNA which acts as a "master" template for damaged "slave" genes. The slave genes that are damaged have a master template that can be brought into use for repair by various mechanisms.

The major error and repair systems found in E-coli are listed in the following chart –

Repair and Error Avoidance Systems in *E. coli*

General mode of operation	Example	Genetic loci	Types of lesion repaired	Mechanism
Detoxification	Superoxide dismutase	*sodA,B*	Prevents formation of oxidative damage	Converts peroxides to hydrogen peroxide, which is neutralized by catalase
Direct removal of lesions	Alkyl transferases	*ada, ogt*	O^6-alkyl guanine O^4-alkyl thymine	Transfers alkyl group to cysteine residue on transferase
	Photoreactivating enzyme (PRE)	*phr*	UV photodimers (cyclobutane)	Splits dimers in the presence of visible light
General excision	Excision repair	*uvrA,B,C*	Lesions causing distortions in the double helix, such as UV photoproducts and bulky chemical adducts	Makes endonucleolytic cut on either side of lesion, and the resulting gap is repaired by DNA polymerase I and DNA ligase
Specific excision	AP endonucleases	*xthA, nfo, nth*	AP sites	Makes endonucleolytic cut; exonuclease creates gap, which is repaired by DNA polymerase I and DNA ligase
	DNA glycosylase	*ung, tag, alkA, mutY, mutM*	Certain altered bases, such as deaminated bases, certain methylated bases, ring-opened purines, and oxidatively damaged bases; also G:A mispairs	Removes bases, creating AP site which is repaired by AP endonucleases
During replication	Editing by ϵ subunit of DNA polymerase III	*mutD*	Mispaired bases	Excises mismatched base

	MutT protein	*mutT*		Prevents incorporation of certain A:8-oxodG mispairs by hydrolyzing the triphosphate of 8-oxodG
Postreplication	Mismatch repair system	*mutH, mutL, mutS, uvrD (mutU), dam*	Mispaired bases that result from replication errors	Recognizes which strand is correct by detecting methyl-A residues that are part of the 5′-GATC-3′ sequence and then excises bases from the newly synthesized strand when a mismatch is detected
	Very short patch (VSP) repair	*vsr*	T:G mismatches in sequence contexts such as CTAGG/GGTCC and CTTGG/GGACC; corrected to C/G	Heteroduplex excision repair of tracts of not more than ten nucleotides
	G:A mismatch repair	*mutY*	G:A mispairs, when G is the correct base; 8-oxodG:A mispairs	Glycosylase: Removes mispaired A, creating AP site which is repaired by AP endonucleases
	Recombinational repair	*recA, recF, recB,C, uvrB, ssb*	Lesions that block replication and result in single-stranded gaps	Recombinational exchange
	SOS system	*recA, umuC,D*	Lesions that block replication	Allows replication bypass of blocking lesion, resulting in frequent mutations across from lesion

Nonsense suppressors

A category of substances have been found in nature and have been produced synthetically that are used to force amino acid substitutions of a specific kind which creates specific new polypeptides and proteins. [If the amino acids have a comparable effect as the one they are substituting, they can leave the protein-toxin active. The change in the amino acid in the antigen of an active toxin means that the recipient or host of the

toxin producer will have weakened or no immunity even if they have been vaccinated for it.]

Nonsense suppressors used in laboratories included –

Suppressor	Codons recognized	Amino acid inserted	Efficiency (%)
A. Natural			
Su1 (*supD*)	UAG	Serine	6–54
Su2 (*supE*)	UAG	Glutamine	0.8–20
Su2-89 (*supE*)	UAG	Glutamine	32–60
Su3 (*supF*)	UAG	Tyrosine	11–100
Su5 (*supG*)	UAA, UAG	Lysine	0.2–2 6–30*
Su6 (*supP*)	UAG	Leucine	30–100
Su9	UGA	Tryptophan	0.1–30
B. Synthetic			
$tRNA^{Phe}_{CUA}$	UAG	Phenylalanine	48–100
$tRNA^{GluA}_{CUA}$	UAG	85% Glutamic acid 15% Glutamine	8–100
$tRNA^{Cys}_{CUA}$	UAG	Cysteine	17–51
$tRNA^{HisA}_{CUA}$	UAG	Histidine	16–100
$tRNA^{ProH}_{CUA}$	UAG	Proline	9–60
$tRNA^{Lys}_{CUA}$	UAG	Lysine	9–29
$tRNA^{Ala}_{CUA}$	UAG	Alanine	8–83
$tRNA^{Gly1}_{CUA}$	UAG	Glycine	39–67
FTORI 26	UAG	Arginine	4–28 4–47*

Producing Ultraviolet (UV) Light Mutations

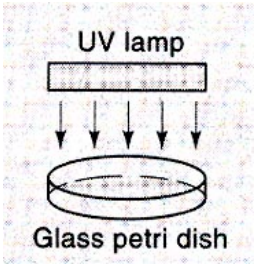
UV irradiation induces numerous frameshifts and base substitutions which stimulates recombination and other genetic rearrangements. Some pre-mutagenic lesions caused by UV are photoreversible in visible light so the use of UV in producing mutations is accomplished in the absence of strong visible light.

[The following is not an official laboratory procedure. It is adapted for home use with common items.]

1. The culture to be modified is grown overnight on Jell-O, agar or in a liquid soup.
2. The solid mass of organisms is obtained by scraping from the solid medium and transferring to a container or by filtering off a liquid soup and using the solid residue mass.
3. The container will hold a solution of water with magnesium sulfate or epsom salts. The amount of the salt should be 1/10 that of the volume of the cells added to the liquid solution. An example would be –

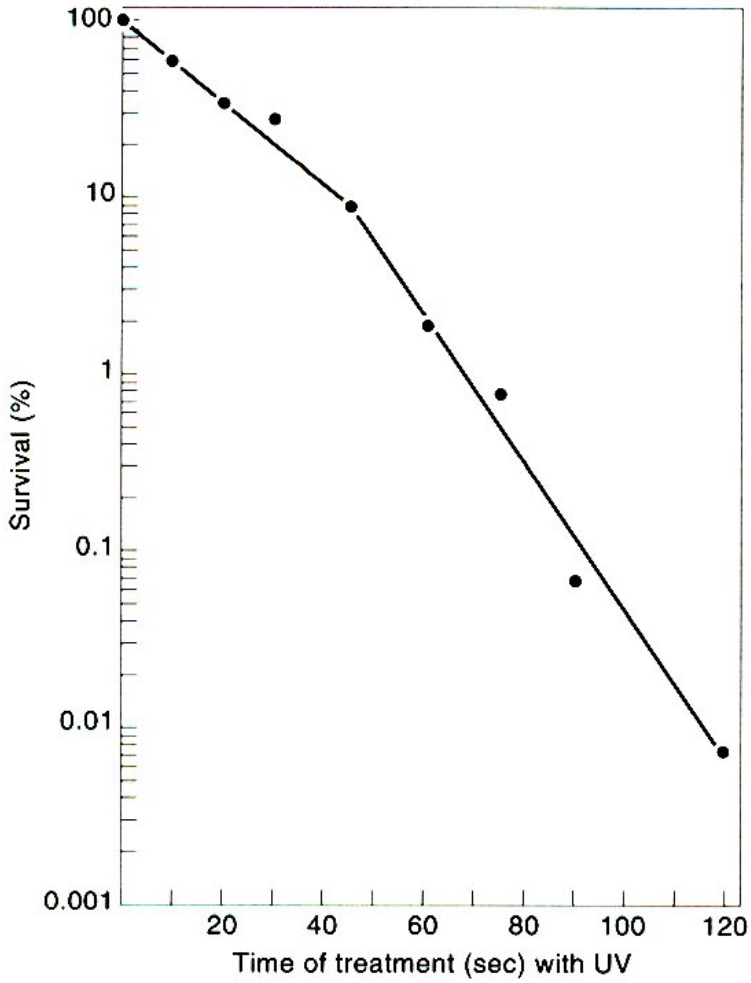
   One quart of water
   One tablespoon of bacteria culture mass
   $1/10^{th}$ tablespoon of magnesium sulfate

4. The bacteria are mixed into this liquid solution and suspended by mixing. Turn on the UV lamp one hour before the experiment.
5. Place the container on ice to cool the mix for 5-10 minutes.
6. Take a small sample of the mix (about 5 ml.) and place it onto an enpty petri dish or other container such as a **glass** bowl.
7. Rock the dish back and forth to spread the liquid and then place it on a flat surface under the UV lamp or bulb. For most bacteria, the bulb is placed 37 cm. from the cells.



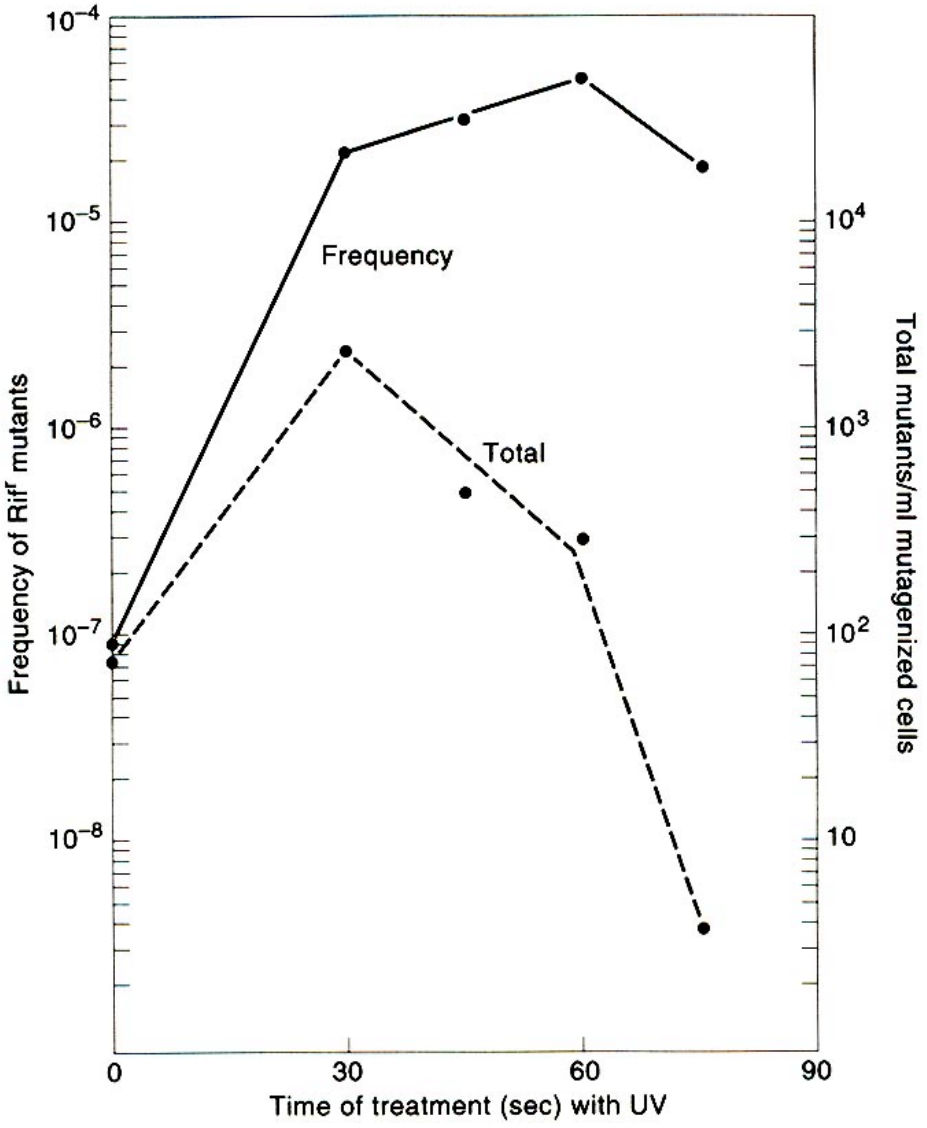
8. The cells are exposed to the UV for 30, 45, 60, 75, and 90 seconds for each batch to be tested. By this method, you can determine the lethality of the dose as well as the mutation rates. The dish is gently rocked occasionally during dosing.
9. The room lights should be dimmed to prevent photo-reversing effects and if the strains are pathogenic, precautions must be taken to contain all of these procedures and protect the operators.
10. Each of the samples for the different time exposures are then grown on culture growth medium. This can be a standard food for the organism or it can be made selective with antibiotic (for antibiotic resistance) or other substances.

On the second day, the cultures are examined. A chart of the typical survival rates for E-coli are listed below. Survival usually drops from 100% with no UV exposure to less than 1% after 75 seconds.



Almost all bacteria have significant mutations after only 10 seconds exposure.

The following charts show the mutation rates for E-coli. The mutation for the specific resistance to the antibiotic rifampicin is also described. The plates the mutants are grown on contains rifampicin and only the mutatns with the mutation for antibiotic resistance will grow. The number of mutant cells for rifampicin peaks at 30 seconds after which a significant number of cells die off.



The above method describes a simple way of causing mutations to produce antibiotic resistance in organisms. Multiple antibiotic resistance is accomplished in a similar manner with repeated subcultures.

Producing EMS Mutations

Ethylmethane sulfonate (EMS) is a common laboratory chemical (sold by Sigma and Aldrich chemical companies). It is a volatile organic solvent (like gasoline) that is carcinogenic and mutagenic. Wear protective clothing and gloves when working with this substance. All materials that come in contact with EMS should be immersed in sodium hydroxide or bleach solutions.

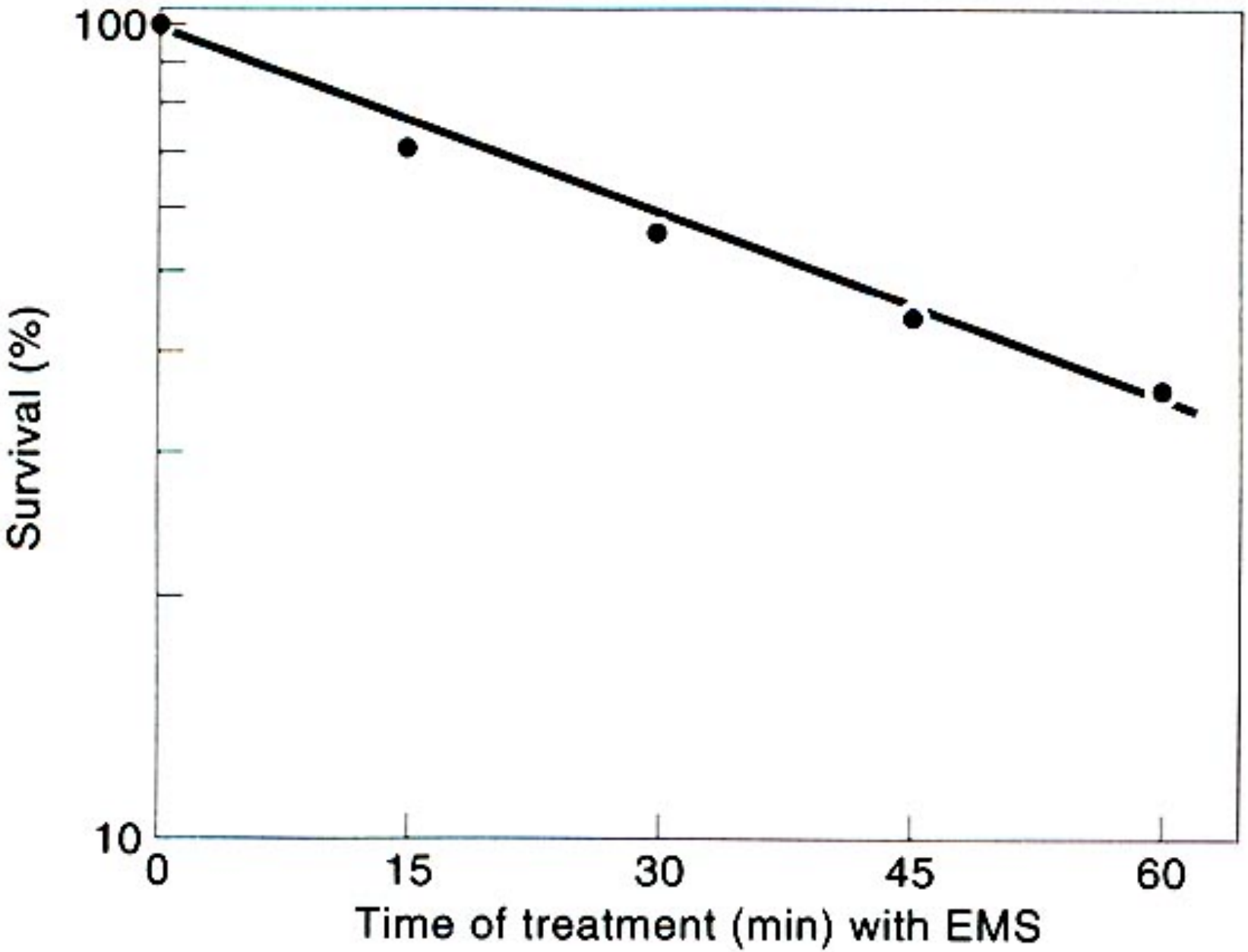
EMS is an alkylating agent that adds an ethyl group to many positions on bases of DNA. It causes G-C to A-T transitions almost exclusively in E-coli. The method is to expose washed cells to EMS under conditions where there is little lethal effect (20% + survival). The mutagen is then washed out and the surviving cells are grown in rich medium overnight to allow for expression of mutant types and segregation of the desired traits.

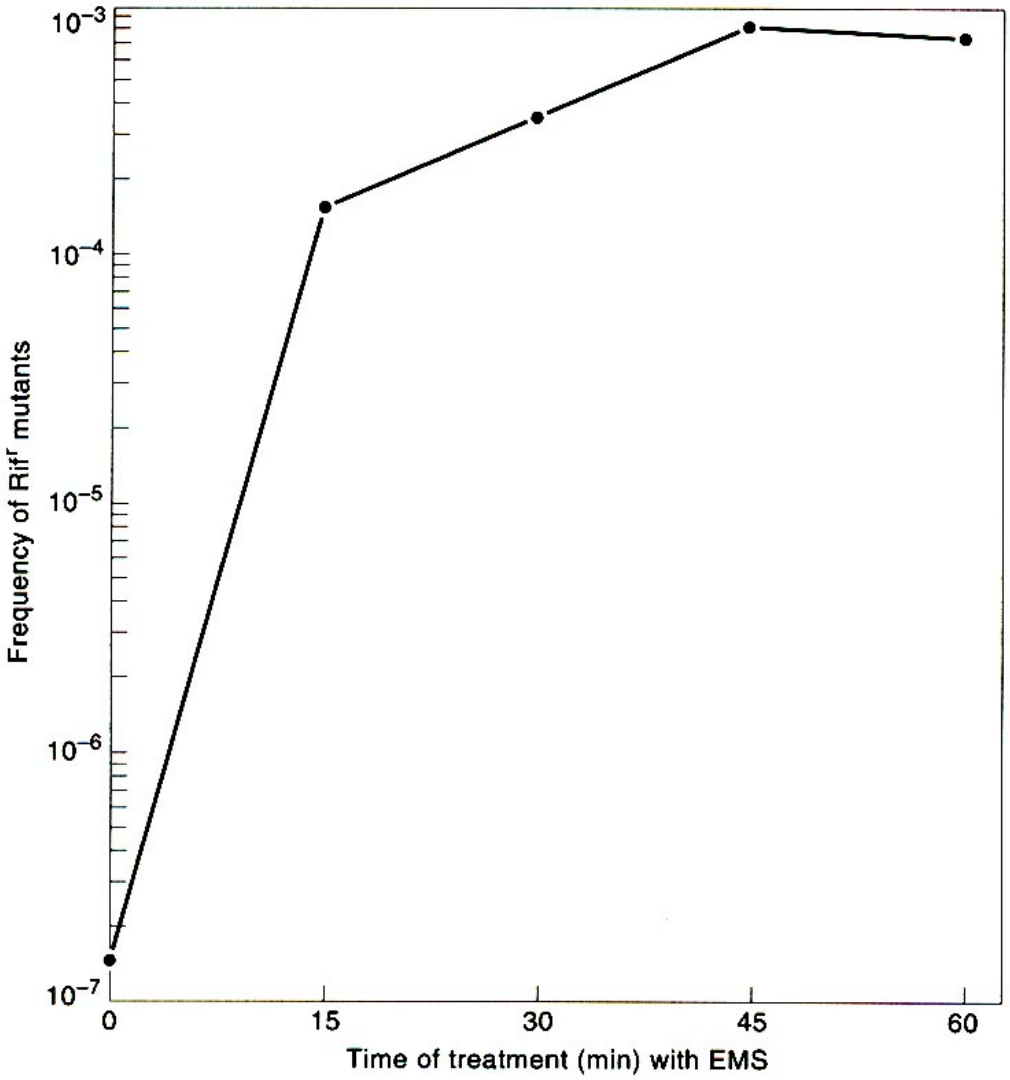
It is useful to use test tubes with screw on caps. If these are not available, any glassware that can be sealed at the opening will suffice. You will need to prepare a buffer of 1 quart of distilled water mixed with 40 grams of baking soda and 2 grams of sodium citrate (from vitamin C tablets). You will also need a rich growth medium for the culture. This is a prepared Jell-O mix or soup with the desired powdered soup mix added. Blood and/or meat meal, or powdered milk formula may be used depending on the needs of the organism you wish to modify.

1. Grow a culture of the target strain of cells overnight in a culture medium.
2. Prepare up to 6 test tubes or containers with 5 ml each of rich growth medium. This is done to test 6 different levels of exposure to EMS.
3. If the culture medium is a solid gel, scrape the growth off of the surface and into a glass container. The container should have 5 ml of buffer added (for each 1 ml. Of cells) and then is shaken thoroughly to mix the cells and suspend them in the buffer. The cells are then spun in a centrifuge or allowed to settle into a solid mass or pellet in the bottom. This is called washing the cells. Professional laboratory buffer and equipment are preferred. You can use coffee filters to separate the cells from the liquid if needed.
4. Wash the cells a second time and then re-suspend the solids in buffer at 2ml of buffer added for each 1 ml of cells. Cool the cells on ice for 10 minutes (when doing time curves with many test tubes) Mix and then place 2 ml samples in each of the six test tubes to be tested.
5. Add .03 ml of EMS to each tube. You need to use some method of shaking each tube or container to maintain mixing for the test. Vibrators can be used to shake test tubes on a tray or in a holder.
6. The cells are treated for 5, 15, 30, 45, and 60 minutes with EMS in each tube while vibrating. Each tube must be sealed to prevent the EMS from evaporating away.
7. On completion, wash the cells in buffer twice as described above to remove the EMS. (All wash water with EMS should be discarded in a bottle of liquid Drano)
8. Re-suspend the cells in 2ml of buffer and then add each one to a solid culture plate for growth. The solid culture should contain the growth nutrients desired plus any test materials like an antibiotic (to test for antibiotic resistance). If the mass of growing cells is solid on the plate, then you can dilute the test tubes at 10:1 and 100:1 and 1,000:1 to reduce the numbers so you can see individual colonies that do not run together. (This is called titer samples when you run various 10 fold dilutions like this)

9. Viable cells (still living and growing) appear on the culture plates when grown at 25-37C overnight. These can be counted in the dilutions for comparison. Survival rates will decline for each longer treatment period and will often decline to 50-20% of the original untreated sample.

Typical survival and mutation rates for rifampicin resistance in E-coli are given in the charts below.


100
10
Survival (%)
0
15
30
45
60
Time of treatment (min) with EMS



The untreated samples typically have no mutant strains that are antibiotic resistant.

Producing Mutations With MNNG

This method is identical to that of EMS just described. Like EMS, MNNG is a powerful alkylating agent. It causes G-C to A-T transitions. Bacteria are cultured and then washed in buffer and then exposed in the buffer solution to MNNG. A sample of the exposed cells are then washed out and grown overnight to recover those with the desired mutations.

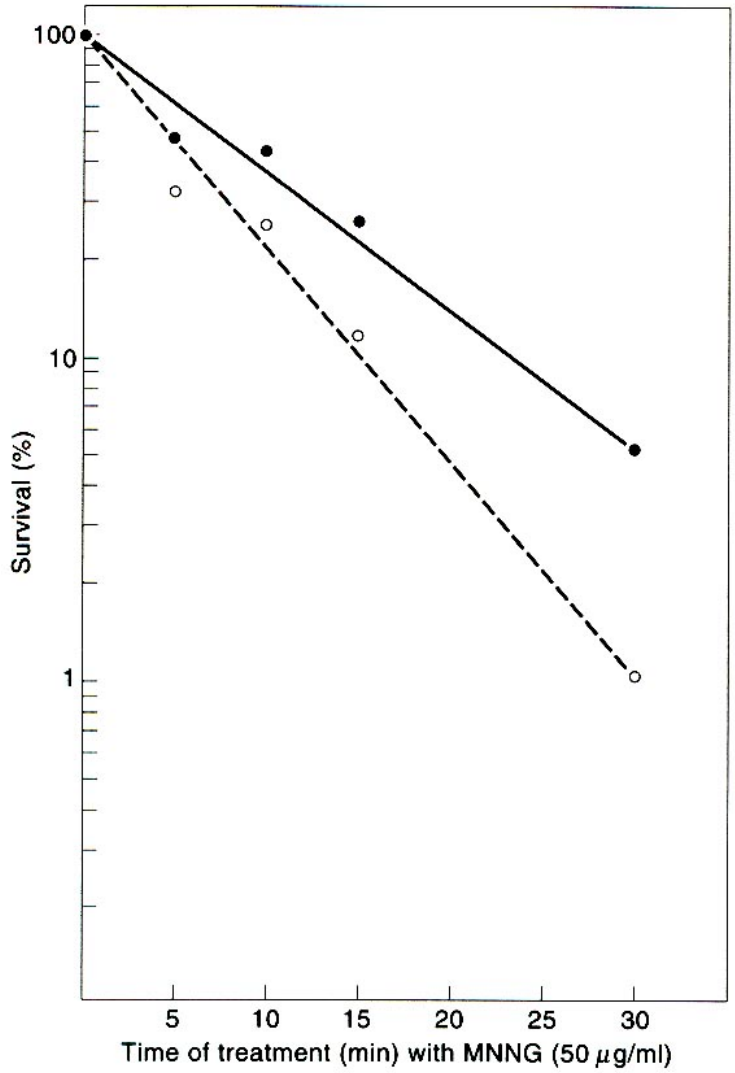
The buffer in this case is made of 40 grams of citric acid, and 20 grams of caustic soda mixed into a quart of sterile water. The pH is adjusted to 5.5 with added caustic soda. The buffer used in the previous method with EMS should also be prepared.

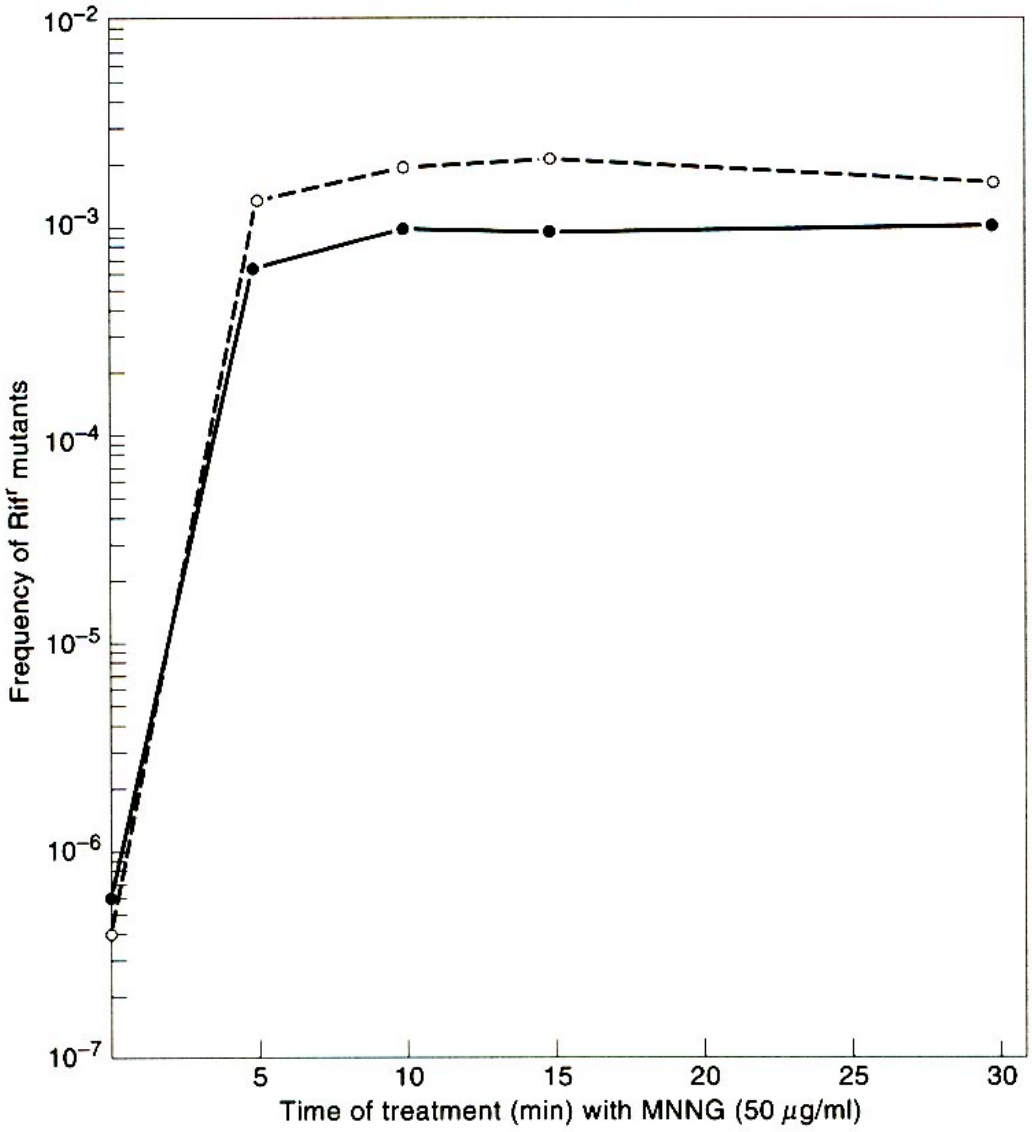
MNNG is a solid crystalline powder. When the bottle of it is opened, it can be dispersed and inhaled which can be harmful. Add 35-50 mg of MNNG to 10 times that amount of citrate buffer and dissolve by shaking thoroughly. This is the MNNG solution.

Following the procedures for EMS, the cells are grown overnight, washed out twice and then suspended using the citrate buffer. These are cooled on ice for 10 minutes. A dose of .1ml of the MNNG solution is then added to each test tube to be tested.

The immersion times are the same as for EMS and you can then plot the survival and mutation rates. The cells can be washed in the baking soda buffer after treatment and the added to a solid plating medium.

Survival and mutation rates for MNNG treatment of E-coli appear in the charts below. Survival rates drop rapidly and most mutations occur within 5 minutes exposure.





Producing mutations by direct application of the mutagenic chemical (Shortcut Method)

All chemical mutagens can be tested using the methods just described. A simpler approach that accomplishes screening and mutation effectively is also possible. The following method does not produce the mathematical measurements for survival and mutation as in the previous experiments but is a shortcut for field use.

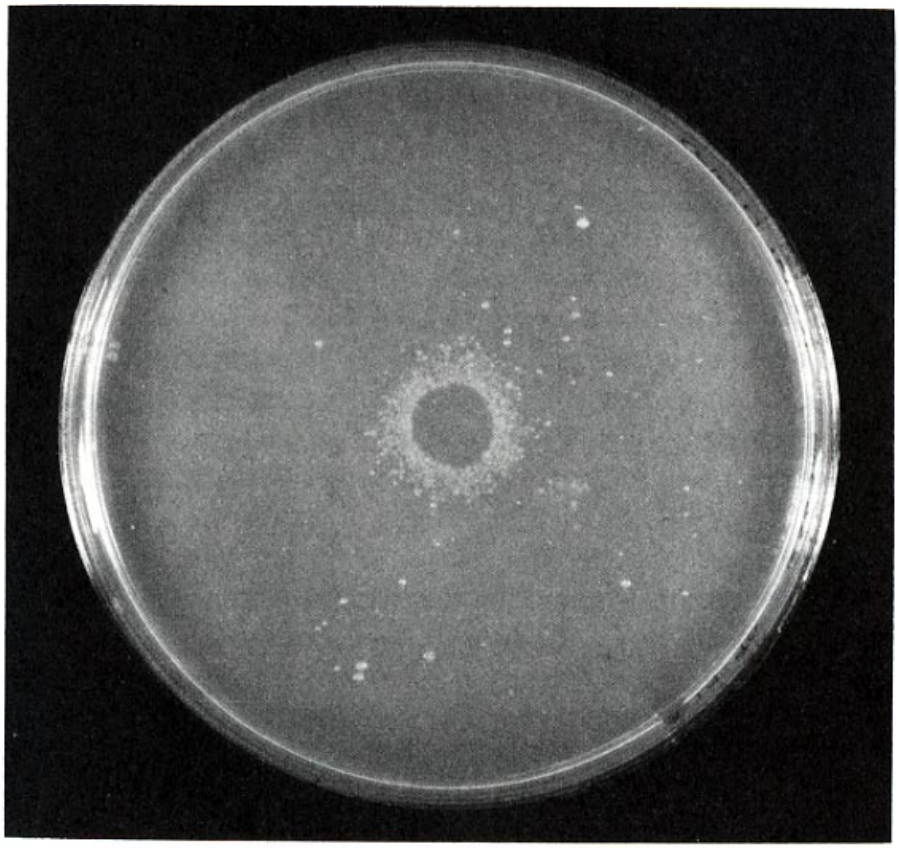
The chemical mutagen is mixed and dissolved into a solution of distilled water or alcohol and diluted 10:1. One drop of this solution is placed onto a cloth disc (such as filter paper cut into tiny discs). This disk is placed onto the culture plate (Jell-O/Agar) that has been inoculated with the bacteria you wish to grow and mutate.

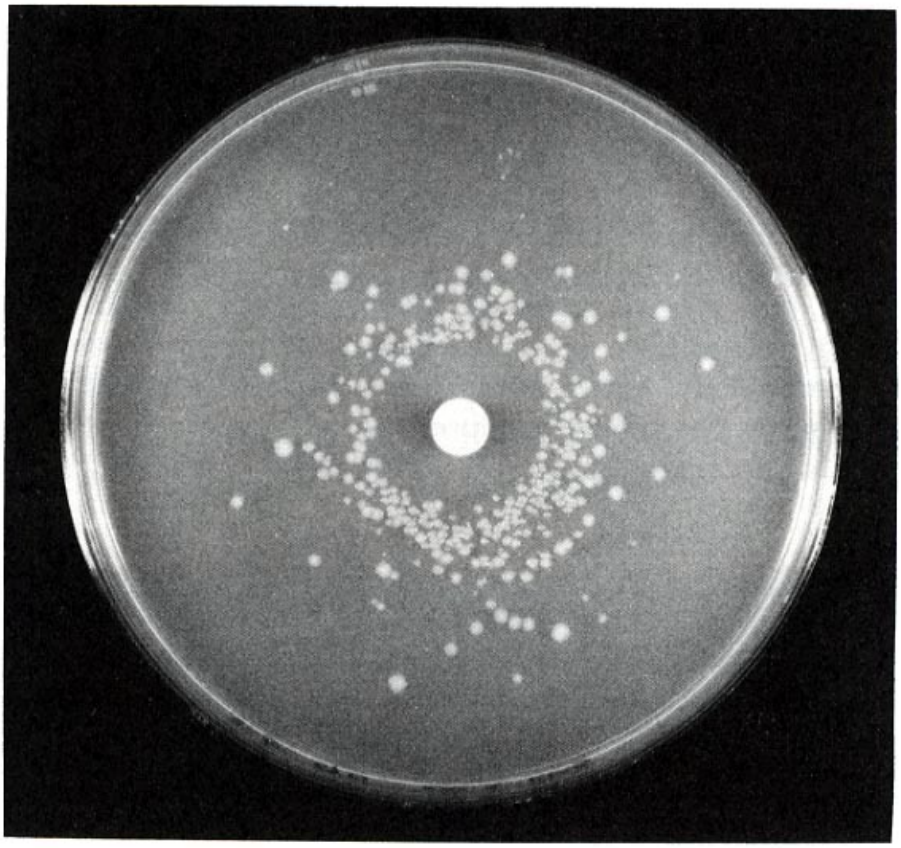
The chemical from the disc diffuses into the semi solid gel and as it spreads out from its central location, it dilutes itself as well. Antibiotic discs can be purchased or can be made by purchasing the antibiotic, dissolving it and then treating a cloth or paper disc with a drop of it.

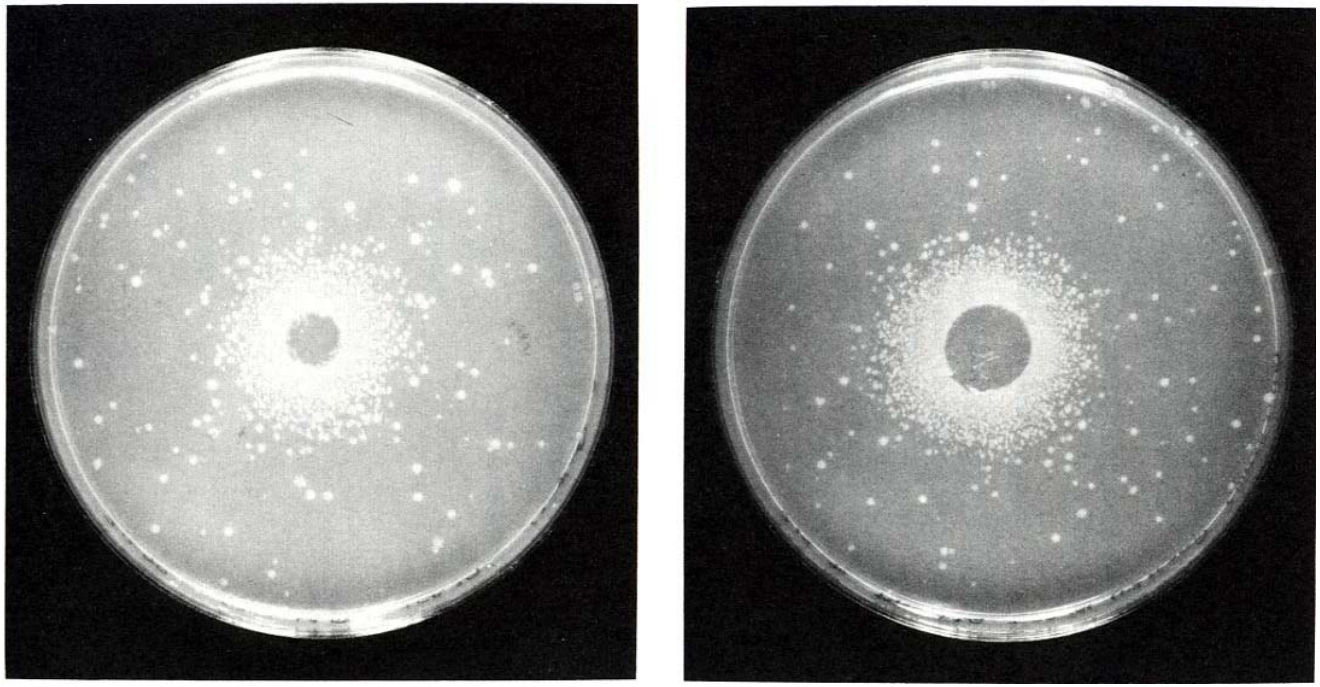
Bacteria growing next to an antibiotic disc or mutagenic chemical are usually killed by the high concentrations next to the disc. The farther away from the disc, the more dilute the chemical becomes and the less toxic. A halo of cell growth forms around the disc when the toxic concentration drops to a level where it no longer kills all the cells. In a normal growth of cells on a culture plate with an antibiotic disc, growth improves as you get further away from the toxic chemical disc. (See page 180 Volume 6-A Bacteria based weapons for an example of an antibiotic sensitivity disc and growth)

In this method, you can mix a very small amount of the desired antibiotic (if you want to produce antibiotic resistant strains directly) into the Jell-O. The culture is streaked onto the plate and the result should be no colony growth because all the cells are inhibited by the antibiotic. In this case, we add the disc with a drop of the chemical mutagen in the center. The mutagen dilutes out into the medium with the cells and causes mutations. It kills the closest cells because it is toxic, but at a sub-lethal level of mutagen, a halo forms around the disc. In this area, cells have survived the mutagen and mutated into antibiotic resistant strains. These cells begin to grow and form colonies. As the chemical dilutes even further, fewer and fewer cells are mutated so that fewer antibiotic resistant strains are produced. The portion of the plate that receives no diluted mutagen chemical from the disc will have no growth at all because no cells have mutated to a resistant form and are killed or prevented from growing by the antibiotic.

The photos below illustrate this method using a medium filled with dilute antibiotic and a mutagen drop on a disc placed in the center.







An alternative to this is to simply use one disc with antibiotic on one side of the plate and another disc with mutagen on the other side of the plate. The mutated strains will grow in the combined zone between the discs and these are antibiotic resistant mutants.

This method allows for the rapid production of antibiotic resistant strains. An example would be the anthrax used in the highly publicized postal attacks of 2001. This strain was sensitive to Cipro and other antibiotics. These anthrax cells could be grown on a culture plate with a Cipro disc on one part and a mutagen on another part. The anthrax will not grow around the Cipro until it is diluted to a point where the halo forms. The chemical mutagen disc diffuses into the plate and mutates the anthrax. Those cells that

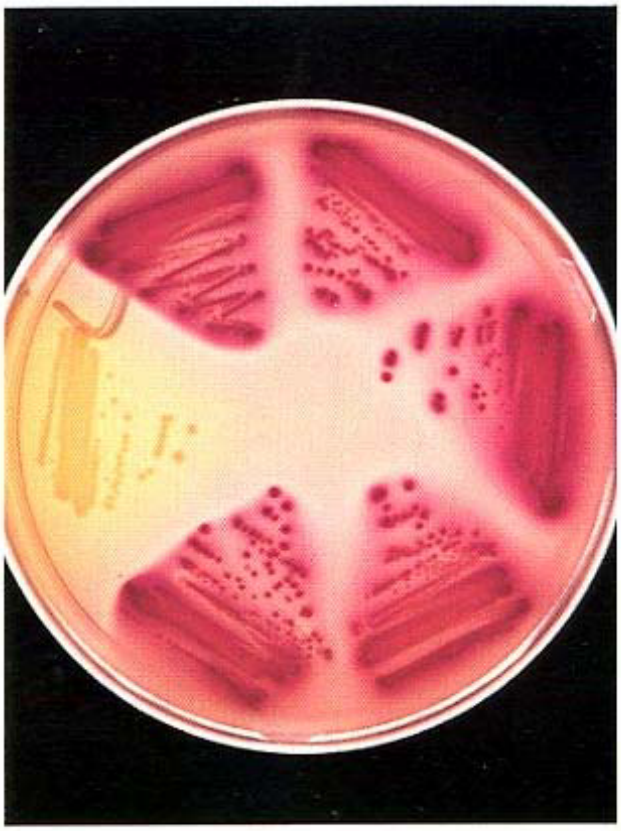
become resistant to the Cipro grow inside its halo in another halo that is formed from the mutagen. [A visible example of what combination effects look like can be seen on pages 260,119 and 136 of Volume 6-A.]

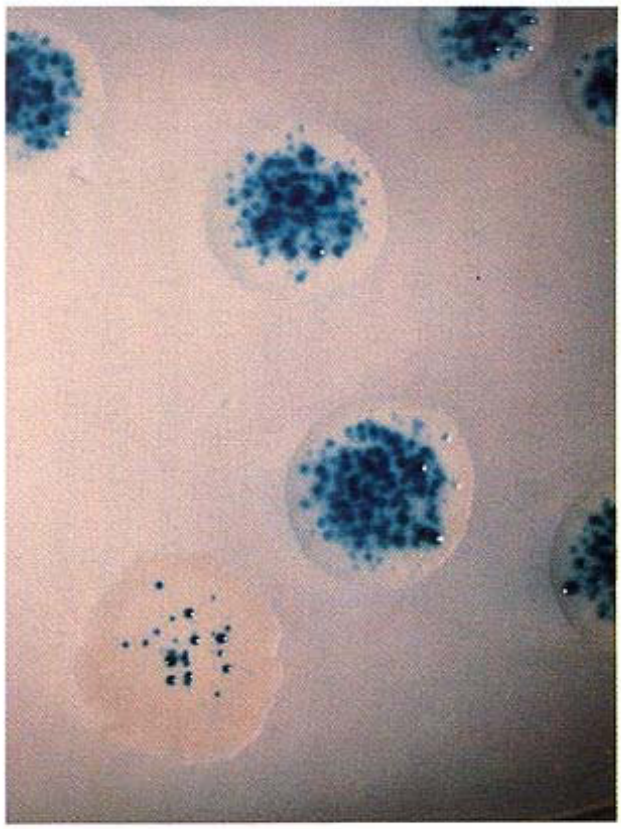
This method can be used to produce multiple antibiotic resistance but requires some patience. Each new resistant strain can be passed through a test animal (mouse) and recovered from the spleen to insure it remains virulent.

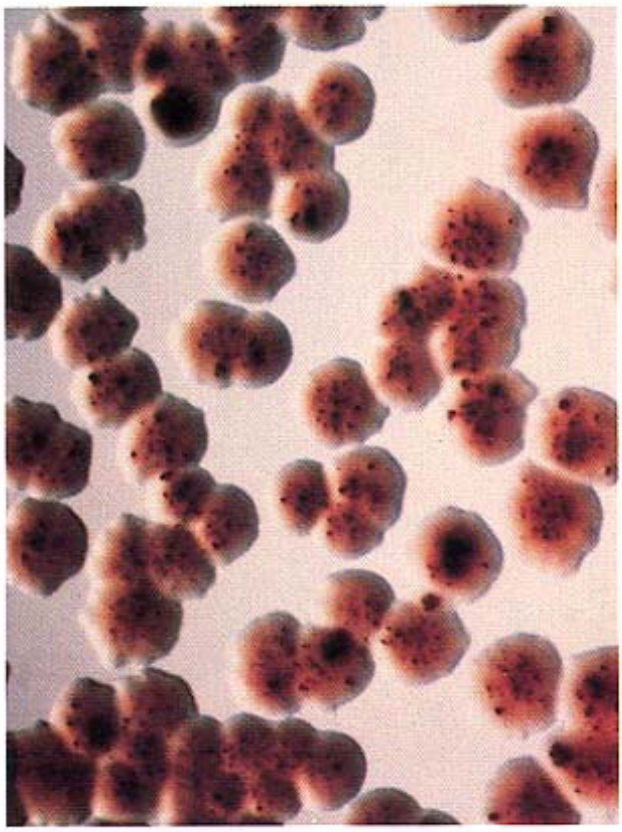
Screening Mutations by other methods

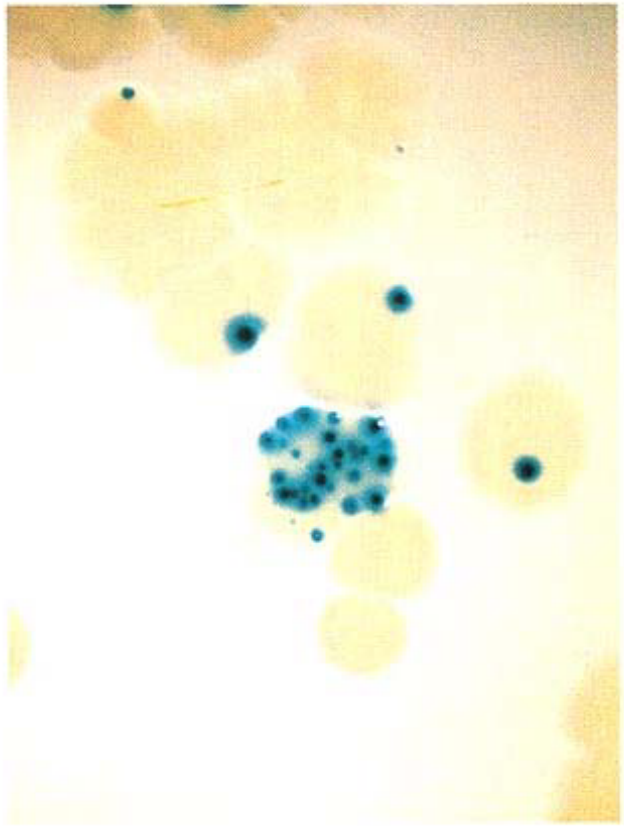
Mutation effects other than antibiotic resistance can be screened for. An example is when a cell has lost the ability to use lactose in nature or in the lab. You can add a mutagen to a culture plate and those cells that mutate to recover this ability will be the only ones that will grow on a plate with lactose as the sole carbon source. This can be seen when a special plate is prepared with lactose and an added color indicator dye like MacConkey agar or EMB agar.

On these special mediums, cells that cannot use lactose (lactose negative or lac-) produce white colonies while lac+ cells produce red colonies. In the course of natural mutation, some of these cells can revert back to lac- and this effect can be seen in the clear colonies that form blue spots. These blue spots are daughter cells that have just mutated and lost the ability to use lactose. This effect can be seen as these cells form their own mini-colonies in the larger colony.









Another method for screening that is used in weapons laboratories is live test animals. Lab mice are basically living culture media or food for the bacteria. Those strains that are most effective at infecting this living food and overcoming its defense mechanisms will grow inside the mice and spread to many organs. These superior infecting cells are recovered from these organs. They often increase in virulence as those individual cells that are better at growing inside the mice and infecting it will grow preferentially. Cells that have been mutated with a chemical mutagen and washed can be used directly in test mice by inoculation. [Rats are a better choice because they are much more resistant to anthrax from the start-They require up to 1.5 million spores for a lethal dose while mice die off at 5 spores]

An interesting method of producing anthrax vaccine resistant strains is to vaccinate the mice with your anthrax strain or feed weakened or killed anthrax cells and toxin (killed in formaldehyde or by heat) to them and then use the surviving mice. Anthrax can also be grown at 42-44C to produce inactivated strains used in animal vaccination. These survivors have some immunity and will often survive challenge doses of the same strain. By mutating the anthrax and then feeding the new strains, a new anthrax strain can be found with modified capsule and/or toxin antigens that the body can no longer recognize. This makes vaccines worthless against the new strains. If these are also made antibiotic resistant, they can become devastating weapons.

Practical Mutation Options

Methods of creating mutant strains can be combined. It has been found that ultraviolet light sensitizes bacteria to caffeine and that the caffeine is then highly mutagenic. Treating cells with UV and then growing them on a plate in the presence of a caffeine disc or .1% caffeine added to the gel will yield higher numbers of mutants at the low UV exposure times. UV irradiation sensitizes all bacteria to chemical mutagenic effects. This is due in part to the UV creating a leaky cell membrane that permits the chemicals to obtain entry into the cell more quickly and easily.

Germicidal UV lamps are considered the best for producing light induced mutations and are available at local stores. Mutant cultures should be stored in the dark or under poorly lit conditions to avoid photo-reversion effects. Most chemicals used to mutate bacteria are available at any college lab or from chemical supply houses. Those with a chemical background can produce their own stocks.

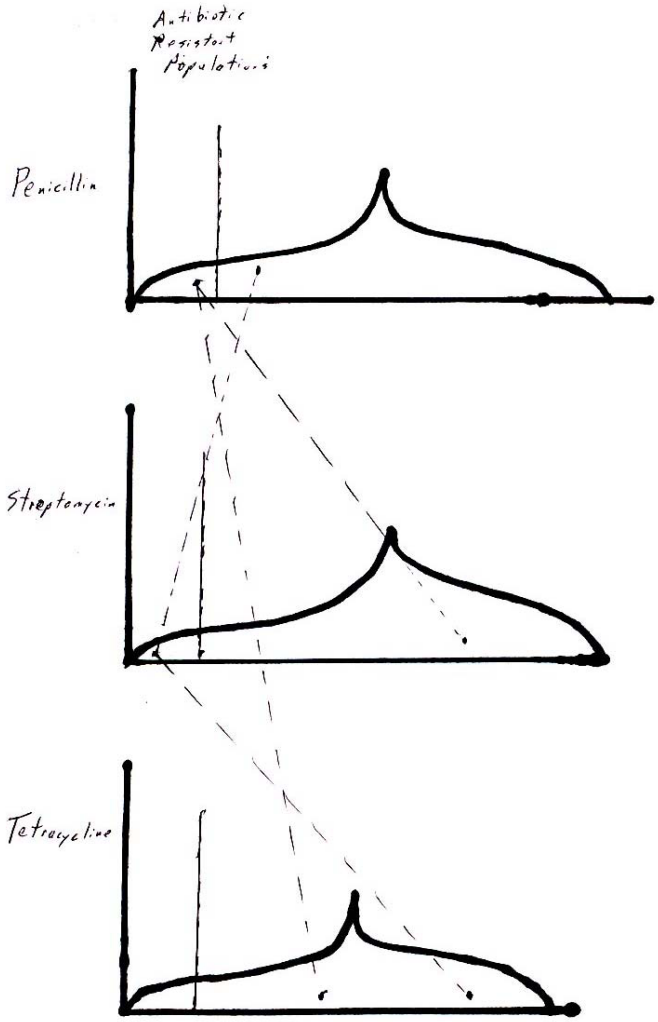
Mutation Weapons Theory

In nature, bacteria mutate at a known rate. This figure may be 10 (-4) or whatever figure is applicable. This is for a single mutation. For a particular mutation at a specific base pair or codon, the events are much rarer. When they are on the surface of soil and exposed to the Suns UV radiation, these rates increase substantially. The result is a large variety of mutations. Most of these are lethal. The remaining mutants that survive in the soil will be those that can still live using the surrounding food and water and reproduce. Those with a mutation that favors using the surroundings more efficiently will be the ones that produce colonies and spread more efficiently.

Prepared mutations, like those described where all the cells have mutations, speed up this process considerably. By using selection, desired traits can be acquired and mass produced. New weapons can be produced with these new traits and properties. Lets take antibiotic resistance as an example.

A small percentage of cells mutate to become resistant to the desired antibiotic in the processes we have shown you. You can see them on the culture plates as the colonies that will grow in the presence of the antibiotic. All other cells that were not resistant could not grow and were not seen. Some of these other cells would likely have had mutations that made them resistant to other antibiotics. A cell that has resistance to rifampicin may not be resistant to streptomycin. The same holds true for cells that can become resistant to streptomycin. In a few cases, cells may be resistant to several antibiotics at the same time if the biology is right.

The antibiotic resistance for each cell can be plotted on a bell curve. All the cells in a given sample fall on some part of the bell curve. Those at the extreme left are most resistant. Those in the middle which comprise most of the cell mass did not have changes making them resistant while those at the extreme right had harmful mutations which made them more sensitive to the antibiotics. The bell curves for three different antibiotics are displayed below.



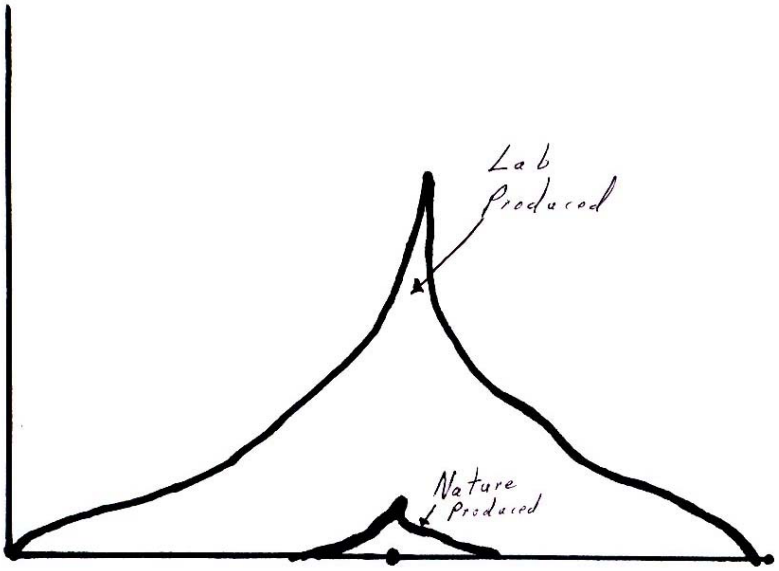
A cell that is resistant to the first antibiotic may fall anywhere on the remaining charts. This is the same way as saying that a tall man in a given population can also be heavy or light and that he may have blond or black hair. These are all measured traits. Our colony is like a city of different people with different appearances.

If you look at Anthrax cells, when a colony is formed, all of the daughter cells will be identical to the parent until the mutation point is reached where a single mutation may occur. There is no difference between the daughter and parent at all until this occurs so there is no bell curve of different traits or antibiotic resistance. (Our city of people-the colony- are all identical twins.) Once all the colony cells have been treated with radiation or chemicals, they will all have a mutation or multiple mutations (except for the app. 25% of cells that died). This produces the bell curves seen above with billions of cells filling all parts of each bell curve. They are all genetically different now.

Using this understanding, you can now take anthrax cells, mutate a very large population and then do one of several things. You can select the anthrax with a particular antibiotic resistance, (to Cipro for example) and then mass culture this as a weapon. You can also use the entire mass as a weapon. If billions of these mutated cells are released in a target area, you will have a large range of antibiotic resistance. If several thousand cells are inhaled by a host in the target area, many of these cells will germinate and begin an infection. The target goes on antibiotic treatment. An enemy quickly discovers which antibiotic will kill the infecting bacteria and uses this antibiotic to treat their affected populations. In this case however, once the main anthrax is killed by a new antibiotic, another strain that is resistant and also growing, continues the infection and kills the host. This is because you have delivered a mix of weapons that cover the bell curves of all the likely antibiotics and the normal defenses of using selected antibiotics may not work.

This new weapon which uses all the mutated forms in its design will be called a "**bell curve weapon"** in this book. The concept is simple. Simply mutate the selected parent cell and the remaining colony of identical cells with radiation. This produces billion of new mutated forms that cover the entire spectrum of changes that are possible. You can use UV radiation for one plate of cells, nitrous acid for another, MNNG for another and so on. Each one of these different methods produce different types of changes such as GC-AT transitions, frameshifts, additions, deletions and so on. A huge range of variations and resistant strains can be produced. All of these can be mixed together and used in the final weapon. (Chemicals and radiation can also be used in combination to increase the variation)

These mutations occur in nature and produce a small bell curve of variation. By using all these methods, a huge variation is produced that will not occur in nature. The bell curves are compared below.



Anthrax has other important properties besides antibiotic resistance that are also mutated in the same mixtures. The most important of these are –

Capsule formation and antigens
Virulence
Toxin effectiveness and antigens

These factors may be affected as well. Some may lose the ability to form capsules. Some may produce superior capsules which have better resistance to phagocytes and immune responses. Each of the cells may have an improved or declined ability to use the host for food and spread to surrounding tissues and other organs. The toxin may improve or lose its ability to kill the host and assist in invading surrounding tissues. Each of these factors will form their own bell curves so that there may be dozens of important bell curves present for each mutated batch.

The cells which have the improved virulence ability will be the best at spreading to all the internal organs. If these have the same toxin or an improved toxin, they will kill the host. If they lost the ability to produce the toxin they will likely lose the fight against the host immune system over time and disappear. Cells of all these types will be present in a mixed bell curve weapon. This means that both weak and strong virulent cells can invade the body. As you will see in later chapters, some of these cells can pass on their superior traits during the infection so that if they lack one capability, their good parts are not necessarily lost. This forms the concept of the bell curve weapons. When all the genetic material is present in a cell mass, both good and bad, these traits are present in the weapon and can be exchanged inside the host during infection. That makes this weapon concept promising. All the parts of every bell curve are represented in the delivered weapon.

Bell curve weapons can be enhanced by selecting a superior trait to begin with. Virulence can be tested for by using mutated anthrax cells to infect a resistant species, like the rat. A rat requires over one million anthrax cells to initiate a successful lethal infection. In this large mass, only the superior cells that have been mutated with improved virulence will invade the rats superior immune system and spread to the internal organs. These superior cells can be recovered from the organs and used as parent cells for the bell curve mutation weapons.

One of the most important mutations in the anthrax cells will be changes in the toxin proteins that change the surface antigens. Vaccines used for protection against anthrax build up immunity to the toxin. By changing the toxin antigens, you make the vaccine worthless and the weapon far more effective. [There is probably one particular type of mutation method that is superior for changing the toxin antigens over all the others but this is unknown to the author at this time. It will be the subject of future research I am sure.]

In the 1970's, in a (now) well publicized secret test, the US Army spread bacteria agents around some US cities. A particular species released off of San Francisco infected and hospitalized over 300 people with one fatality. It was a species that was supposedly harmless. In mass producing and spreading a huge mass of the bacteria, a small amount of mutations occurred and some of these filled particular niches on the bell curve that allowed them to infect specific humans that inhaled the cells. The army had mass produced and tested a crude bell curve weapon. They had not deliberately produced a mutated mass but the mass was so large that the normal rates in nature took a hand. The large number of cells insured that large numbers would be inhaled in the target populations so that a few of the cells with bell curve superior traits landed inside the lungs of some of the people.

This concept allows bell curve weapons to be produced from bacteria that normally live on the body and are harmless. These can be turned into potent weapons directly via mutation.

This concept also allows for the formation of a new combination weapon. In Volume 6-D, the author describes the production of multiplier effect weapons in which soil samples containing anthrax (or other desired organisms) can be grown directly on Jell-O and delivered while growing into the target area. By adding a mutagen, new cells with widely differing properties can be mass produced and delivered. The wide variation insures some superior virulence, some wide ranging antibiotic resistance, and in this case attacks by multiple organisms.

As we have already seen, changes in antigens allows some organisms to have superior infective ability. In the case of treponema and some bacteria, they exhaust the immune system enabling them to completely infect and ultimately kill the host. By mass producing a huge range of antigenically different strains, the same exhaustion can be produced while other infective traits also take hold. [Aids has a mutator gene which allows it to mutate at a far superior rate than other virus species which is why it continually fools the bodies immune system and becomes drug resistant with great frequency].

Bell curve weapons allow a huge range of antigens and other traits to be applied as part of the weapon. Each new cell that has a superior trait will be the one to express it inside the host. All of its daughter progeny will also carry these superior traits which makes the weapons reproducible and reliable. The mutations that yielded the superior strains can be reproduced from the new superior daughter strains or from the original parent. Mass variation of each bell curve weapon insures that antibiotics and vaccines will generally be ineffective (as well as host defenses).

When bacteria invade human skin, the mutation rate of the bacteria usually declines slightly because the skin blocks UV radiation. Inside a human body, the process of selection takes over as only those cells that can use human fluids and tissues as food will do so and will produce progeny that do so as well.

Another infection that somewhat mimics the bell curve weapons is gas gangrene (volume 6-A). These infections occur primarily when multiple bacteria species are present at the site rather than a single species. These different Clostridium each produce different toxins. Some lyse the surrounding tissues. Some cause damage further away and so on. This mix of toxins acts like a different type of military unit. Long range toxins act like artillery destroying defenses far away. Short range toxins act like tanks blowing holes in nearby cells (door openers) and some act like infantry, invading cell interiors and fighting inside (like urban warfare). The bodies defenses work at recognizing each one of these and attacks them. Combination attacks from multiple toxins and organisms seems to be very effective in this instance.

The more different species of Clostridium, the more likely a successful gas gangrene infection occurs. With different species, the local immune system has less likelihood of identifying and eliminating each one in sufficient numbers to be effective. The local immune system becomes exhausted and cut off and the infection spreads. Now add the potential of bell curve weapons. Antibiotics are used to stop gas gangrene, usually with success. When millions of Clostridium have been modified and some now have antibiotic resistance, the infection is no longer stopped. Those strains that are resistant take over and continue the infection. Some of the Clostridium will be modified so that the local immune system that recognizes and fights off the first strains it encounters, will not be able to recognize and fight off the strains coming up right behind it. These cells can be considered the equivalent of stealth bombers. They are invisible to the immune systems radar temporarily and if the immune system is cut off by the action of the toxin, this becomes permanent.

If better capsules are produced by the mutations, it is like producing better tank armor so the enemies shells or bullets cannot penetrate. If the toxins antigens (botulinum) are changed, it becomes the ultimate stealth bomber in that the toxin reaches its target nervous system and destroys it unmolested. The toxoid for this specific botulinum no longer works. In nature, different types of botulinum toxin have already evolved with different antigens. Multiple antibiotic resistance can be compared to different body armor that stops a specific kind of bullet (the antibiotic).

The lesson is that the more species with different toxins that are used, the more effective the infection (weapon). The more strains of species that are used, the more effective the infection. Combined species which have large numbers of mutations should produce very effective weapons which is why the multiplier effects weapons combined with the bell curve weapon concept may be so effective.

These weapons can be produced in a variety of ways. A soil sample can be obtained and treated with UV or chemicals, or both. It can then be directly grown in the Jell-O mix and delivered. The Jell-O can also contain the mutagen so that all growing cells are mutated. This is the easiest and least expensive way to produce effective weapons without a laboratory or training in this field. It can also be accomplished using sunlight in place of a UV lamp in third world settings (no electricity). This process would yield photo-reversions but still produces significant mutation rates.

With a small lab and some experimentation, you can quickly discover which mutagens best produce changed toxin antigens, improved virulence, capsule changes and so on. It is known that UV and most chemicals reliably produces antibiotic resistance. Some will produce toxin antigens better than others. This may be specific to only the species or particular toxin and needs to be researched in each case. In the case of plasmid exchange, cell fusion and genetic engineering, the desired traits can be produced directly without mass production systems.

By combining mutagens, you increase the variability of the weapon. In some cases the variations may become so great that overall effectiveness is reduced, although you may find exceptionally effective individual cells that kill some targets with far greater efficiency. These cells produce epidemics when they occur in nature and may account for the different outbreaks of plague that have occurred historically. [This happens with influenza annually]. When a superior strain of plague occurs, it often spreads first in animal populations and then to humans. Imagine a plague cell with mutator genes added via genetic engineering. The potential for large scale depopulation is considerable. Common infectious agents such as those associated with colds, flu, pneumonia, and so on can be drastically enhanced via mutation.

In nature, when a cell mutates due to sunlight or other causes, it must be able to use the mutation and then survive in nature. This may be in the soil or it may in manure. If an anthrax cell has mutated and produces a new toxin antigen, it might die in the sun if it cannot produce a spore or simply die off form heat, cold, or lack of food before it can produce a spore or spread to a friendly environment. It is lost forever. This goes on every day all over the planet. The mutations that improve its ability to live in its surrounding (usually soil, manure, pond water, etc.) environment are those that are passed on to future generations. Most of these have rare contact with humans and animals and unless they are of a contagious variety or have reliable vectors to re-infect, they rarely mutate to favorable infective forms. The ability of people (and the Army off San Francisco in the 1970's) to modify and select them specifically for human infection has changed this forever.

The bell curve concept applies to all bacteria. Almost any bacteria can be made infective eventually by selecting and modifying continuously. An unlimited number of possible weapons can now be produced by anyone willing to take the time to learn this science. These will be the army builders of the future. The ability to reliable produce an unlimited number of new bio-weapons, each with vastly different properties is now possible using bell curve techniques. Ordinary citizens can never be disarmed by their government ever again as long as they possess the education to understand this and pass it on.

Notes on Mutation

A few facts which may be useful regarding mutation are included here.

Mutations can be induced by adding the mutagen to the food or water of a target population. EMS has been added to the sugar solution of *Drosophila* at .4% to yield 25% survival and 70% sex-linked lethal mutations among offspring. At .031% in food fed to flies for 18 hours, a sex linked lethal mutation was observed at a 100% level.

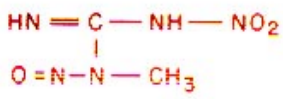
Acridine compounds tested as mutagens in laboratories –

Compound	Basic pK	Optimal Mutagenic Concentration (μg/ml)	Mutagenicity
Acridine	5.3	90	Moderate
1-Aminoacridine	4.2	150	Weak
2-Aminoacridine	7.7	16	Strong
3-Aminoacridine	5.6	80	Weak
4-Aminoacridine	5.7	80	Strong
5-Aminoacridine	9.6	8	Strong
2,5-Diaminoacridine	11.1	8	Strong
2,7-Diaminoacridine	7.8	16	Moderate
2,8-Diaminoacridine (proflavin)	9.3	5	Strong
10-Methylacridinium			None
5-Amino-10-methylacridinium			None
5-(N-piperidino)-acridine			Moderate
2,8-diamino-10-methylacridinium (acriflavin)			Strong
2,8-diamino-3,7-dimethylacridine (acridine yellow)	9.8	2	Strong
2,8-bisdimethylaminoacridine (acridine orange)	10.1	20	Weak
Phenanthridine			Moderate
1-Aminophenanthridine			Weak
6-Aminophenanthridine			Moderate

By varying the concentration of the mutagen, the kinds of mutations can be changed. Nitrous acid produces A-T and G-C mutations. Sometimes these will crossover to A-G and T-C. At higher levels of concentration, it produces frameshift mutations. Many of the mutations produced by nitrous acid are also known to be leaky or

temperature sensitive. Mutation rates can also be increased by lowering the pH of the medium while the nitrous acid is being used. A drop in pH from 5.0 to 4.2 produced an increase in mutation rates of 80 times in bacteriophage (as well as a thirty fold increase in lethality).

Mutagens can mutate all life, from viruses to bacteria to plants to all animals including humans. Among the most effective are the alkylating agents in the following chart –

CHEMICAL NAME	COMMON NAME	STRUCTURE
DI-(2-CHLOROETHYL) SULFIDE	MUSTARD GAS	$Cl-CH_2-CH_2-S-CH_2-CH_2-Cl$
ETHYLMETHANE SULFONATE	EMS	$CH_3-CH_2-O-SO_2-CH_3$
ETHYLETHANE SULFONATE	EES	$CH_3-CH_2-O-SO_2-CH_2-CH_3$
DIETHYLSULFATE	DES	$CH_3-CH_2-O-SO_2-O-CH_2-CH_3$
N-METHYL-N'-NITRO-N-NITROSOGUANIDINE	NG	

Ultraviolet radiation usually means the 253.7nm mercury emission obtained from low pressure germicidal lamps. The effect of the UV is considerable on virus particles inside of cells as well as bacteria. Virus particle survival rates actually increase inside of cells that are exposed to UV as well as the mutation rates. (Virus particles do not mutate outside of a cell with UV). It appears that virus particles preferentially repair lethal

mutation hits over non-lethal hits. The effect of UV is partially reversible in bacteria and viruses using intense white light afterwards (blue wavelengths are the most effective).

Dry cells are mutated best in UV in conditions of low humidity and these are not easily photo-reactivated. A variety of common dyes can also be added to the fluid that the bacteria are immersed in during UV treatment to increase the mutation rate –

Organism	Mutation	Dye
Serratia marcescens	aberrant colonies	ER
Penicillium notatum	aberrant colonies	ER
Sarcina lutea	penicillin resistance	MP,TB
Proteus mirabilis	*phe* → *phe*$^+$	MP,TP
Escherichia coli	resistance to T7	ER
	resistance to T5	AO
	try → *try*$^+$	AO,AY,MB,TB
Bacteriophage kappa	plaque morphology	MB
Bacteriophage T4	$r^+ \rightarrow r$	PF
		PS,TP
		(many)
	$rII \rightarrow r^+$	MB
		MB

AO = acridine orange; AY = acridine yellow; ER = erythrosine; MB = methylene blue; MP = 8-methoxypsoralen; PF = proflavin; PS = psoralen; TB = toluidine blue; TP = thiopyronin.

High temperatures can also produce mutation rates of 10%. E-coli heated to 60C or dried and heated to 135-155C yield these mutation rates among surviving cells.

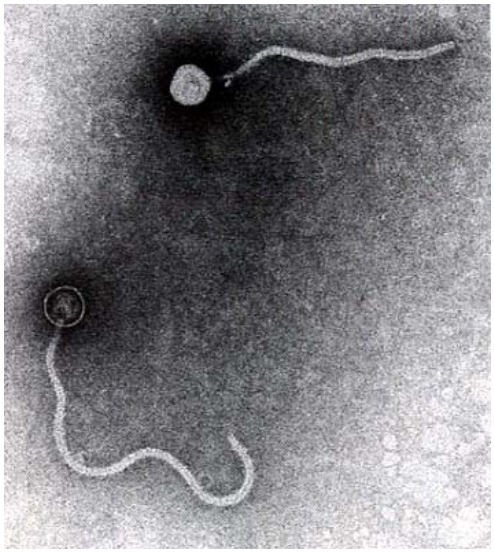
Chapter 5
Bacterial Phage

The first written description of viral action in bacterial cultures was made in 1915 by F.W. Twort. Soon afterwards, Felix d'Herell isolated these particles from dysentery bacillus in which he observed that infected cells broke up. These cells released what he called *Bacteriophage* or "phage" which passed to other cells and would also pass through filters that held bacteria. The phage would infect and lyse (destroy) the host bacteria releasing more phage into its surroundings.

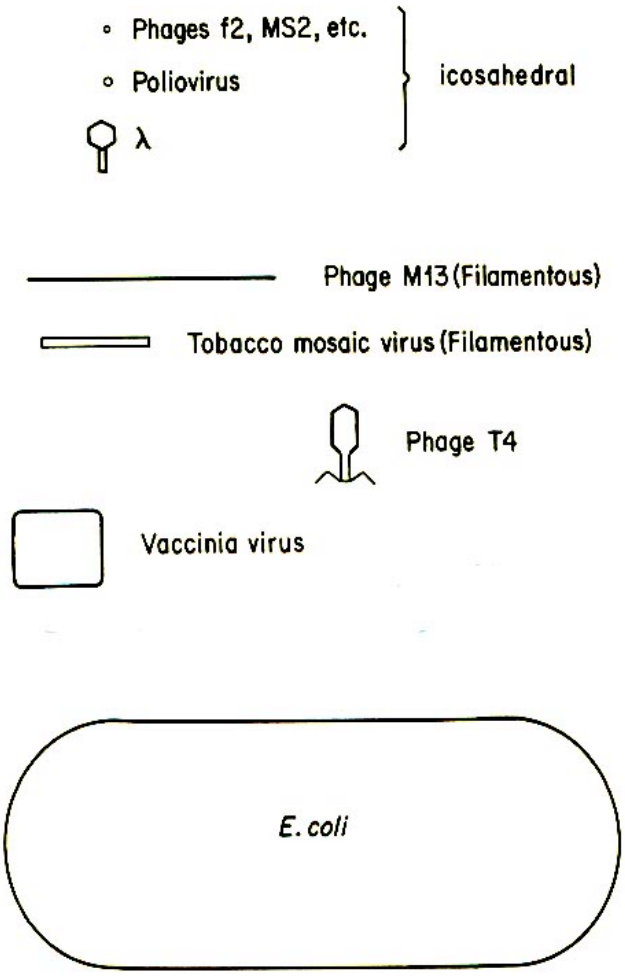
It is now known that every bacteria species can act as a host for phage (virus) species. Not all phage is lethal to the bacteria. Some enter into symbiotic relationships with the bacteria and the infected cell often acquires new properties. This kind of phage is called a **temperate phage**.

The study of bacterial phage has led to the discovery of most of the early knowledge of mutations, gene expression and macromolecular assembly. Today, phage are used as vectors (carriers) of cloned genes in which they carry the gene inside of target cells and this gene becomes part of the DNA of the cell. This technique is used to produce bacteria that manufacture insulin, proteins, growth hormones and interferons.

Many temperate phages also code for genes that instruct the bacteria to produce toxins. This gives the bacteria an advantage in which it can then use the toxin to turn its surroundings into more food. The bacteria that cause diphtheria and scarlet fever have been infected with phage that instruct the production of specific disease causing toxins. The photo blow shows an electron micrograph of the phage (virus) that infects Corynebacterium and instructs it to produce diphtheria toxin. Without this phage, the bacteria is harmless.



An example of the relative size of some common virus particles to E-coli is given in the chart below. Some phage are very tiny (poliovirus) while others are large (vaccinia).



Many phage can infect only one kind of bacteria and cause a single disease with that strain of bacteria. This kind of specific host-phage relationship can be used to identify pathogens in hospitals via **phage typing**.

Phage physiology

Phages are compact particles that do not consume food and reproduce by themselves. They are inert until they come in contact with a suitable host bacteria. Their "body" is made up of a dehydrated chromosome strand which is covered by a protective shell called a **Capsid** which is also referred to as a head or coat. The capsid acts as a

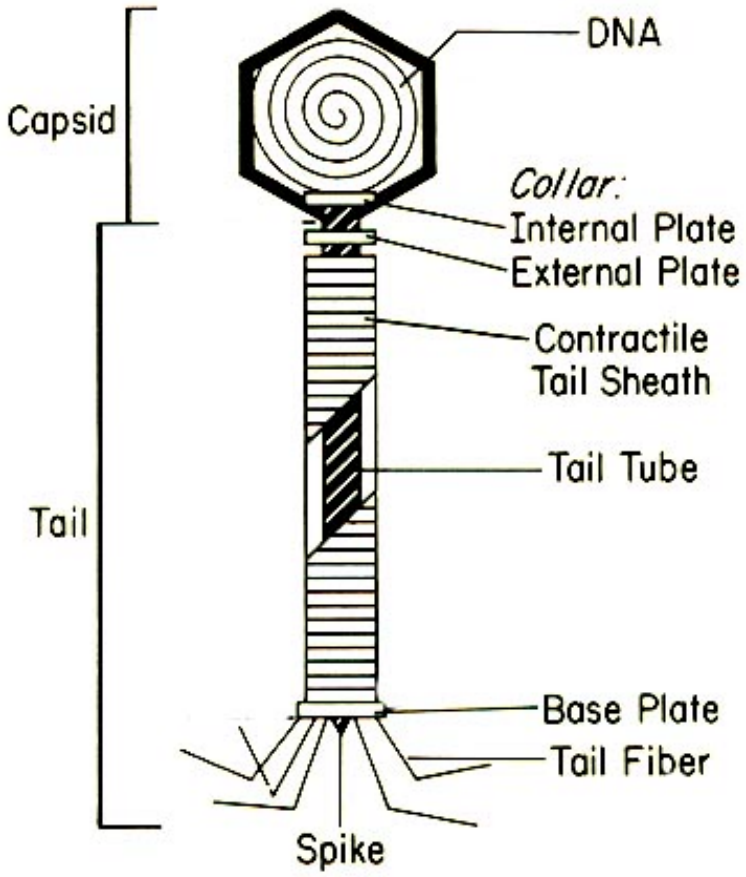
container for the genome of the phage and has proteins that help in packaging the genome inside, and bind to (adsorb on) bacteria host cell walls or membranes.

The capsid encloses a single copy of the viral genome which is usually one molecule of double stranded DNA, single stranded DNA, double stranded RNA or single stranded RNA. In the case of phage 6 which is a virus that infects Psuedomonas, three different strands of double stranded DNA are found which allows it to recombine different genes by reassortment. This chromosome alone may make up 50% of the mass of the phage.

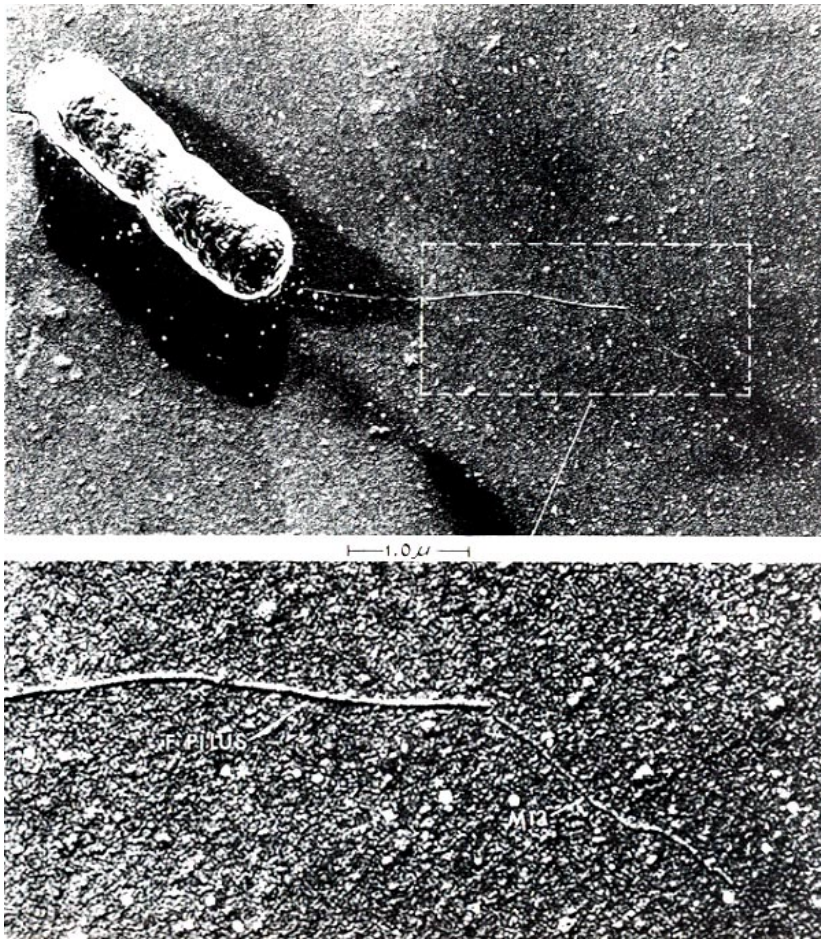
A phage can be very small and simple like the E-coli R17 phage which has 3,600 base pairs, and these make up only four genes. The E-coli PB51 phage has over 240 genes with 2.5x 10(5) base pairs making it much larger in size and effect.

One type of virus has a shape similar to that of a geodesic dome and are called spherical viruses. They have a **icosahedral** symmetry like the geodesic dome and the capsid has 60 protein parts or in larger phage, has multiples of 60 proteins (120, 180, 240 etc.)

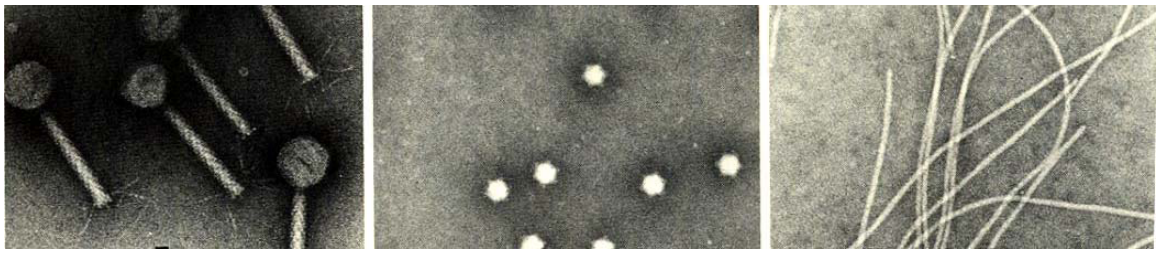
Another type of capsid has an icosahedral "head" with collars underneath attaching the head to a tail tube, base plate tail fibers and spike.



A third type of capsid has a filamentous structure which is a hollow cylinder with helically arranged protein parts. Some of these only attack male bacterial cells using the sex pili as receptors and may use the host cells plasma membrane as cover.



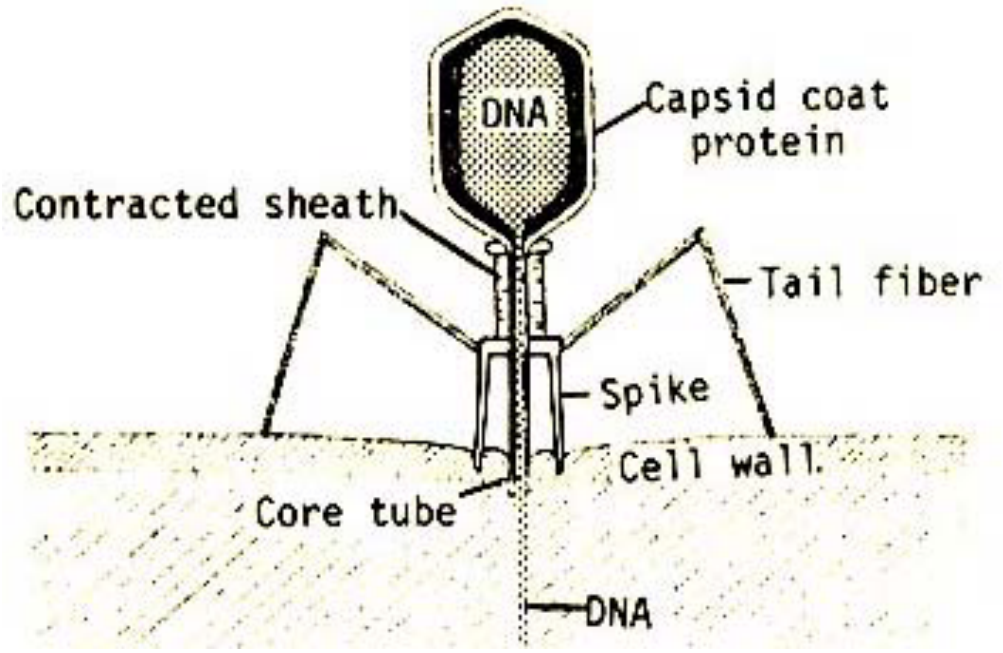
The above photo shows a filamentous bacteriophage (M13) attached to the sex pilus of an E-coli.



Several kinds of phages are shown in the above micrograph

The phage uses its protein parts to attach to a similar receptor protein on the bacteria cell surface. The bacteria may have many suitable receptor proteins which also have important functions for the cell. Sometimes the phage must anchor or bind to more than one receptor to invade the bacteria. This is why some phage are specific for only one bacteria species or strain.

Several different systems are employed by phage to inject their chromosome into bacteria. In T4 phage, once it has attached to the cell surface, the tail sheath contracts and forces its hollow inner tube into the cell through several cell envelope layers. This action causes changes in the phage which releases the genome through the tube into the cell.

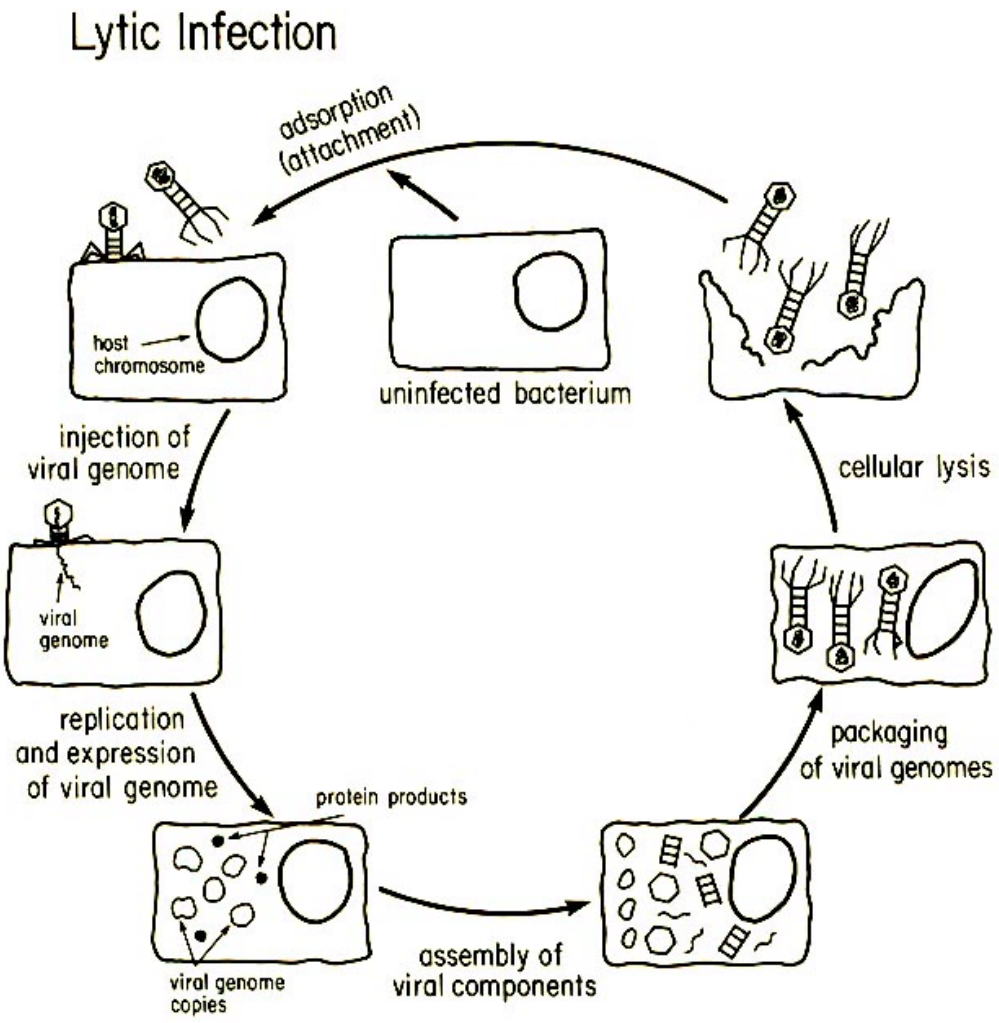


The empty capsid often remains attached to the cell surface after the DNA enters the cell. Filamentous phage appear to enter the cell by being drawn into the inner membrane of the cell envelope while being uncoated. The DNA is released inside the cell as the coat protein breaks apart into proteins which remain in the membrane.

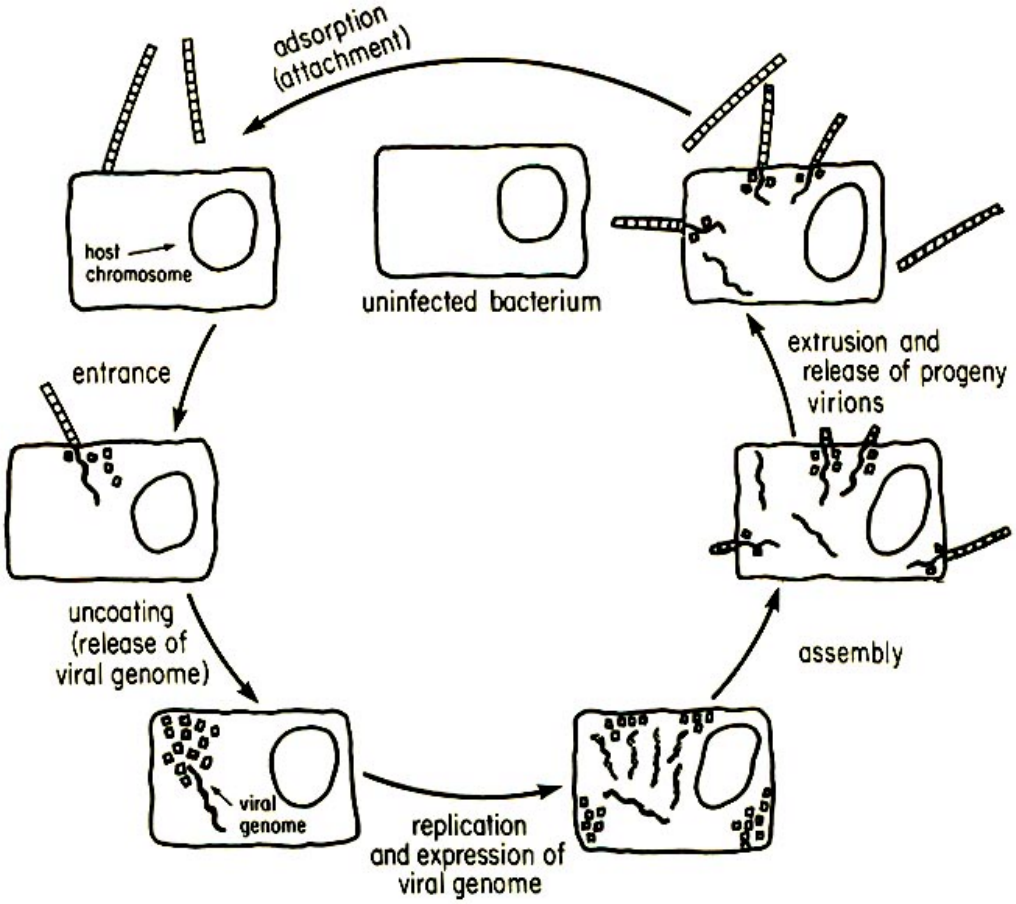
At this point the phage chromosome begins a four step process –

1. “Early” genes instruct the cell machinery to code for proteins which are used to replicate the viral genome. They also modify the cellular machinery to only make copies of the viral genes.
2. The remaining genes are then used to initiate the synthesis of capsid proteins which are assembled into intermediate structures.

3. The new gene copies are produced and packaged within capsid and ancillary proteins and the mature virus is finished.
4. The phage instructs the cell to produce the enzyme lysozyme which disintegrates the cell wall and the new phage particles are released.



In temperate phages, this lytic phase may be delayed with the virus remaining dormant inside the cell. They integrate their DNA into the host cell (lysogeny). In the case of some filamentous phage, the host cells intermittently produce more filamentous phage and extrude the phage outside the cell wall through pores



Growing Phage and Bacteria

When bacteria are infected with a virus, they go through the processes described above. This process can be seen while growing bacterial cultures. A petri dish is filled with a bottom layer of agar (or Jell-O) which has a nutrient mix for the bacteria to be grown. This is identical to the mixtures described in Volume 6-A. Another mix of Jello or agar is prepared using only ½ of the amount of Jell-O or agar so that it does not solidify and is maintained as a liquid at 45C.

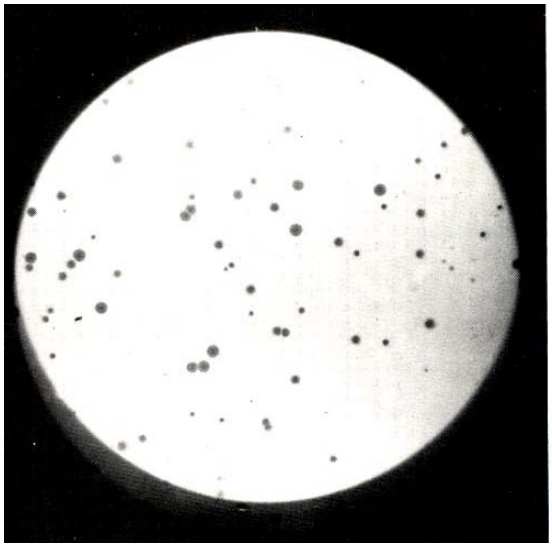
Just before use, your bacteria culture is added to this liquid in high concentrations. A small amount of the bacteriophage is added as well. [For teaching purposes, bacteriophage can be ordered from ATCC and medical supply companies or may be obtained by using this method with bacteria already infected with the phage].

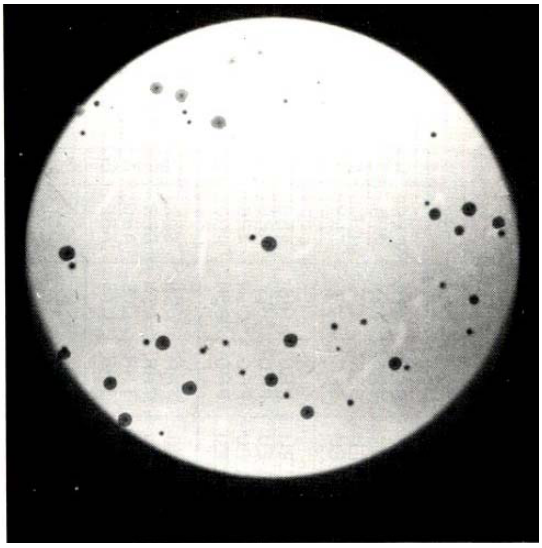
The contents are then mixed together and then poured over the bottom layer in the petri dish (or other container). The mix is cooled at 75F for Jell-O or 95F for agar so that it solidifies and incubates.

The bacteria in the mix grow in a continuous lawn across the surface. At the same time, the virus particles become active and infect a living bacteria next to it. As the

bacteria grow and become visible in a few hours, the virus spread to surrounding cells (slowly due to the solid mass of agar or Jell-O). This produces holes in the lawn of bacteria that can be seen. At the center (foci) of each virus infection, the bacteria cells have died and lysed leaving a visible hole called a **plaque**.

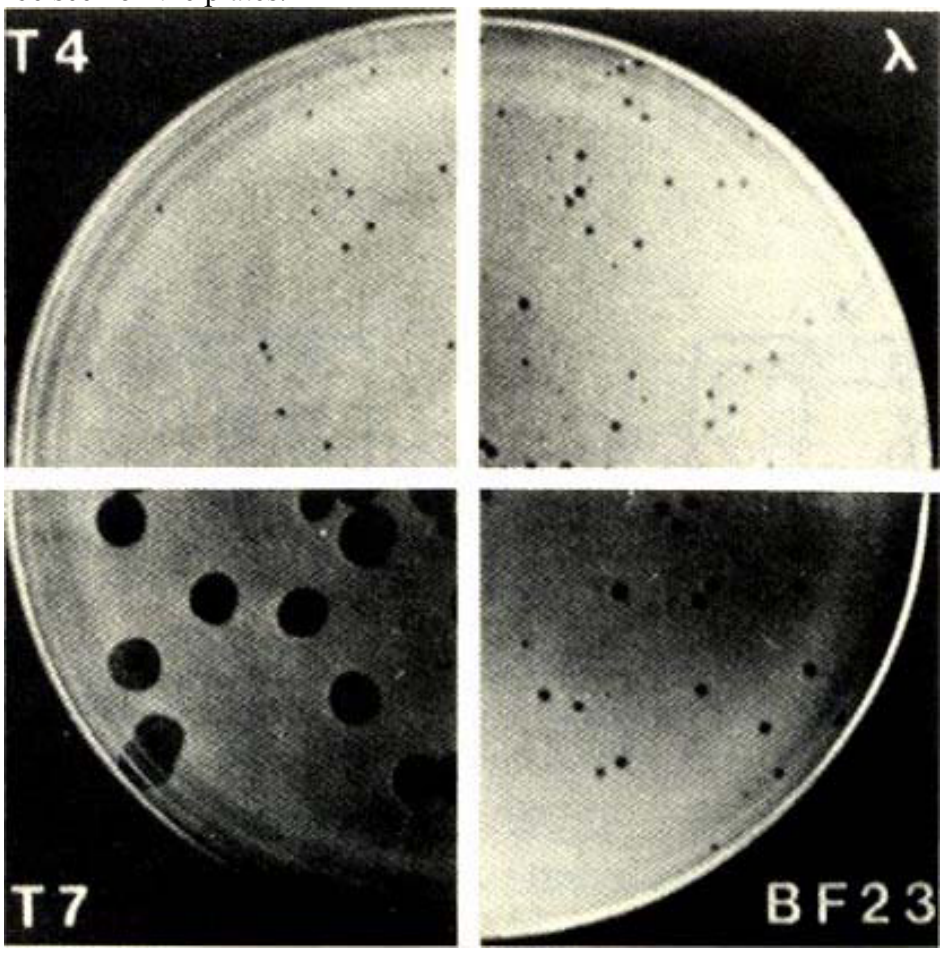
Plaques are produced by single virus particles so a count of the number of virus particles can be made by simply counting the number of holes in the lawn. Each plaque can contain from 1,000 to 1,000,000 virus particles. The plaques do not spread to cover the entire plate because they usually only infect growing cells. When the bacteria have filled the plate and used the available nutrients, they quit growing so this stops the phage from overrunning the entire plate.





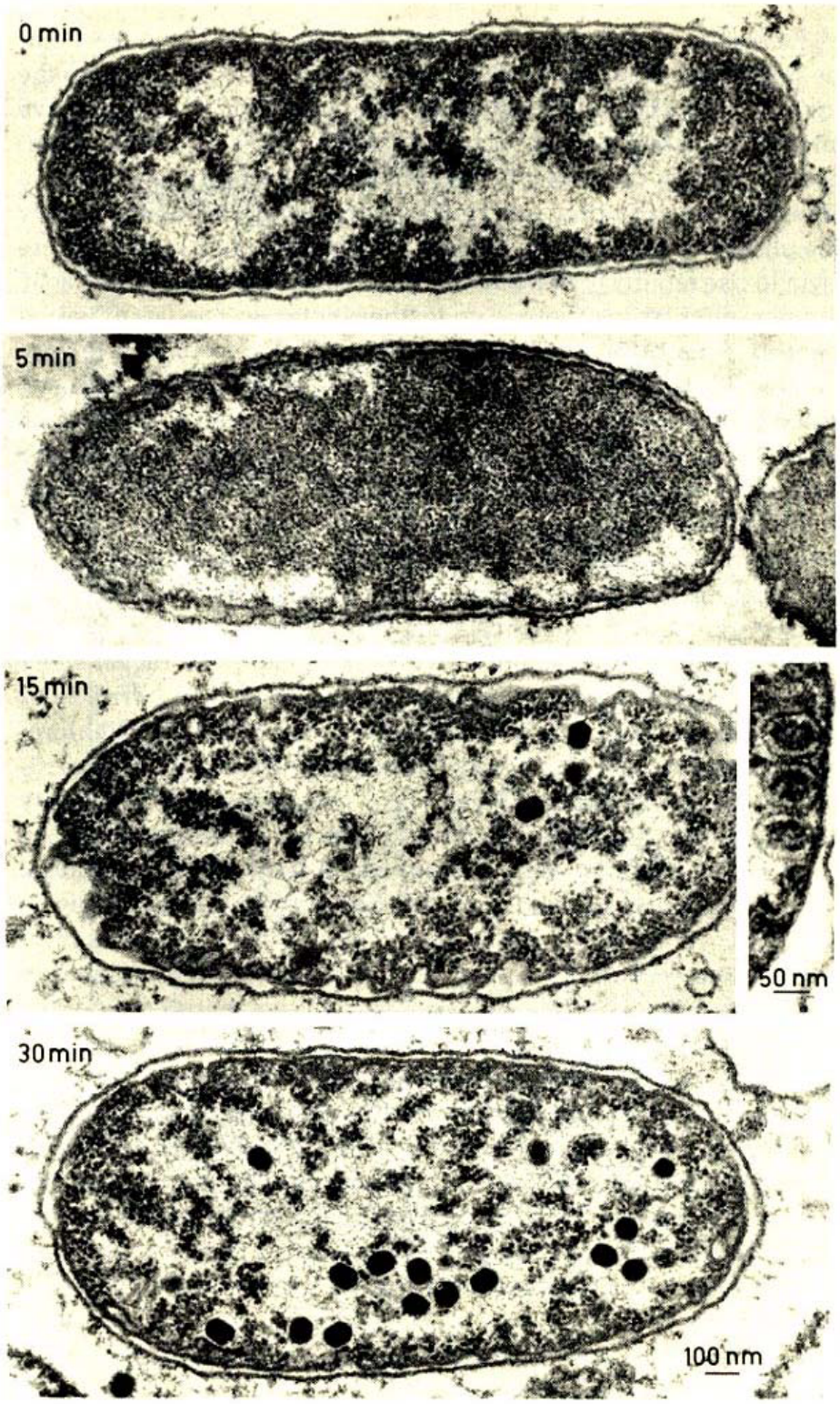
In the photos above you can see differences in three strains of phage. These differences in morphology (appearance) helps identify different phage infections. The

appearances can change with temperature, agar thickness, pH and so on. The identification of virus by morphology is a laboratory science in itself. The photo below illustrates several different types of plaques. The size of the plaque may vary depending on the speed of the viral multiplication and size of particles released by each bacteria. A change in plaque size by the same species of virus usually indicates a mutation by that virus and can be seen on the plates.



Virus particles can also be observed and counted in electron microscopes. These phage particles have specific and known rates of growth, recombination and mutation. They are often recovered from nature and observed using the plaque technique. Many E-coli phages have been discovered using samples obtained from raw sewage and then observed by sterilizing the sewage with chloroform (to kill all living cells) and then mixing the sample into an E-coli batch and growing the plaque forming plates. Phage are very resistant to chloroform so they will be the only substances that survive to grow with the stock E-coli cultures.

When a virus first enters a bacteria, it may take 15-60 minutes for the entire cycle of growing and release of virus particles to take place. Some bacteria may be infected by many virus particles on its cell surface at the same time because they have many receptors for the suitable virus to attach to. This entire single phase of infection to cell lysis and release is called **one-step growth**. This cycle is illustrated in the photos below –


0 min
5 min
15 min
50 nm
30 min
100 nm

It takes about 7 minutes to produce a single mature particle from scratch. The inset for 15 minutes in the above photo show the immature heads (capsids) of the particles without the chromosome inside. The dark spots illustrate the mature virus particles. When the bacteria releases its virus package, the number of virus particles present is called the **burst size**. The burst size can range from 2-2,000 phage depending on the phage-host system and the physiology at the moment (enough food to grow and produce necessary proteins).

Cells can be treated with chloroform any time during the cycle and this kills the cells stopping production of virus without killing the virus. This allows phage counts to be taken at any stage to identify how far production has gone over any time period. If the cells are killed immediately after the virus infects it, then no new phage are recovered because the cell did not have time to produce new particles. The chromosome of the virus cannot infect any further bacteria without its capsid and proteins to enable it to infect another cell.

Temperate phage infect the cell but do not take over the machinery tom produce more copies of itself right away. Viral DNA is replicated with the host DNA due to a common nucleotide sequence in the chromosomes. At this point, the bacteria produces a combined chromosome and is said to be in a **prophage** state. The functioning of the viral part of the gene is repressed so that the bacteria does not produce virus copies. At some later point, such as external signals that the cell may run out of food or is in danger, the genes activate and the lytic cycle of producing more virus particles begins. Until this point, the bacteria are immune to further infection by the same strain of virus which is the virus (parasites) way of protecting its host temporarily.

Most bacteria strains are believed to carry the chromosomes of one or more temperate phages (prophages). All daughter cells carry the DNA for the prophage genome so all descendents will have the ability to go into the lytic phase and produce more phage when the conditions are right. A phage carrying repressed prophage is called a **lysogen** (able to lyse). The lysogens are immune from further infection by the same phage types. These show up on turbid plaques grown on the bacteria lawn. The turbidity results from the outgrowth of the many microcolonies of lysogens. (The temperate phages actually kill most of the host cells so this is a good method of identifying and selecting prophage cells).

Lysogens are not very stable. The repression I spontaneously lost at a rate of about 10 (-3) in any population of cells. By using chemical and radiation mutation, it is possible to produce lysogens that have the repressor gene altered so that it does not enter its lytic phase and the bacteria can survive permanently with part of the viral DNA intact (and mutated).

When one lytic phage has infected a bacteria, it begins the cycle of ordering it to produce copies of itself. If another lytic phage reinfects the cell at some point before cell lysis, it delays the lysis of the cell so that intracellular production of phage continues with

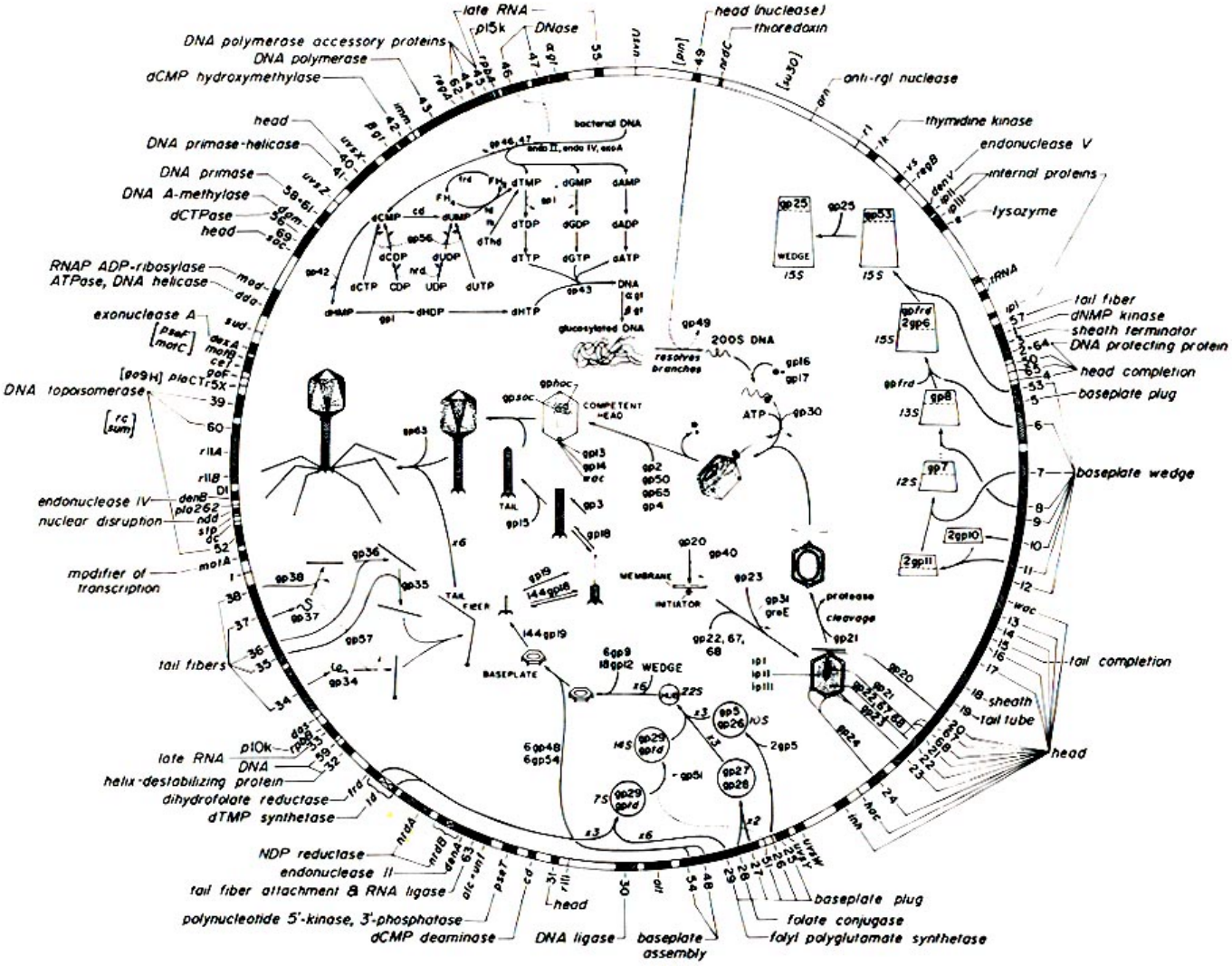
its own chromosomal package. This usually causes very large numbers (burst sizes) to be produced.

By using this method of infecting a bacteria with one phage, and then adding another simultaneously or shortly afterwards, a recombination takes place in which the genetic material is recombined to produce **genetic crosses**. This type of cross is very successful with virus that infect the same cell type and are the same species. They do not work well with different virus species because the chromosome ends do not often have common nucleotides which line up the genes perfectly.

This is the case with Influenza and Aids. If both Aids and Influenza are introduced together simultaneously into a human cell, their chromosomes could mix (and do) and produce a hybrid that could become contagious and transmissible by air. The machinery in Influenza that permits this are long genes (blocks of base pairs) that do not match up well with Aids genes to produce a contagious progeny with the traits of both parents. Mutation, cell fusion and genetic engineering may make this possible in the future.

The mixing of genetic material occurs in the cell because the cell machinery is producing the virus parts and these form a pool of parts. The new virus is assembled with parts from the pool. It may have a new chromosome that does not code for all the proteins necessary to produce the outer coat and then will not be able to reproduce itself in subsequent infections. Some of the pooled genetic combinations may produce new species if the biology is correct. Using same species of virus with different strains, genetic maps can be produced that describe what each gene does by observing the recombination pairings and effects. Mutant phages are often used for genetic mapping purposes.

A map of the bacteriophage T4 is shown below. Each of the genes code for a different function and the entire chromosome in the circle is represented a s a single circular strand. The center of the circle shows the assembly pathways for a mature virus (virion) and the pattern of host DNA degradation and recycling.



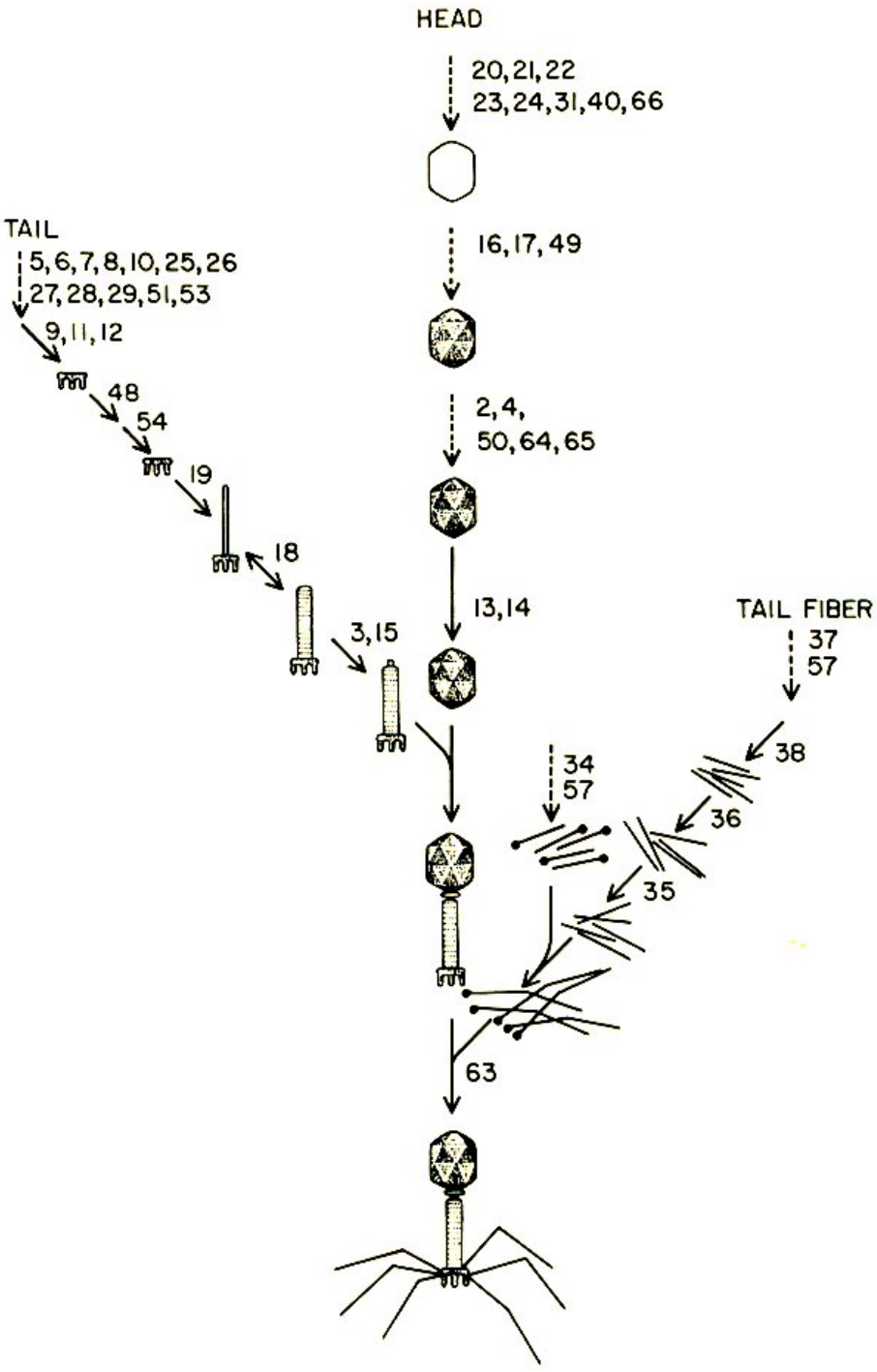
All of these points on this and other genetic maps were discovered by producing mutant versions of the gene. In the case of T4, several thousand plaques are derived from a common ancestor and several of these appear with distinctly larger or smaller or more turbid appearances. The sizes and shapes change with each mutation and most these variants breed true indicating they are true mutants. Some mutants lyse the cells rapidly. These are designated *r* for "rapid lysis". If they can infect cells normally resistant to phage infection they are designated *h* for extended host range. These specialized strains are available from ATCC and other laboratories.

It has also been found that multiple viral parents can be used with each contributing genetic material. Genetic exchange occurs repeatedly even after DNA replication has begun. The DNA floating in the bacterial cytoplasm can be replicated again, it can be packaged into a phage particle to produce the mature virion or it can "recombine" with other DNA before packaging or replicating again. The pool of DNA

molecules remains constant inside the cell during this entire processing and the recombination is generally random with respect to time and partner.

Separate biochemical pathways are used to generate the parts of the virus such as the head, tail, tail fibers and so on. Separate genes contain the instructions for each.

The following chart illustrates how each of the parts are assembled individually and then pieced together –



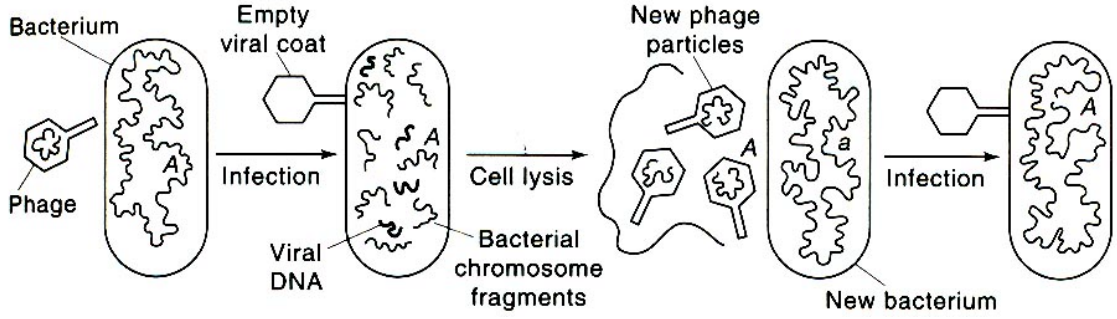
Some phages have only RNA and are very simple. They attach to the sex pilus of only male bacteria. Once its RNA reaches the cytoplasm of the bacteria, it codes for the production of as few as four proteins. These are the coat protein, the maturation protein, the replicase protein and the lysis protein. Sometimes, overlapping proteins which combine parts of the above four are produced. RNA phages are very simple and do not recombine like DNA phages.

Late in the infection cycle, phage DNA is packaged into capsid structures. One of these forms of packaging is called site to site. In this type of packaging, the DNA is cleaved at specific sites with certain DNA sequences rather than the overall size or length of the DNA molecule. When this type of packaging is used, DNA from bacteria with identical starting and ending sites, or DNA sequences are picked up during the packaging process and inserted into the phage capsid instead. When this phage particle leaves the host bacteria cell after lysis, it may find another bacteria and inject this into the host. The host then incorporates this bacterial DNA from the first bacteria into its own DNA. This is a virus can move a gene from one bacteria to another.

This process of moving a "headfull" of DNA from one bacteria to another inside phage capsids is called **generalized transduction**. Any DNA including bacteria DNA, that is present in the host bacteria during the later part of the infection may be enclosed in a capsid. The phage particles that are best at this do not produce enzymes that attack and degrade the host DNA. These are the E-coli phage P1 and S. typhimurium P22. These two species do not produce nucleases that degrade the host DNA so that the host DNA can be present in large amounts during infection and capsid packaging. This is another way that E-coli and other bacteria exchange genes between strains.

These fragments of DNA cannot replicate themselves but can be incorporated into the host chromosome by recombination.

Another form of DNA exchange can take place called **specialized transduction**. Nearby DNA with correct starting and ending sequences can be integrated into the viral DNA. These are rare and carry a mix of both bacteria and viral DNA. These special phages occur at a rate of about 1 per 10,000 of cell populations. These can be selected and recovered by using them to infect bacteria populations and selecting for the desired gene.



In the above drawing, the letter A represents a gene in the first bacteria's chromosome. You can see how it is packaged and transferred via phage and capsid.

When the chromosome is formed in the donor bacteria, it displaces some of the virus DNA that is then lost. The virus loses whatever functions that were replaced. The

maximum amount of bacteria DNA that can be carried is about 5% because that is usually the maximum that will fit in the head of the capsid for these types of phage.

It has also been found that infection with with some prophage also causes changes in the host cell surfaces making them more resistant to infection by other unrelated phage. Usually this is accomplished by a small chemical change in a surface antigen receptor (that also changes its ability to infect humans and not be immediately recognized by the immune system). Salmonella commonly experience this type of phage change. This type of change can prolong the length of infection and in some cases make it lethal (when many changes in antigens occur).

There are also phages that carry antibiotic resistance, and add virulence factors to the host bacterium. Bacteria modified with these phage and then used as part of a multi strain bio-weapon are very superior in ability to infect and injure or kill.

The best example of a temperate phage at work is the prophage of Corynebacterium. When it infects the Corynebacterium diphtheria, the gene to produce diphtheria toxin is turned on. This gene becomes active in an iron deficient environment (the human body) and the toxin is produced and excreted. The toxin causes necrosis of the surrounding tissues (usually in the upper respiratory tract) and the broken down cell material becomes bacteria (and virus) food. This allows more bacteria to grow until the process stops.

Obtaining phage suitable for uses described above is easily accomplished via ATCC and other lab supply companies. Recovering, identifying and selecting the correct phages useful in this regard from nature would be a daunting task for any single professional. This method of modifying bacteria for use as weapons requires a minimal laboratory and understanding of what is trying to be accomplished,

Chapter 6
Genetic Changes Using Plasmids

Plasmids and other segments of intact genes can be transferred between bacteria a number of ways. In earlier chapters we described the discoveries of these methods. Here we will cover them in greater detail.

Transformation

In 1928, Griffith made the first discovery of a mechanism of gene transfer. He observed *Streptococcus pneumonia* which causes classic bacterial pneumonia, and found that smooth (S strain) and glistening colonies of the bacteria were the virulent ones that caused the disease. Under the microscope, these had a polysaccharide capsule which protected the bacteria from host defenses. Colonies of the same streptococcus with a rough (R strain) colony appearance had no capsule and did not cause a fatal infection due to the ability of the mouse immune system to destroy the invading bacteria. If the smooth colony bacteria were killed by heat, they did not cause infection when injected into the mice.

Griffith found that when he injected live R strain and killed S strain bacteria into mice, the R strain converted to S strain and would kill the mice with a septicemia. When he cultured the strain he found that they now produced smooth colonies and had a capsule. Inside the mice, during the course of infection, the rough strain adsorbed the gene from the killed smooth strain and integrated it into its own genes. It could now produce a capsule to protect it from the mice immune defenses. This process is called transformation.

Griffith also learned that he could kill the smooth cells and inject them directly into rough cells and they would convert to the S type. He would also do this in a test tube successfully. He would kill the S strain cells using heat and the cells would lyse. Intact strands of DNA containing 10 or more genes would be released when they cells are killed by heat and recipient bacteria species absorb the DNA by a special uptake process. Cells that can absorb the DNA are called **competent**. Bacteria cells that cannot do this can be made temporarily competent by exposing them to a high concentration of Ca++ ions which cause transient changes in the cell envelope permitting uptake of DNA in surrounding fluid.

Some bacteria become competent with a change in temperature such as B. subtilis which becomes competent when taken from 42C to 37C in culture. The number of competent cells in a culture can range from 15% in B. subtilis to 100% in S. pneumoniae. Competent S. pneumoniae cells have a new protein antigen on their cell surface. This competence factor is released into the surrounding medium and can make cells competent that it comes into contact with. This requires a high cell density 10(8) per ml.

When this process occurs, other genes are also exchanged and transferred from the donor cells. DNA nucleases also exist inside of recipient cells which break down free DNA. This makes the transfer and survival of the desired DNA a rare event. Many thousands of cells are treated to produce one with the desired trait for virulence. In the case of the mice, thousands of cells were injected and all were killed by the immune system except the one rare strain that obtained the virulence gene. This one strain multiplied by the millions until it eventually killed its host. [This is the concept behind the bell curve weapons-You do not need thousands of cells or spores to initiate successful infection, you only need the right one and nature selects it for you.]

Once a cell becomes competent, it develops up to as many as 50 new DNA binding sites on its cell surfaces. Any type of DNA can bind to these sites including human, plant and viral DNA. The DNA is cut by an enzyme into segments about 15,000 bases long and is then drawn into the cell. The cutting enzyme requires magnesium or calcium ++ ions to perform this task artificially.

While outside, the DNA forms an **eclipse complex** which then interacts with chromosomal DNA. As much as 25% of this donor DNA eventually becomes part of the recipient DNA. A single strand of DNA is formed and then extruded into the interior of the cell. This process protects the strands from DNA nuclease enzymes inside the cell. This single strand is directly substituted for one of the DNA strands of the host DNA. Transformation frequency is low when the DNA is not homologous. [This means that base pairs do not line up from both types of DNA to produce a working set of genes in the offspring.] When the genes line up satisfactorily, there can be many successful transformations.

Haemophilus influenza cultures become competent without an exterior binding protein being produced when placed on a starvation medium that severely limits growth. Once rich medium is added competence is quickly lost. The competent cells are selective in the type of DNA that they take in. This is due to a minimum binding sequence" of 11 base pairs. DNA lacking this binding sequence are not usually admitted into the cell. H. influenza has a series of membrane extrusions that appear on the cell surface. The DNA is cut on the cell surface and is then transported into the cell membranous extrusions (not the cytoplasm) where they bud off to form independent vacuoles containing double stranded DNA. The vacuoles are then released into the cytoplasm. The single strand DNA is processed to yield gaps so that short segments are used in the recombination process.

Cells that have lost part of their cell walls can take up DNA and be transformed by it. When cells are shocked (exposed) to high concentrations of Ca++ ions, they become competent. The addition of cobalt chloride and dimethylsulfoxide enhances the effect. The procedure is done with the cells "on ice" and the transforming DNA is added to the chilled solution. A time period of 20-60 minutes is allowed for the DNA to be drawn up onto the cells and then the solution is warmed to 37C and then up to 42C for 90

seconds to heat shock the cells and stimulate uptake and entry of the DNA into the cell. As many as 95% of the cells are killed by the treatment.

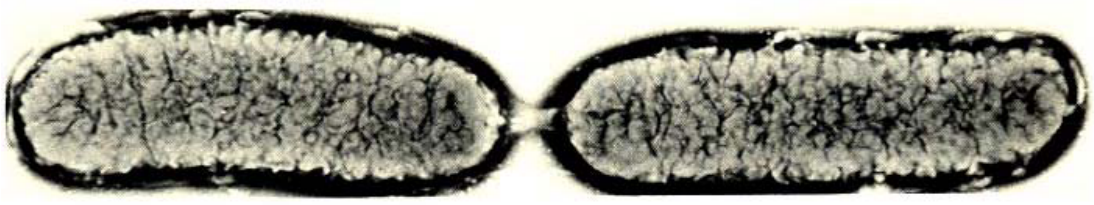
Conjugation

Bacteria transfer genetic material between themselves in a manner similar to humans. They use a sex pilus for a male strain of a bacteria to transfer DNA to a female strain of bacteria. This process was discovered by Joshua Lederberg who developed the following process.

1. Intact living donor and recipient cells were used. Close cell contact between the cells was required.
2. E-coli strains used in the experiment would be classified as F+ (for fertility or male strain) and F- (for recipient or female strains). It was found that F+ and F- recombination's occurred in which genetic material was exchanged. In rare instances F+ and F+ would combine to produce recombinants. F- and F- never produced exchange of genes.
3. When mutants of F+ and F- were crossed, most of the characteristics were those of the F- strain which indicates that the genetic contributions of the parents is unequal and favors the female strain.
4. The fertility characteristic (making an F- into an F+ in F- descendents) was transmitted at a high frequency while other characteristics were transferred at a low frequency.

It was discovered that the fertility characteristic was associated with a small extra chromosome called the **F plasmid** which is about 2% of the size of the entire E-coli chromosome. The F plasmid floats in the cell cytoplasm and replicates independently of the bacterial chromosome. Each bacteria F+ cell has one or two copies produced with each of its own chromosomes. When the cells divide, an occasional daughter cell fails to acquire the F plasmid resulting in an F- offspring. This occurs in about 1 per 100-1,000 descendents.

A set of genes on the F plasmid codes for about 30 proteins some of which produce an F pilus which can make contact with receptor cells on the surface of F- cells. This causes the two cells to form a conjugational bridge.



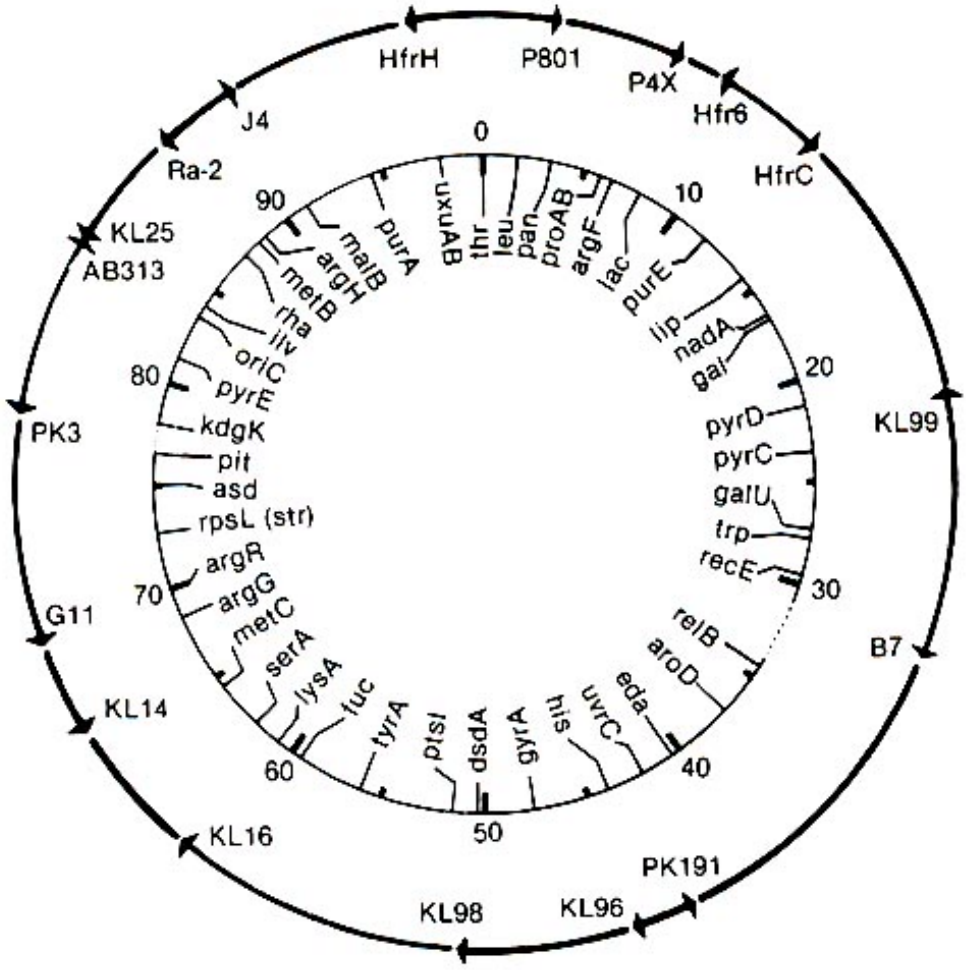
When a small number of F+ cells are added to a large liquid culture of F- cells, almost the entire batch will convert to F+ overnight. This is because the F plasmid is self transmissible and it mediates the transmission of DNA between F+ and F- cells. When conjugation takes place, an F plasmid is driven from the donor F+ cell through the pilus and into the recipient cell. It is replicated in the recipient cell which now has two complete F plasmids. A remaining strand of F plasmid in the donor is also replicated at this time so that it also has a second strand. This second strand or copy of DNA is called a **concatemer**.

It is possible for mating to take place on well mixed solid surface cultures. Some types of plasmids are only exchanged this way and have been observed doing so on membrane filters.

Mutants strains of F+ cells have the ability to transfer other genes besides the F plasmid to recipient cells. Mutants which transfer the bacterial chromosomes at high frequency with the F plasmid are called **Hfr strains** (for high frequency). These strains do not seem to contain the F plasmid as an independent gene and rarely convert F- to F+ cells. The Hfr strains do carry all the F plasmid genes intact in its chromosome. This complete chromosome is much larger than the F plasmid so it takes about 2 hours for the chromosome to be transferred from an Hfr cell to an F- cell. The conjugation bridge between the mating pairs is not very stable so they often break and the chromosome transfer is usually partial. This results in a partial gene transfer. By interrupting the gene transfer at different intervals, the recombinants can be studied to discover which genes were transferred in which order and what each one does.

When gene transfer is interrupted at time intervals to discover which genes have been transferred and in what order, it is called **mapping by interrupted mating**.

In the map below, the genes for E-coli K-12 strain are listed. On the inner circle are numbers from 0-90 (100) which represents the app. 100 minutes it takes to transfer the complete chromosome by conjugation from the donor to the recipient. The arrow tips on the outside ring indicate the first DNA transferred by Hfr strains in transfer experiments.



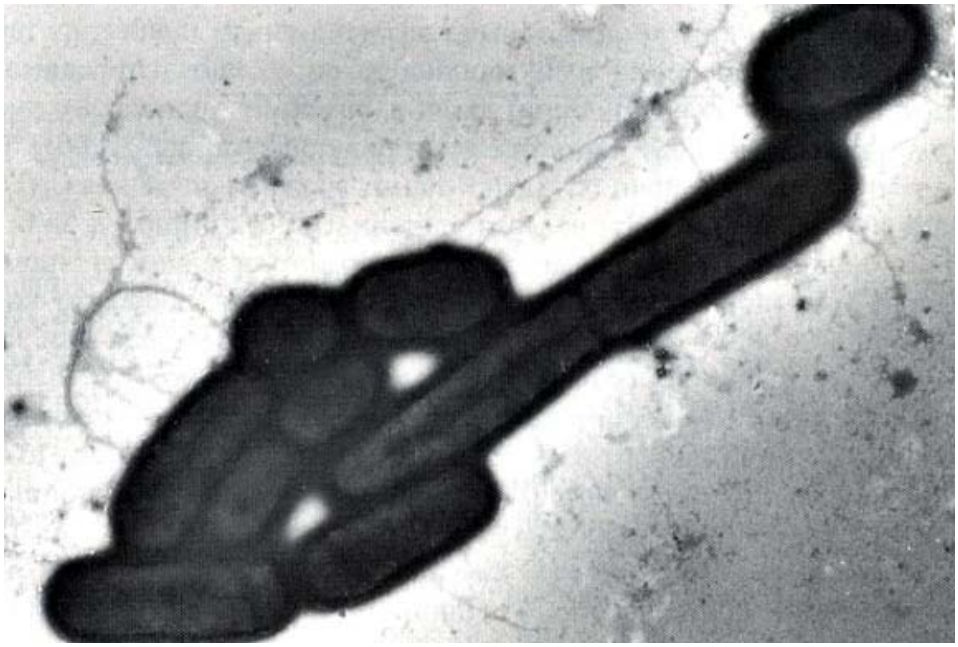
Genetic maps are constantly being changed by mutation and evolutionary processes. Sometimes these changes can be dramatic and involve up to 35% of the map while others are not noticeable.

Certain genes are always first in the transfer and they will appear in most recombinants. Genes are often lost in the crossover due to degradation, dilution and the action of nuclease enzymes. The order of gene transfer during conjugation is consistent with the above circular map and the one in the previous chapter. The genes on this map can be inserted clockwise or counterclockwise so that one part of the circle is injected first in one transfer but may be last in the next transfer.

The effect of the mixing of the gene pool is that many different Hfr strains can be produced once the F plasmid is integrated into the bacterial chromosome. The reason that the F+ gene is rarely transferred to a recipient cell is that most of the F plasmid is located at the end of the DNA circle and is the last gene sequence to be transferred. If the transfer is not complete, genes from the F plasmid are lost and the cell remains F-.

Occasionally, an aberrant crossover occurs in which the F plasmid is broken off from the main DNA chromosome and carries with it some of the DNA of the cell. This broken off strand of F plasmid has all the machinery instructions to function as a normal F plasmid in F+ strains but it also now carries some additional chromosomes from its parent. These strains can now transfer large blocks of DNA (up to 20% of the genome) during conjugation. This is often large enough to transfer significant machinery from one cell to another. [In the case of anthrax for example, it could carry the complete toxin instructions from a toxin producing strain to one that does not produce toxin and convert it.]

F pili are only produced on the cell surfaces when the cultures are growing above 33C in liquid media. The pili disappear when the cells are grown at lower temperatures or when they enter the stationary phase of growth. Donor cells that lack F pili due to these conditions act like recipient cells during conjugation experiments. The mating between F+ and F- and Hfr cells usually occurs in aggregates of the cells as shown below-



Plasmids

The F plasmid is not the only plasmid produced in bacteria cells. Other plasmids exist which are circular, double stranded, extra-chromosomal DNA molecules that have specialized genes and instructions to self replicate in the cell. They replicate with cell division and frequently exchange themselves with other bacteria of their own species and occasionally with other species. Transposons are associated with plasmids that allow for recombination so that plasmids can change their makeup with new genetic material.

Plasmids which transfer antibiotic resistance were discovered about 40 years ago in Japan during an epidemic of *Shigella* dysentery. Four antibiotics were in wide use-

streptomycin, chloramphenicol, sulfonamides and tetracyclines. Strains resistant to all four drug classes became quite common (70-80%) by 1971. This pattern of multiple drug resistance could not be obtained in the laboratory by mutating sensitive strains. It was discovered that a multiply resistant strain of harmless E-coli was donating its resistance to the dysentery bacilli by conjugation. It was found that plasmids carried the resistance factors for all four antibiotic classes and these were called **R plasmids**. It was also found that this particular plasmid combination could transfer between E-coli, Klebsiella, Proteus, Salmonella and Shigella but not Pseudomonas. The plasmid also produced F-pili.

When bacteria with drug resistant plasmids are grown in culture, some of the descendents may lose one or more of the resistant genes due to mutation and recombination. In order to maintain strains with all drug resistance, all the antibiotics must be maintained in the culture medium.

Plasmid carried antibiotic resistance is much more common in nature than mutation of the chromosome drug resistance. This is because plasmids code for large proteins which inactivate or attack the antibiotics. Mutations usually modify an essential drug target site in the bacteria. It is difficult to produce multiple antibiotic resistance by mutation alone because the combinations needed to effect all the sites under attack all at the same time is nearly impossible. The plasmids can instruct the cell to produce substances which counterattack the antibiotics making the bacteria much better protected.

Mutation alone almost never produces new enzymes that will attack antibiotics. Nature has had millions of years of evolution and recent selection in modern hospitals to do just that. The ability to detoxify an antibiotic may have originated in the soil since bacteria in the soil are the source of most known antibiotics. [Nature conducts warfare and weapons development daily at the level of the bacteria].

Hospitals which use antibiotics to treat skin infection are the richest source of R-plasmid containing bacteria. Livestock herds fed antibiotics are another good source. These bacteria can be used as donors for weapons grade bacteria. The bell curve weapons can be enhanced by the inclusion of large numbers of plasmid carrying strains with various toxin, virulence and antibiotic resistance genes.

R plasmids code for proteins which inactivate antibiotics. Some R plasmids have been discovered which produce proteins that protect bacteria from heavy metal toxins like mercury, lead and silver. Bacteria growing in water with photochemical pollutants have been found with plasmids that code to reduce silver ions to a harmless metallic silver form. This plasmid is passed between bacteria species allowing them to live in pollution of this type.

Another class of plasmids are those with virulence factors. Virulent E-coli strains can have the ability to produce pili which allow them to attach to and colonize the upper intestine. They also have the ability to produce and excrete a toxin that damages intestinal

tissues. Both of these traits are borne on different plasmids. One or both can be transferred during conjugation.

Some bacteria produce plasmids called **bacteriocins**. These are a special class of toxins that the bacteria produce that kills other bacteria around it but not itself. The host bacteria is protected from the toxin action while members of its same species or other species without the protective gene are killed. There are many of these toxins. In E-coli the E1 bacteriocin damages the cytoplasmic membrane, E2 attacks DNA, E3 cleaves ribosomal subunits and thereby stops protein synthesis. In E-coli the toxins are called colicins while in Pseudomonas they are called piocins and so on. The same bacteriocin can be produced in slightly different forms by different strains of the same species.

All bacteriocins form two groups of similar acting agents. The first is represented by the colicins which are produced as a pure protein molecule or a molecule attached to the outer membrane of the producer cell. It is often found complexed at a surface antigen of the host cell. Pyocin R from *Psuedomonas* closely resembles the structure of a bacteriophage except it has no DNA associated with it. It has been assumed to be the remnants of a defective prophage.

Bacteriocins are produced all the time in mixed culture growths (and in pure growths as well). They inhibit the growth of other bacteria around them that are not resistant to this particular substance.

All these plasmids confer on the bacteria improved survival. Many of these are produced in rare environmental conditions and are often lost to the bacteria as conditions change. The plasmids are reproduced at different time intervals in the cells and the same number of copies may not be made in each cell during cell division. This results in occasional daughter cells losing one plasmid while retaining another. F plasmids generally exist at 1-2 copies per cell but some may have many copies. Plasmids other than F plasmids may be copied many times. Some plasmids in the mold *Streptomyces* may have copy numbers as high as 800. The plasmids in these cultures usually cause “pock formations” from a delay in the production of aerial mycelia and spores.

Some of these plasmids transfer at rates different than the F plasmid and may only be transferred by conjugation in about 1 per 1,000 cells. Others may transfer to nearly all other nearby cells. Some small plasmids are not self transmissible but can transfer over the conjugation bridge with other plasmids. Others are ready made for packaging and transfer by bacteriophage.

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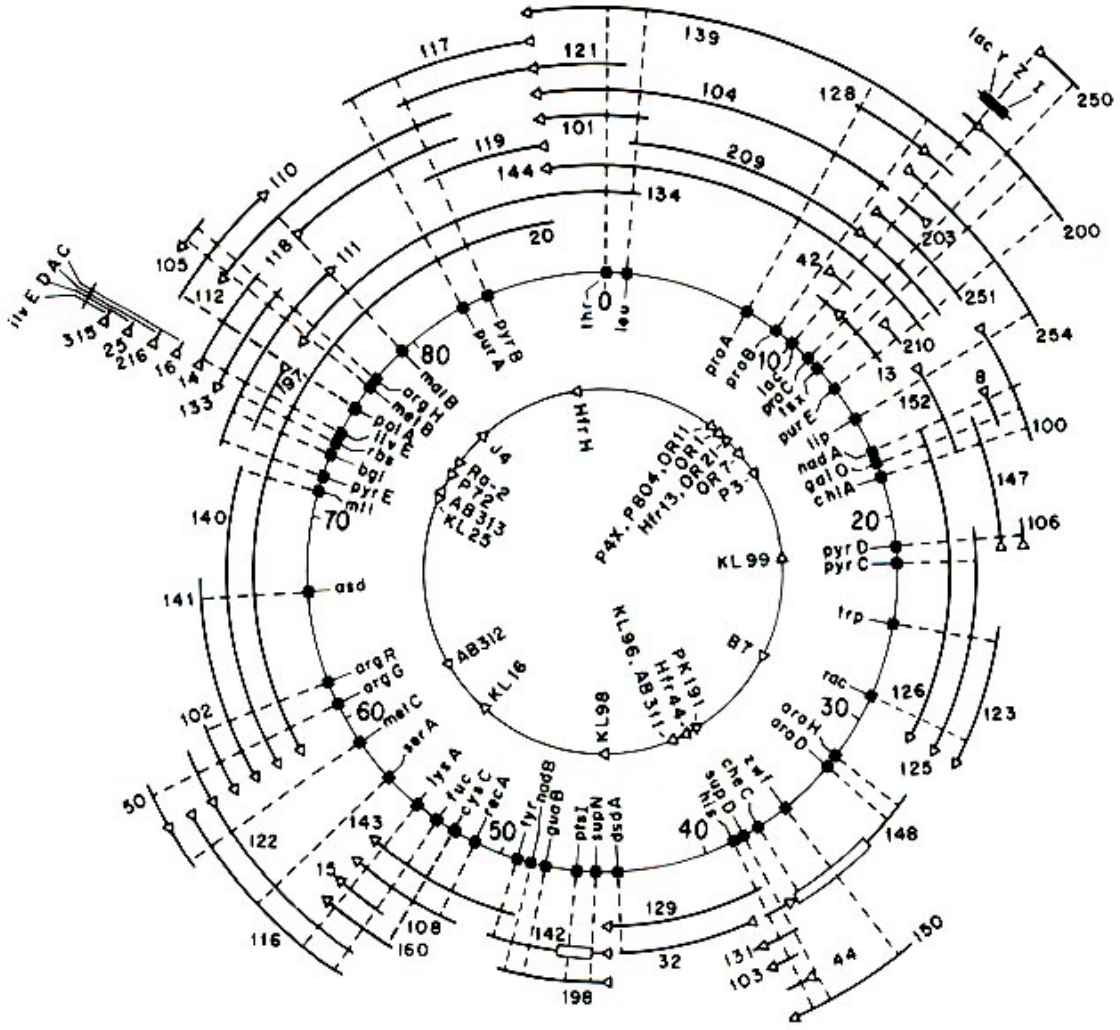
There is a preference for shorter plasmids to be selected in nature and produced in cells from larger plasmids. This is due to large plasmids retarding effect on cell growth. Shortening of the plasmids occur from recombination events.

E-coli are the most popular species for plasmid exchange and genetic cloning experiments. This is due to its ability to be transformed by a wide variety of plasmids from many different bacteria species. The entire E-coli genome can be carried by

different plasmids and the chart below shows the F plasmids that carry extra chromosome information. These are represented by each of the segments with an arrow showing the point of origin from the original Hfr strain. The dashed line which extend out from the genetic markers indicate the approximate termini for each of the F plasmids. The black rectangles represent deletions from the original DNA. As you can see, the entire E-coli genome is carried by different plasmids and can be exchanged among E-coli constantly.



E-coli are reported to have more than 300 naturally occurring plasmids found among its species. Some of these produce no visible effects on the bacteria (or other species) and are called **cryptic plasmids**. Most plasmids are capable of mobilizing part and in some cases all of the bacteria DNA for transfer under the right circumstances.

The Hfr gene can be transferred from E-coli to other enteric bacteria such as S*almonella* species. By transferring the F plasmid from E-coli to S. typhimurium a new Hfr Salmonella cell can be produced. The genetic map is longer for Salmonella than E-coli and takes about 135 minutes to run instead of 100 minutes. Many of the parts of the

two genetic maps are homologous (easily recombined because they code for similar proteins and have the same starting and ending sequences).

The genus Pseudomonas has a great nutritional diversity among its species. It harbors many types of plasmids and it has been determined that every Pseudomonas harbors at least one prophage. The genome size is smaller than that of E-coli at about 75 minutes.

The genus Streptococcus has some conjugative plasmids that also direct the production of a pheromone that elicits mating aggregates to form around the plasmid carrying cell. This pheromone increases plasmid transfer by about 200-300%.

Agrobacterium are unique in that they transfer DNA from their own bacteria genome to the plant that they inhabit. In the case of *Agrobacterium tumefaciens*, a gall or tumor develops near the crown of plants that it infects. If the bacteria does not carry the tumor inducing plasmid, there is no tumor produced. It has been found that a small portion of the DNA from the plasmid is actually incorporated into the plant DNA causing formation of the tumors during infection by the bacteria. This was the first known example of prokaryotic DNA being transferred to the eukaryotic cells. It is an exception to the rule that genetic exchange in nature usually occurs only between closely related species. The bacteria uses amino acids produced by the tumors as food and are a parasite to the infected plant. This particular example has led to many interesting genetic engineering experiments between plants and bacteria.

Plasmids in general use host machinery to replicate and function. The larger plasmids may provide a few of these functions via its own DNA. The dye acridine orange will cause the plasmid to be lost to the cell if treated for a long period. Heating cells will also eliminate plasmids such as growing E-coli above 42C but below 46C which kills the bacteria. Plasmids can be mutated into runaway replication in which it continuously copies itself until it fills up the cell.

Some plasmids produce substances which prevent other plasmids from functioning inside a cell at the same time. In some cases, conditions occur in which these same plasmids will permit a plasmid with a different part of the bacterial chromosome in its makeup to co-exist.

Sex pili produced by plasmids can be broken off by treating a culture with a detergent (sodium dodecyl sulfate-SDS). This detergent is used over time to gently break apart the cell wall in a blending machine. This also releases the internal plasmids from inside the cell so they float in the medium. These are then added to living cultures as donor DNA.

Plasmids arrange themselves so that they are equally distributed in the cell so that each daughter cell receives at least one copy during cell division. They regulate their copy numbers so that the host cell does not use all cell resources solely for plasmid

copies. This allows the cell enough resources to grow and divide itself while still supporting copies of the plasmids.

Field Experiment

One of the best way to teach these principle is to obtain a manure sample from a livestock population on a farm that includes antibiotics in its feed. All the bacteria should be resistant to the antibiotics that are fed. These bacteria can be grown on Jell-O or agar and recovered as mixed colonies on a plate.

Soil bacteria (gram negative-enteric) can be grown to provide "natural" strains which can be killed by the antibiotic. These are tested colony by colony to see which ones will not grow in the presence of an antibiotic disc (antibiotic used on the farm).

A colony from the antibiotic resistant farm sample is then grown in a liquid medium with a colony of the antibiotic sensitive strain. If the antibiotic resistance is plasmid generated, the antibiotic resistant strain should donate a plasmid for this resistance to the other species in some of the cells. By plating the mixed colonies in the presence of the antibiotic (via a disc or mixed in the solid medium), you should be able to see if the antibiotic sensitive strain obtained an antibiotic resistant plasmid. If you do not have medium that allows you to see color changes in the bacteria, you can most often tell the strains apart by colony morphology (see Volume 6-A).

The above method is a simple way of obtaining antibiotic resistant plasmid carriers. Samples can also be obtained from hospitals and culture collections (ATCC). Virulence factors can also be obtained and screened for via live experiments in mice.

In the case of multiplier effects and bell curve weapons, these can be delivered directly as mixed weapons. Those cells carrying plasmids and virulence factors can be mixed into the primary populations as part of the mixed weapons. These may be less effective overall than selected strain weapons but they are also harder to defend against as each separate target may be affected with a different pattern of virulence, antibiotic resistance and vaccine resistance. These characteristics can be passed between cells during infection and the combinations of cell types allow for many recombinant possibilities.

These types of weapons could also be called combination and selection weapons since the environment inside the target selects for the most effective combinations of genetic material and characteristics. Although theoretical, these weapons should include plasmid carriers for antibiotic resistance and virulence factors as well as separately produced mutant populations. This allows for the greatest degree of core genetic material to be provided in the delivered mix.

Such a weapon would consist of prepared mutant cultures of the desired original species and strains. Plasmid carriers would be mixed into the weapon prior to use (distribution). Alternatively, experiments could be done comparing the delivery of both

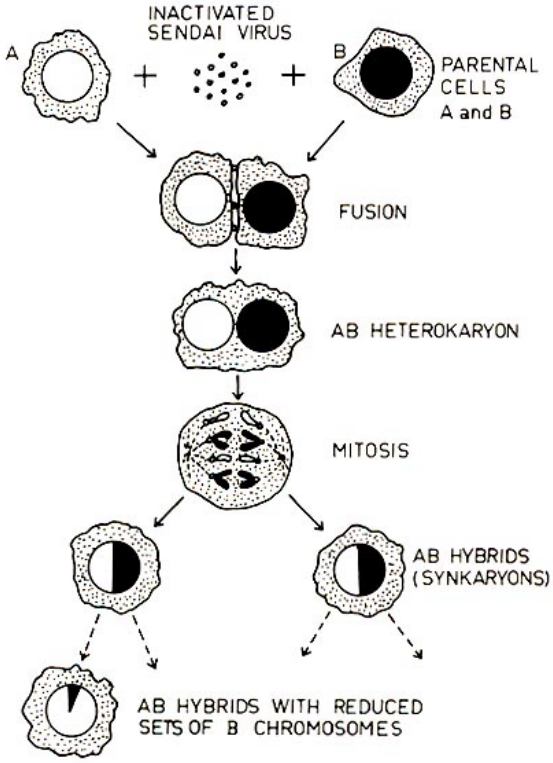
sets of organisms and a batch in which the plasmids were already exchanged with the mutant populations. [An interesting set of combinations of experiments for some future scientist to earn a PhD with].

Chapter 7
Cell Fusion & Hybridization

A wide variety of human, plant, animal, insect and microbial cells have been used in the last fifty years as parents to produce new types of organisms. Through a process of spontaneous or induced **fusion**, cells of virtually any type can be combined to yield a **cell hybrid**. Cells from different species have been fused (mouse and man, rat and mosquito, etc.) as well as different cell types of same species (mouse fibroblasts and mouse lymphoblasts).

Spontaneous fusion of cells occurs rarely in laboratory cultures and occasionally in living organisms. The earliest successful lab procedure for inducing cells to combine (fusion) utilized inactivated *Sendai virus* in a culture containing the different cell types. Using this procedure, two groups of multinucleate cells (**polykaryons**) are produced. One of these groups are called **homokaryons** and have nuclei from the same parental type while the other is called **heterokaryons** and have nuclei from both parent cell types and only these are the cell hybrids.

Once a heterokaryon cell is formed, it either grows in a culture medium, dividing into two mononucleate cells, or it dies. The daughter mononucleate hybrid cells cells are called **synkaryons**. Many of these have been found to be capable of cell division over long periods of time. The general process is illustrated below –



When synkaryons are produced in culture, they can sometimes be obtained in pure populations because they grow at a superior rate to their parents (hybrid vigor) and overrun the medium. In other cases, they grow slowly and tend to be overwhelmed by their parents growth. In the latter case, the cells are separated out by selective culture techniques using a growth medium that only allows hybrid cells to grow. They can also be separated mechanically by various techniques. Single cells can then be selected for producing clones which ensures pure populations of the hybrid cells.

Fusion has been used to learn much about cellular biology. Cells exhibiting or lacking known properties have been fused together to reveal their inner workings (which parts of the cell control a specific characteristic). Fusion has also been used to produce new biological agents for warfare. Defecting Soviet scientists reported that they fused Ebola and smallpox virus during the 1990's to form a new hybrid capable of a general depopulation of the planet.

Virus induced fusion could also fuse cytoplasms lacking nuclei (chromosomes) with other cells containing nuclei. Bare chromosomes have also been fused with anucleate (no nucleus) cytoplasms and even whole cells.

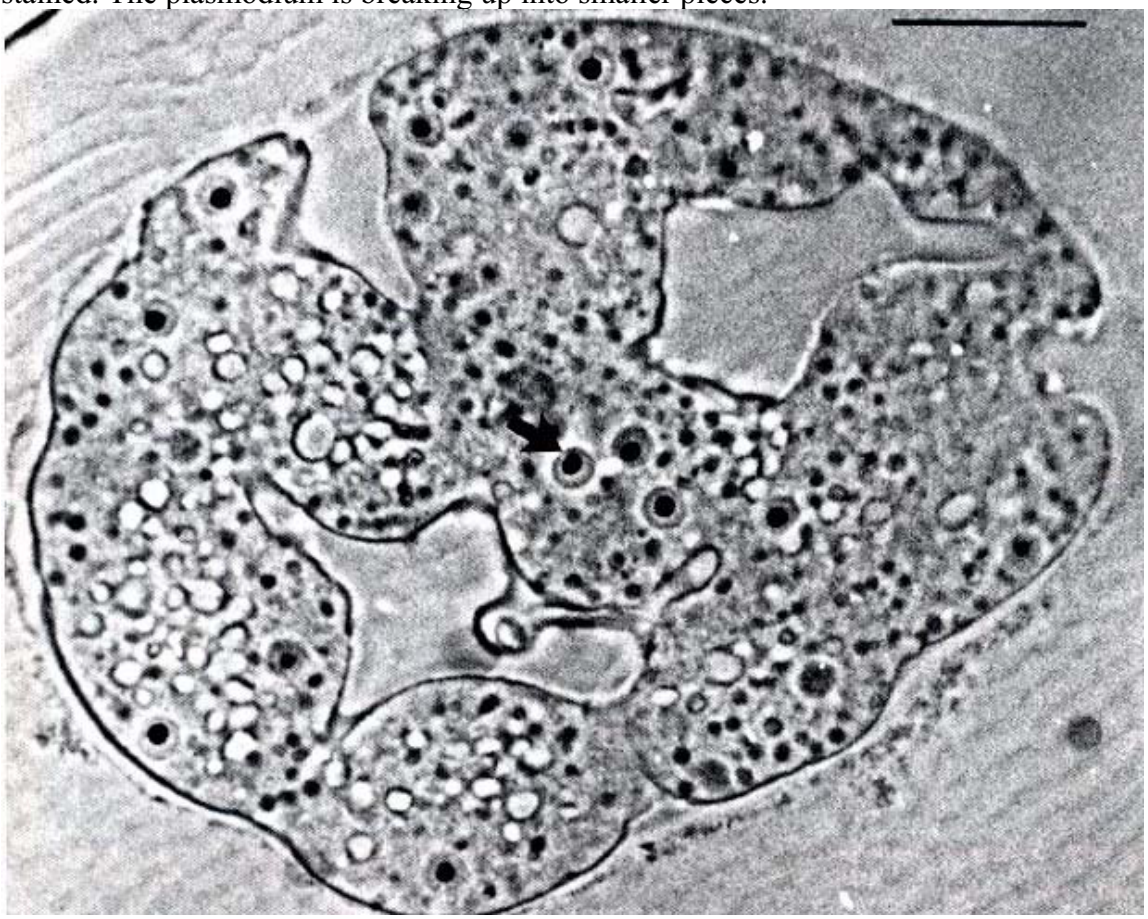
The long lived and proliferating synkaryons from different species of parents usually experience a reduction in chromosome numbers as they divide. Same parent hybrid cells usually retain the original number of chromosomes. In a human and mouse hybrid, the human chromosomes are gradually eliminated over time and this rule generally applies to many cell combinations. The chromosome of one of the parents is eventually segregated out. Knowledge of this allowed scientists to identify which chromosome (in mouse-human hybrids) would produce certain human proteins when it was the last human chromosome remaining in the hybrid.

Tumor cells have also been fused with healthy cells in efforts to locate the particular chromosomes which carry the instructions for malignant growth. This technique has also been used to locate cancer inducing virus (prophage) by activating the genes for the tumor growth in normal cells fused with the cells containing the dormant tumor inducing virus.

History of Cell Fusion

Polykaryons were observed and reported in the 1800's and were usually seen in microscopic sections from a variety of disease tissues including tuberculosis, variola, rubeola, and varicella. In 1868, Langhans described his theory that polykaryons may rise from fusion of preexisting different cell types (as well as being formed from cell division-mitosis). In slime molds, polykaryons can be formed by mitosis and these cells are called plasmodium. Among higher organisms, cell fusion is rarely found without being associated with disease.

In the photo of a plasmodium below, there are twelve nuclei (arrow) darkly stained. The plasmodium is breaking up into smaller pieces.



In 1937, Michel used a mechanical technique for using plant protoplasts to produce short lived heterokaryons from cabbage and onions as well as cabbage and algae.

The modern age of discovery in cell fusion began in 1960 when a group of scientists found cell hybrids that formed in lab cultures spontaneously. During 1957-1962, another scientist (Okada) used ultraviolet (UV) inactivated Sendai virus to induce cell fusion and generate the first homokaryons in the lab. By 1964 the scientists were using mutants and selective media to isolate specific hybrid cells and in 1965, Okada and Murayama use the inactivated virus to produce the first heterokaryons. That same year, the first proliferating synkaryons were produced by spontaneous fusion.

1965 Harris and Watkins (1965) and, independently, Okada and Murayama (1965) produce the first interspecific heterokaryons using inactivated Sendai virus. Ephrussi and Weiss (1965) produce the first interspecific proliferating synkaryons by spontaneous fusion.

1966 Yerganian and Nell (1966) demonstrate that Sendai virus can be used to produce proliferating hybrids. Davidson, Ephrussi, and Yamamoto (1966), using melanoma and unpigmented cells, demonstrate that a phenotypic marker of histiotypic differentiation (i.e., pigmentation) can be selectively extinguished in hybrids.

1967 Weiss and Green (1967) discover preferential elimination of human chromosomes in man–mouse hybrids and demonstrate that this phenomenon can be used for gene assignment in man. Watkins and Dulbecco (1967) and, independently, Koprowski *et al.* (1967) demonstrate virus rescue when active SV40 particles emerge from hybrids derived from transformed cells and permissive ones.

1969 Harris, Klein, and their colleagues (Harris *et al.*, 1969a) begin experiments showing that malignancy need not dominate in synkaryons derived from oncogenic and normal cells.

1970 Lucy and his colleagues (Lucy, 1970) begin experiments with lysolecithin-induced fusion. Cocking and colleagues successfully fuse plant protoplasts (Power *et al., 1970).*

1971 Ruddle, Bodmer, Miller, Siniscalco, Bootsma, and their co-workers begin the systematic use of cell hybridization as experimental system for mapping human chromosomes (for references see Chapter XIII).

1972 Carlson, Smith, and Dearing (1972) produce an interspecific hybrid plant from fused protoplasts.

Since 1972, an explosion of advances have taken place with entire periodicals devoted to the reporting of advances in the field.

Most of the earlier discoveries were made because the hybrid cells happened to outgrow their parents. In 1964, scientists began to develop media that would inhibit either parent strain of hybrids because both would lack a different particular enzyme, but some daughter strains would inherit the enzyme producing genes (one from each parent) and would be the only cells that could grow on the selective media. This process was tedious and complex, and yet successful. An example of a weapons selection of this type would be the fusion of anthrax bacilli and the mold that causes athletes foot. Daughter cells would be screened for the ability to infect skin tissues and simultaneously produce the anthrax toxin. Both sets of genes would have to be present in the cells to do both simultaneously.

In 1965, the first hybrids from different animal species were produced using rat embryo cells and mouse L cells. This same year, Sendai virus, dead, alive, and

inactivated (all three forms) were used to produce polykaryons from cells in culture. Human HeLa cells were fused with tumor cells from mice. Human cells were also fused with mouse and pig cells in separate experiments as well. The next year, long lived strains of synkaryons were discovered and the use of inactivated virus became the standard laboratory method.

These advances were important because the genomes of both parents were expressed in the daughter cells, and the long lived interspecies hybrids would tend to slowly lose one parents chromosomes one at a time. This allowed for the mapping of the characteristics of each chromosome as it was lost.

From 1970 onwards, efforts were made to find other techniques to produce cell hybrids. Their was always a risk of infection with Sendai virus not completely inactivated so experiments began using high calcium ion concentrations which had already been successfully used to create leaky cell membranes and transfer phage and plasmids. In the 1970's polyethylene glycol and derivatives of lipids were successful in yielding hybrids. Plant cells were also be fused by removing their cell walls and using inorganic salts and other agents in treatments.

It was also discovered in the 1970's that fusion of malignant and normal cells did not always produce hybrids in which malignancy was dominant. This led to a discovery in 1972 that tumor causing cells fuse with normal human cells in the body during cancer with a high frequency. It was also discovered that the drug cytochalasin B could be used to treat cells and remove the nuclei only via the process of **nuclear extrusion**. These enucleate cells could then be fused with other cells using the Sendai virus. The nuclei that were removed had a thin layer of cytoplasm and plasma membrane surrounding them. It was found that they could be fused in this form with virus particles to produce cell and virus hybrids.

Lab procedures for nuclear extrusion and fusion are located in the following-

Ladda & Estenson (1970), *Proc. Natl Acad. Sci.* ***67,*** 1528-1533
Poste & Reeve (1971), *Nature (London), New Biol.* **229,** 123-125
Poste & Reeve (1972), *Exp. Cell Res.* **73**, 287-294

Ege, T., and Ringertz, N. R. (1974). Preparation of microcells by enucleation of micronucleate cells. *Exp. Cell Res.* **87**, 378–382.

Ege, T., and Ringertz, N. R. (1975). Viability of cells reconstituted by virus-induced fusion of minicells with anucleate cells. *Exp. Cell Res.* **94**, 469–473.

Ege, T., Carlsson, S-. A., and Ringertz, N. R. (1971). Immune microfluorimetric analysis of the distribution of species specific nuclear antigens in HeLa–chick erythrocyte heterokaryons. *Exp. Cell Res.* **69**, 472–477.

Ege, T., Zeuthen, J., and Ringertz, N. R. (1973). Cell fusion with enucleated cytoplasms. *In* "Chromosome Identification" (T. Caspersson and L. Zech, eds.), pp. 189–194. Academic Press, New York.

Ege, T., Hamberg, H., Krondahl, U., Ericsson, J., and Ringertz, N. R. (1974a). Characterization of minicells (nuclei) obtained by cytochalasin enucleation. *Exp. Cell Res.* **87**, 365–377.

Ege, T., Krondahl, U., and Ringertz, N. R. (1974b). Introduction of nuclei and micronuclei into cells and enucleated cytoplasms by Sendai virus induced fusion. *Exp. Cell Res.* **88**, 428–432.

Ege, T., Zeuthen, J., and Ringertz, N. R. (1975). Reactivation of chick erythrocyte nuclei after fusion with enucleated cells. *Somatic Cell Genet.* **1**, 65–80.

Ege, T., Sidebottom, E., and Ringertz, N. R. (1976). Preparation of microcells. *Methods in Cell Biol.* **15**

The applications for new weapons with varying properties was obvious. Harmless bacteria cells that live on human skin could be fused with highly contagious species such as plague. Cells that slowly lose the chromosomes for the plague would infect only a small portion of a target population before the loss of the virulence genes and would "burn out" before spreading across the planet. These self destructing infectious agents could, ideally, selectively wipe out a narrow geographic area without posing a threat to the attacking country. This is the "stuff" of science fiction novels made real by modern science. These sciences translate directly to an interesting combination of revolutionary, political, military and in some cases even evolutionary power.

The military did not pursue these types of weapons because they could not be stored for long periods once they were produced. An interesting concept would be the use of binary (trinary, etc.) weapons in which the component parent cells and the fusing chemicals are stored and ready for use as specific packages. The time for fusion and release would be slower than ordinary biological weapons, but that can be advantageous in invisible warfare as well (especially when time is required for large scale covert delivery). The weapon would be a mix of hybrids with potentially unpredictable effects and the military tends to avoid these types of weapons and warfare concepts.

Another potential weapon is one in which a human cell has its nucleus removed and foreign weapon DNA is introduced. This human cell has surface antigens which tell the donor person that it is part of him and should not be attacked by the bodies defenses (like cancer cells). If the weapon DNA is an anthrax or other toxin producing nucleus, it can produce anthrax toxin and release it inside the host as it reproduces itself and spreads. This ensures killing the host. This type of weapon would kill only the donor since his cell would likely be identified as foreign by any other human body. This makes it possible to take a cell from a target (hair, blood, muscle, etc) and produce weapons that are specific only for a single human target.

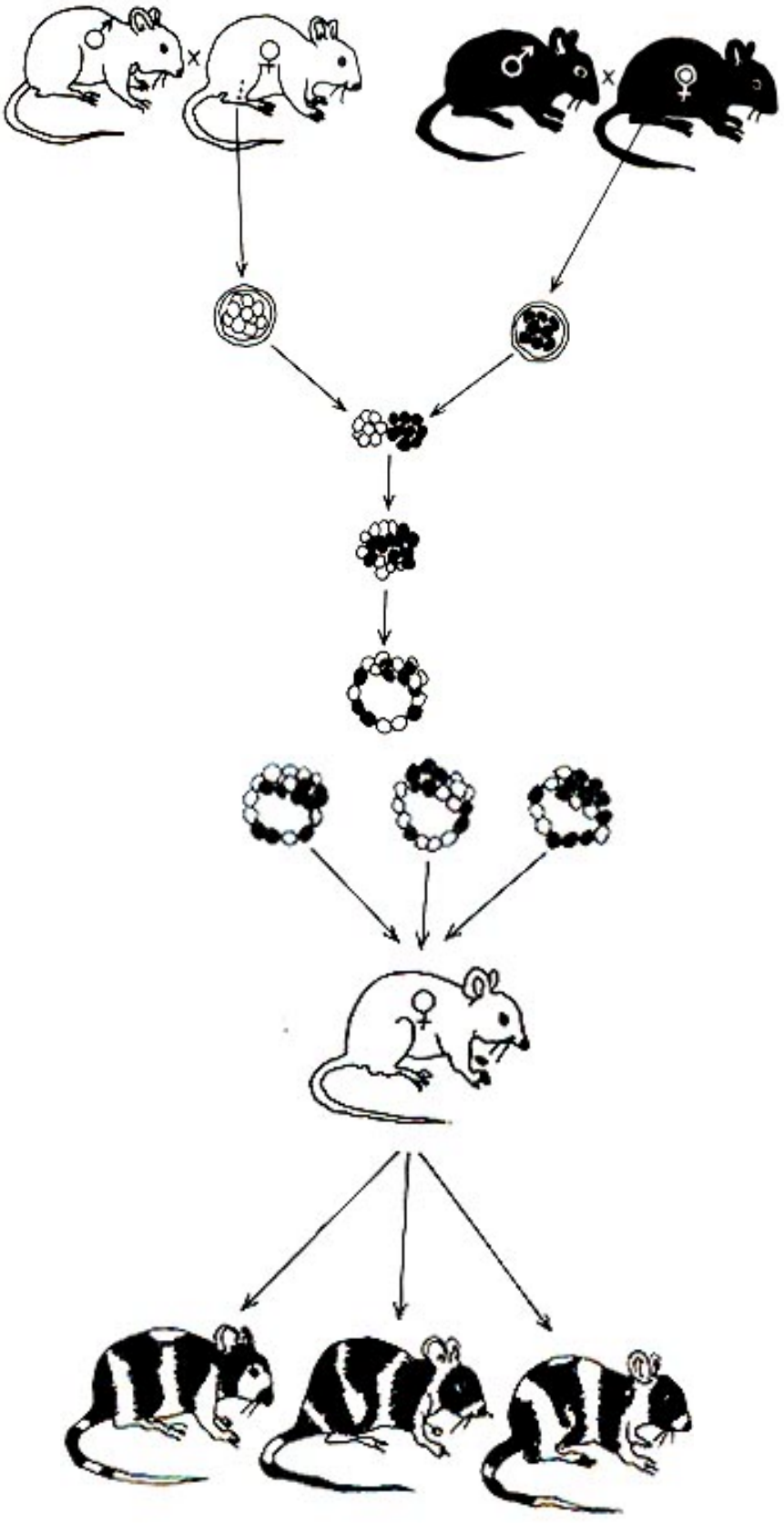
By extension, different cell lines can be used to produce equivalent weapons for target populations. A cell type that can be recognized as self (provoking no immune response-histocompatible for example) can be used for entire closely related families. A different cell type can be confined for use only with selected ethnic groups and even entire races can be uniquely targeted using this method. As long as the cell types have antigens unique to that group, the group cells can be modified via nuclear extrusion and fusion to produce specific target group weapons.

Spontaneous Cell Fusion

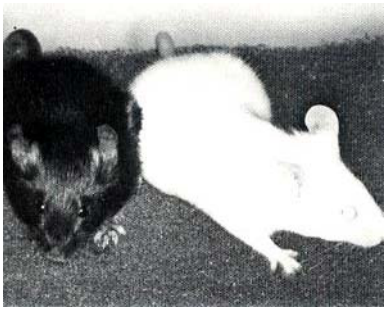
Some cells actually fuse in nature all the time. This is observed when sperm fuse with an egg to form a new progeny. Sperm cells have been used in the virus induced fusion to fuse with cells other than the same species egg. In cells other than these, the event is rare in nature. As mentioned, the higher fungi cells with a single nucleus (mononucleate) join to form binucleate (**dikaryon**) cells. These cells proliferate by mitosis and always remain dikaryotic.

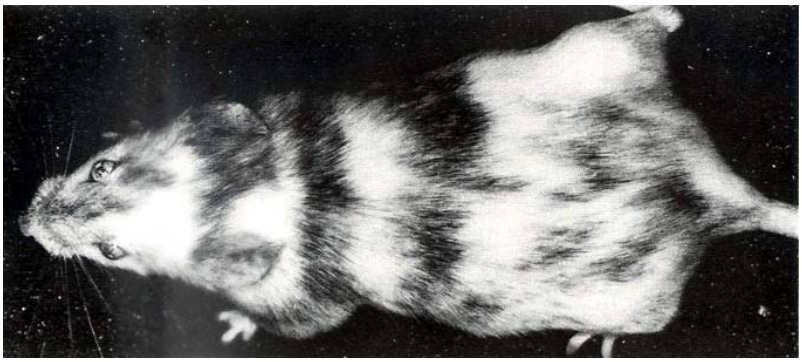
Cell fusion also takes place in mammals. In a well known experiment, fertilized eggs from a white haired mouse and a black haired mouse are “denuded” by pronase digestion and then placed into direct contact with each other at 37C. They fuse into a double sized early embryo. This cell is grown in tissue culture into a cell mass of 128-260 cells in which the “black & white” cells are intermingled. This cell mass is surgically introduced into the uterus of a foster mother that has been made artificially receptive-pregnant with injection of hormones.

This process is illustrated in the diagram below –



The result is a newborn mouse with fur that is a mix of the parents. The internal tissues and organs are also mosaics, or mixes of the two parents rather than the genetic trait of a single parent that is normally expressed. This chimeric mouse is a hybrid and the experiment is a tool used to teach hybridization and genetics in many institutions. The photos below show the parents and offspring of the experiment.





Myoblasts (single nucleus muscle cell) can be fused with each other if they are of the same species and this occurs in nature. It has been found that if they are in the same stage of development, they can fuse with myoblasts of another species. Chick and rat myoblasts have been grown together in tissue cultures and when they touch each other during the same stage of development, they fuse into myotubes with both rat and chick nuclei. In these myotubes, muscle proteins of both rats and chicks are produced in the same ratio as the different nuclei.

As mentioned in previous chapters, high levels of calcium ions (Ca++) can be added to culture medium to produce cell membranes that are leaky or porous. (Calcium Chloride is the most popular source of this type of calcium ion used in labs, it is the material used in winter to de-ice your sidewalk). This allows introduction of phage and plasmids into the cells. It increases the ability of cells to fuse, and also is required to fuse a number of cell types.

The older the cells in a culture, the less likely they are to fuse on their own. This is due to changes which take place on their cell membranes during development which make them less receptive to signals on other cell surfaces.

Fusion of cells takes place with some frequency in the uterus when cells are implanted. The mothers immune system does not attack the fused cells as foreign since part of the cell is progeny DNA with recognized antigens and this protects foreign cells from attack.

"Giant cells" are produced during infections with variola (smallpox), vaccinia (cowpox), measles, mumps, herpes simplex and para-influenza. These are sometimes produced spontaneously without disease and are often found during infections with many viruses in human tissues. These multinucleate cells (polykaryons) can contain up to 200 nuclei and are formed at the infection sites from parental macrophages.

While many of these spontaneous events described above do not produce hybrid strains, they have been seen in a few mammals. Twin bulls will sometimes have exchanged primordial red blood cell tissues in the uterus. One of the bulls at birth would exhibit both parental types of erythrocytes but by eight years of age would have only one type, a hybrid of both parents. In these natural instances, the chance pairing of two "chimera" cells at exactly the right time, place and cell surface chemistry is usually an extremely rare event. These cells rarely produce progeny so they are even more rarely seen as a successful cell line.

Cell fusion in the lab using calcium ions and ideal culture media is generally low-10(-2) TO 10(-6). This rate has been improved considerably using virus and chemical methods to induce the formation of cell hybrids.

It is clear that cell membrane architecture and biology allows cells to fuse together in reproduction, and in common cell types. These cells seem to fuse easily when their inner cell membrane surfaces meet. Different species and cell types have different outer cell surfaces such as glycoproteins which prevent fusion from taking place.

Using a Virus to Induce Cell Fusion

The techniques developed in the 1960's to induce cell fusion using Sendai virus were so simple that they were quickly adopted and practiced by scientists the world over. This led to an explosion of research in the field and widespread use of cell fusion as common laboratory practice.

Several different viruses were in fact reported to induce formation of polykaryons in cell cultures.

Some Viruses Reported to Induce Cell Fusion

DNA-containing viruses
- Herpesvirus
  - Varicella (Weller *et al.*, 1958)
  - Herpes simplex (Hoggan and Roizman, 1959)
- Poxvirus
  - Rabbitpox (Appleyard *et al.*, 1962)

RNA-containing viruses
- Paramyxovirus
  - Mumps (Henle *et al.*, 1954)
  - Newcastle disease (Kohn, 1965)
  - Parainfluenza types, e.g., Sendai (HVJ), SV5 (Okada, 1958; Compans *et al.*, 1964)
  - Measles [a] (Enders and Peebles, 1954; Cascardo and Karzon, 1965)
  - Respiratory Syncytia [a] (Morris *et al.*, 1956)
- Oncornavirus
  - Rous sarcoma (Moses and Kohn, 1963)
  - Visna (Harter and Choppin, 1967)
- Coronavirus
  - Avian infectuous bronchitis (Akers and Cunningham, 1968)

Weller, T. H., Witton, H. M., and Bell, E. J. (1958). The etiologic agents of varicella and herpes zoster. Isolation, propagation and cultured characteristics *in vitro*. *J. Exp. Med.* **108**, 843–868.

Hoggan, M. D., and Roizman, B. (1959). The isolation and properties of a variant of herpes simplex producing multinucleate giant cells in monolayer cultures in the presence of antibody. *Am. J. Hyg.* **70**, 208–219.

Appleyard, G., Westwood, J. C. N., and Zwartouw, H. T. (1962). The toxic effect of rabbitpox virus in tissue culture. *Virology* **18**, 159–169.

Henle, G., Deinhardt, F., and Girardi, A. (1954). Cytolytic effects of mumps virus in tissue cultures of epithelial cells. *Proc. Soc. Exp. Biol. Med.* **87**, 386–393.

Kohn, A. (1965). Polykaryocytosis induced by Newcastle disease virus in monolayers of animal cells. *Virology* **26**, 228–245.

Okada, Y. (1958). The fusion of Ehrlich's tumor cells caused by HVJ virus *in vitro*. *Biken's J* **1**, 103–110.

Compans, R. W., Holmes, K. V., Dales, S., and Choppin, P. W. (1964). An electron microscopic study of moderate and virulent virus–cell interactions of the parainfluenza virus SV5. *Virology* **30**, 411–426.

Enders, J. F., and Peebles, T. C. (1954). Propagation in tissue cultures of cytopathogenic agents from patients with measles. *Proc. Soc. Exp. Biol. Med.* **86**, 277–286.

Cascardo, M. R., and Karzon, D. T. (1965). Measles virus giant cell inducing factor (fusion factor). *Virology* **26**, 311–325.

Morris, J. A., Blount, R. F., and Savage, R. E. (1956). Recovery of cytopathogenic agent from chimpanzees with coryza. *Proc. Soc. Exp. Biol. Med.* **92**, 544–549.

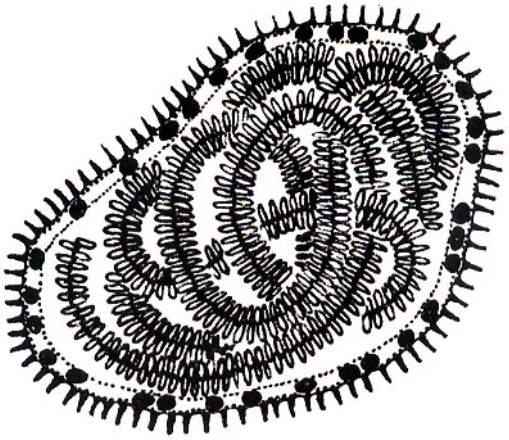
Moses, E., and Kohn, A. (1963). Polykaryocytosis induced by Rous sarcoma virus in chick fibroblasts. *Exp. Cell Res.* **32,** 182–186.

Harter, D. H., and Choppin, P. W. (1967). Cell-fusing activity of Visna virus particles. *Virology* **31,** 279–288.

Akers, T. G., and Cunningham, C. H. (1968). Replication and cytopathogenicity of avian infections bronchitis virus in chicken embryo kidney cells. *Arch. Gesamte Virusforsch.* **25,** 30–37.

The most useful viruses for use in producing cell hybrids are the *paramoxyviruses* which attack cell membrane glycoproteins enzymatically during infection. In this class, the Sendai virus was found to be the best because it caused an exceptionally wide variety of cells to be susceptible to fusion and hybrid formation. It is also known by the names of *parainfluenza virus* and *HVJ-Hemagglutinating virus* of Japan. It was first isolated from mice by Ishida & associates at the Tohoko University School of Medicine in Sendai, Japan.

The Sendai virus has a lipid containing envelope which stains like eukaryotic membranes under the microscope. The envelope has spikes and a helical RNA core which it surrounds.



When Sendai virus particles leave the host cell, they become surrounded by part of the host cell plasma membrane. When glycoprotein and glycolipid antigens are present on host cells, the myxovirus liberated from these cells were found to also have these same serologically defined membrane structures. In other words, the virus adopts part of the cell wall structure of these particular host cells to protect itself from immune responses. In this way, the host cell modifies the virus phenotype.

This effects the viruses ability to hemolyze and fuse cells. Sendai virus grown on fertile chick eggs can lyse red cells while those grown on L cells or chick embryo fibroblasts or bovine kidney cells cannot. The difference is that the specialized cells cause the formation of a larger glycoprotein complex on the virus envelope. Virus grown on eggs produce a smaller version of this glycoprotein making it more potent and able to fuse cells.

Before the virus pinches free from the host cell, the adopted plasma membrane becomes modified in several ways, the most obvious of which is the addition of spikes. Each spike acts as a hemagglutinin which taken as a whole makes the virus a multivalent agglutinating agent. This enables the virus to aggregate many different kinds of cells. The spikes also possess an enzyme activity called nueraminidase which attacks certain glycoproteins on cell plasma membranes. These sites that are attacked by the virus enzyme are also the receptor sites that the virus attaches to.

As we have illustrated in previous chapters, phages attack bacteria like syringes with hypodermic needles which inject their genetic packages. Animal viruses, on the other hand, are generally phagocytosed and then drawn into the cells inside of vacuoles. The paramoxyviruses coalesce with the host plasma membrane while it surrounds the cell or when it is drawn into a vacuole. The membranes formed are often part viral and part host cell.

When the virus agglutinates host cells, they affix themselves to the surface of one cell and then the other side of the virus attaches or sticks to another nearby cell effectively “stapling” the two cells together. Unlike most other viruses, after this aggregation of cells, the virus effects fusion of the adjacent cells. Many viruses are capable of agglutinating cells and coalescing with the cell membranes as described but these properties alone do not enable fusion.

Viruses infect specific types of cells. This host specificity accounts for some of the differences in virus ability to fuse particular cells. The Sendai virus has many receptors capable of binding to a wide variety of different types of cells and membrane surfaces. In mixed cell cultures it has also been found that similar cell types tend to find each other during their movements and aggregate together preferentially. This effect also crosses species boundaries. Chick embryo myoblasts will aggregate with rat myobleasts preferentially when many other chick cell types are mixed in together. This promotes fusion of similar cell types in the formed aggregates because the aggregates are usually similar cell types.

Sendai virus that is exposed to UV irradiation for one minute becomes inactivated and is unable to infect the cell with its genetic package. Its capacity to fuse ascite cells remains close to 100% through two minutes of UV exposure and is about 20% at six minutes exposure. It is always inactivated for fusion experiments to avoid infecting cells and complicating the testing results.

The number of virus particles is important in cell fusion. It has been found that at least 150-300 viral particles is the minimum needed for fusing single layers of cultured cells into polykaryons. This minimal number is higher for cells fused in liquid suspension cultures. If live virus is used, fusion can be achieved in some cases with only a few virus particles but the resulting fusion is often not seen until six hours after infection.

Sendai virus that is used commercially to produce cell fusion is measured in HAU, or *Hemagglutinating units per ml*. They come in a standard amount used to agglutinate a standard suspension of cells [10(7) cell/ml]. The optimum HAU for each cell type is determined experimentally and is usually published with the research data. For bio-weapons, each set of cell combinations will likely need to be tested for different levels. Successfully fused cells can clearly be seen under the microscope with multiple nuclei stained to stand out. Homemade virus preparations can be produced using chick eggs to grow the virus and an ultraviolet lamp to inactivate it.

Virus induced fusion is also dependent on the presence of calcium ions. Concentrations of .1-1 mM or more of Calcium Chloride is usually used in the medium to effect cell fusion. To achieve the maximum frequency, a minimum of .42 mM calcium is required for more than 10 minutes for fusion to begin. A special medium just for cell fusion can be purchased commercially. It is called BSS (Hanks or Earls basal salt solution) which meets the calcium and other fusion requirements.

Optimal pH for fusion experiments have been reported to range from 7.3-7.8 to 7.8 to 8.0. The higher pH will precipitate calcium ions making them unavailable for effect so different buffers are used. The fusion colonies often grow best at pH near 8. In some experiments, the use of the high levels of calcium ions and high pH alone have yielded cell hybrids.

During cell fusion, the cells are agglutinated by virus at 4C during which metabolic and respiration activities are slowed. The cells are then warmed to 37C which initiates the fusion. Warming also speeds up the viral enzyme activity on the cell membranes. A race then begins between successful fusion of aggregated cells and the damage to receptor sites by the enzyme activity which dis-aggregates the cells.

The following are lab reported methods of virus growth and cell fusion by Sendai virus –

Watkins, J.F. (1971) Cell Fusion in the study of tumor cells. *Intl. Rev. Exp. Pathol.* **10**, 115-141

Rao, P. N., and Johnson, R. T. (1970). Mammalian cell fusion: Studies on the regulation of DNA synthesis and mitosis. *Nature (London)* **225,** 159–164.

Rao, P. N., and Johnson, R. T. (1971). Mammalian cell fusion. IV. Regulation of chromosome formation from interphase nuclei by various chemical compounds. *J. Cell. Physiol.* **78,** 217–224.

Rao, P. N., and Johnson, R. T. (1972a). Cell fusion and its application to studies on the regulation of the cell cycle. *Methods Cell Physiol.* **5,** 75–126.

Rao, P. N., and Johnson, R. T. (1972b). Premature chromosome condensation: A mechanism for the elimination of chromosomes in virus-fused cells. *J. Cell Sci.* **10,** 495–513.

Rao, P. N., and Johnson, R. T. (1974). Regulation of cell cycle in hybrid cells. *In* "Control of Proliferation in Animal Cells" (B. Clarkson and R. Baserga, eds.), pp. 785–800. Cold Spring Harbor Lab., Cold Spring Harbor, New York.

Rao, P. N., and Johnson, R. T. (1974). Induction of chromosome condensation in interphase cells. *Advan. Cell Molec. Biol.* **3,** 135–189.

Rao, P. N., Hittelman, W. N., and Wilson, B. A. (1975). Mammalian cell fusion. VI. Regulation of mitosis in binucleate HeLa cells. *Exp. Cell Res.* **90,** 40–46.

Giles, R. E., and Ruddle, F. H. (1973a). Production of Sendai virus for cell fusion. *In Vitro* **9,** 103–107.

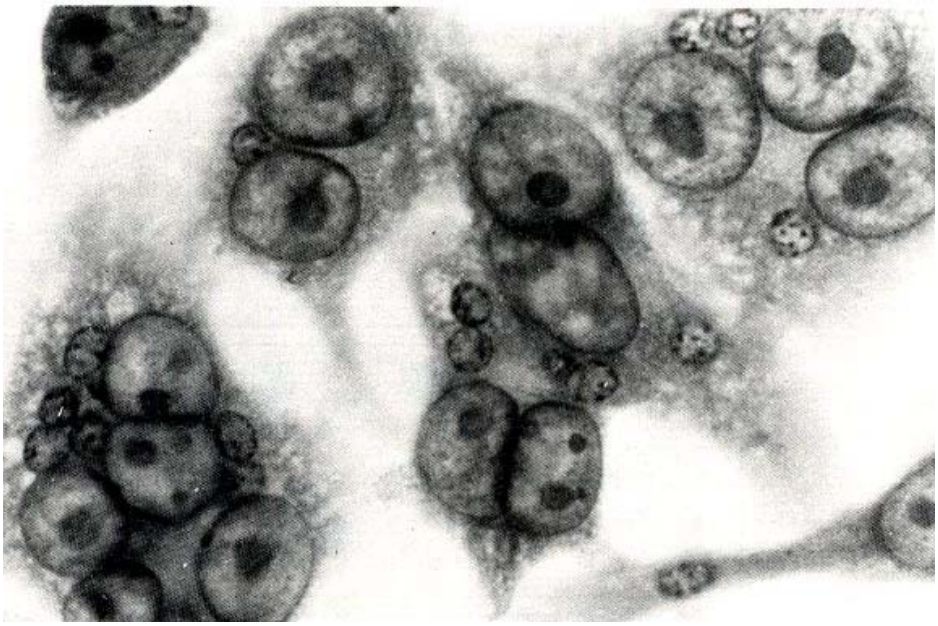
Giles, R. E., and Ruddle, F. H. (1973b). Production and characterization of proliferating somatic cell hybrids. *In* "Tissue Culture: Methods and Applications" (P. F. Kruse, Jr. and M. K. Patterson, Jr., eds.), pp. 475–500. Academic Press, New York.

The results of cell fusion vary among cell types but a sample of multi-nucleate cells formed from a typical mass are listed in the following chart –

"Nucleogram" of 1348 Cells Resulting from Sendai Virus-Induced Fusion of Two Cell Types"

B-Cell nuclei	A-Cell nuclei											
	0	1	2	3	4	5	6	7	8	9	10	11–20
0		466	56	21	8	2	—	3	—	—	—	—
1	421	96	28	20	5	5	—	2	—	—	—	—
2	54	27	13	9	4	—	1	—	—	—	—	1
3	23	5	7	3	2	2	—	—	—	—	—	—
4	10	4	2	5	1	2	1	—	—	—	—	—
5	6	5	—	2	—	—	—	—	—	—	—	—
6	5	—	1	—	1	—	—	1	—	—	—	—
7	3	1	—	1	1	1	—	1	—	—	—	—
8	—	—	—	—	1	—	1	—	—	—	—	—
9	—	2	—	—	1	—	—	—	—	—	—	—
10	—	—	1	—	—	—	—	—	—	—	—	—
11–20	—	1	—	—	1	—	—	—	—	—	—	—
21–30	—	—	—	—	—	—	—	—	—	1	—	1

Only 96 of the 1,348 cells yielded dikaryons with one nucleus from each parent. Many other combinations were produced. In the photo below, you can see the fusion of HeLa cells (having large nuclei) with nucleated chick erythrocytes (small nuclei).



Most of the cells are heterokaryons with 2-10 nuclei.

Chemical Induced Fusion

A number of physical and chemical processes were observed prior to the 1960's to induce fusion. Study of these processes led to the development of several non-viral lab methods for producing cell hybrids.

Calcium ions, most commonly used in the form of calcium chloride can induce fusion as already described. The published methods include

1. Incubating plant protoplasts at 37C with 40-50 mM of calcium chloride which induced considerable fusion
2. Chicken erythrocytes incubated at 37C at pH of 10.5 and then cooled and exposed to 40 mM of calcium chloride and then rewarmed.

Other experiments using similar methods on other cell types have been successful as well. In some cases, magnesium chloride could act as a substitute for calcium ion but usually calcium must be present in minimal amounts.

Lysolecithin and other lipids and lipid-related compounds have been used induce fusion in mononucleate cells. Lysolecithin is and enzymatic degradation product of lecithin (choline phospho glyceride). The enzyme used to convert lecithin to lysolecithin is phospholipase A which is usually prepared from various snake venoms. The resulting lysolecithin is extremely injurious to cell membranes (much like the snake venoms) and as a result, it can be used in small concentrations (100-1,000 mcg/ml) to fuse cells.

It is very toxic to many cells and its toxicity is reduced by adding serum proteins or albumin during treatment or by using it as an emulsion. [J. cell sci. (1972) **10,** 769-787]. Its lethal effect is neutralized after fusing using fetal calf serum.

Glycerol monooleate also induces hybrid cell formation in tissue cultures at rates of 4-7 times that of the spontaneous frequency and does much less damage to cells than lysolecithin.

Polyethylene glycol was introduced in 1974 as an agglutinating agent for plant protoplasts. If it is slowly diluted away with a growth supporting culture medium it yields extensive cell fusion. [Can. J. Bot. 1974, **52**, 1603-1606] Molecular weight of 1500-7500 daltons is necessary for successful cell agglutination.

Principles of Cell Fusion

The first step in producing cell fusion is to bring the cells close to each others membranes. Those agents which cause cell agglutination include Sendai virus and other myxoviruses, polyethylene glycol, polylysine, plant lectins and certain antibodies.

The outer layers of the cell membrane which contain carbohydrates are affected in such a way as to remove them, displace them or damage them so they do not act to repel agglutination and fusion. This is accomplished by the neuraminidase enzyme activity of the virus.

Chemicals effect the lipids of the cell membranes to cause the cell membranes to become “leaky”. Calcium chloride and lysolecithin are the most efficient at causing this effect, with the calcium best used at pH of 8-8.5. Calcium in any ionic form is required in solution to provide part of the necessary cell surface chemistry and process activation in cell fusion. These conditions are not ideal for cell growth but do encourage adjacent membrane coalescence.

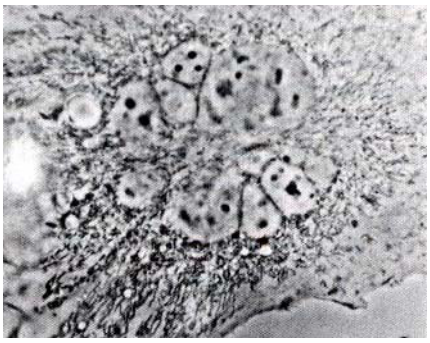
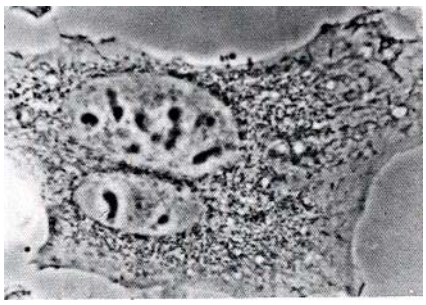
Once the cells are fused, they are returned to the ideal culture medium for growth and selection.

Heterokaryons and Hybrid Cells

The formation of stable and growing heterokaryons is the desired goal of cell fusion. The genetic material from two separate types of cells are present in the cell as intact nuclei. This provides the cell with the material to produce daughter cells with mixtures of the genes from both parents.

The ability to tell which cells are heterokaryons (two different donor cells) from homokaryons is accomplished a number of ways. The easiest (using a microscope) is if the parent cells have different size nuclei. By staining the cells, you can visibly see the nuclei and those with two or more nuclei of different sizes are heterokaryons.

Staining properties, antigens, chromatin morphology and other techniques are used by modern laboratories for identification. In many instances, this technology is not available to ordinary individuals (educated or not). The use of selective media to grow the cells and differentiate them by toxin, virulence, or other factors is often the most suitable.

Left- A large heterokaryon with one melanoma nucleus and many macrophage nuclei. Right- A 1:1 heterokaryon nucleus two days after fusion.

Many heterokaryons display co-dominance traits of both parents as in the case of the white and black haired mice described earlier. Studies indicate there is a mixture of surface antigens from both parents of fused cells. Weapons derived from fused cells would often have mixed antigens. The effect would be to weaken or possibly eliminate the effect of immune responses due to previous exposures or vaccines to the new hybrid (hybrid vigor in this context).

Heterokaryons divide and often form mononucleate hybrid cells of mixed chromosomal material. Many of these form from binucleate heterokaryons by mitosis in which the two nuclei split the genetic material into separate daughter cells. These mononucleate hybrids continue to multiply giving rise to colonies of hybrid cells. A large number of heterokaryons never reach mitosis or fail to complete the first normal mitotic division due to a variety of reasons.

Multipolar divisions are common from the first heterokaryons formed and these are the hybrids. Subsequent divisions to produce descendents of the hybrid cells are rarer and in the final analysis, only a few of the hybrids actually form successful colonies. Successful mating rates of pairs of parents has been found to be higher when layers of cells are used on solid media rather than in liquid suspension cultures.

The first hybrid cells were discovered because they outgrew either of their parental types still in the culture. This phenomena is referred to as hybrid vigor. Most hybrids will grow slower than their parents and this results in a need of isolating a few slower growing hybrids from a mass of faster growing parental cells. Those hybrid cells can often be isolated from a cell mixture by using selective media or isolation techniques. This usually means creating a medium that inhibits or kills the parental cells or that favors a hybrids growth. An example is making the media deficient in a necessary component that will only be used by a hybrid with cellular machinery from both parents.

A good example would be a clone of a gram negative species that selectively grows in bile salts and is sensitive to the antibiotic penicillin. This is combined with a gram positive species that grows on ordinary blood agar but not bile salts and is resistant to penicillin. A true hybrid of both will be able to grow on bile salts and be resistant to a penicillin disc.

You can also use a brute force method of diluting all the cell in liquid and growing them all in isolated colonies and evaluating each ones new properties such as toxin formation, virulence factors and so on. The new hybrids should also form colonies with morphologies different from either parent so with some skill and practice, these can be quickly separated out by a quick glance at the colony. Using a microscope or powerful magnifying glass helps bring out the different morphological characteristics of each colony.

Some hybrids will still resemble their parents morphology and have desirable properties so these may have to be examined for important factors and separated by other methods as well. These may be separated from parents by changing the temperature, pH, exposure to various drugs in which the parents have different resistance patterns and so on. They can also be evaluated for the presence or absence of enzyme activity. If one parent can lyse red blood cells and the other cannot, then only hybrids and this single parent produce colonies with halo formation when grown on blood.

It has also been found that the chromosome numbers vary in hybrid cells with parents containing different numbers of chromosomes. In these cell combinations, one set of chromosomes is usually eventually segregated out in favor of another set. In some cases, it has also been observed that the segregation patterns favoring one species over another has been reversed. This depends on the particular cell types used for each parent. The following chart shows the segregation tendencies of cell line mixtures –

Preferential Loss of Chromosomes in the Interspecific Somatic Cell Hybrids

Hybrids	Specific origin of eliminated chromosomes	References
Man + mouse	Man	Weiss and Green, 1967; Migeon and Miller, 1968;
	Mouse[a]	Minna and Coon, 1974; Minna *et al.*, 1974b
Man + Chinese hamster	Man	Westerveld *et al.*, 1971; Kao and Puck, 1970
Man + Syrian hamster	Man	Grzeschik *et al.*, 1972; Grzeschik, 1973b
Man + mosquito	Mosquito	Zepp *et al.*, 1971
Mouse + monkey	Monkey	Cassingena *et al.*, 1971
Mouse + mule	Mule	Deys, 1972
Mouse + rat	Rat	Weiss and Ephrussi, 1966a
	Mouse and rat	van der Noordaa *et al.*, 1972
Mouse + Chinese hamster	Mouse and hamster	Scaletta *et al.*, 1967; Graves, 1972b; Labella *et al.*, 1973; Handmaker, 1971
Mouse + Syrian hamster	Mouse	Migeon, 1968
	Mouse and hamster	Wiblin and MacPherson, 1973
Mouse + chick	Chick	Schwartz *et al.*, 1971
Chinese hamster + kangaroo-rat	Kangaroo-rat	Jakob and Ruiz, 1970
Chinese hamster + chick	Chick	Kao, 1973

Bacteria only have one chromosome so that if bacteria species are fused together, this preferential observation does not apply. The two parent chromosomes fuse to form a single chromosome with genes from both parents. If a bacteria species is fused to cells from other cell types such as human blood cells, muscle cells, insect, plant, mold and cells of other origins, the results will have to be examined on a case by case basis.

In an interesting study in 1973, the following chart shows the resulting genes present in different hybrid cells from a man and mouse cell hybridization. The hybrid cells all contained a complete set of mouse chromosomes and the following chromosomes from the human parent cell.

Cell by Cell Analysis of Human Chromosomes in a Hybrid Clone (Cl 6) of Hybrid Cells Obtained by Fusing Mouse A9 Cells with a Near-Diploid Human Lymphoblastoid Line[a]

Cell no.	Number of copies of each human chromosome[b]											
	2	10	11	12	13	17	19	20	22	X	Y	3p
1	-	1	1	1	2	-	-	-	-	1	-	-
2	-	1	-	1	1	-	1	-	1	1	-	-
3	-	1	-	1	2	1	1	-	-	-	-	-
4	-	-	1	1	2	1	-	-	-	1	-	-
5	-	-	1	1	2	1	-	-	-	1	-	-
6	-	1	-	-	2	1	-	1	-	1	-	-
7	-	1	1	1	1	-	1	-	1	1	-	-
8	1	-	-	1	1	1	-	-	1	-	-	-
9	-	-	-	-	2	1	-	-	1	1	-	-
10	-	-	2	-	2	-	-	-	-	1	-	-
11	-	1	1	1	1	-	-	-	-	1	-	-
12	-	-	-	-	2	1	1	-	-	1	1	1
13	-	1	-	1	1	1	-	-	-	1	-	-
14	-	-	-	-	1	2	-	-	1	1	-	-
15	-	-	1	-	2	1	-	-	-	1	-	-
16	-	1	-	-	2	-	-	-	-	1	-	-
17	-	-	1	-	1	1	-	-	-	-	-	-
18	-	-	-	-	2	1	-	-	-	-	-	-
19	-	-	-	-	2	-	-	-	-	-	-	-
20	-	-	-	-	1	-	-	-	-	-	-	-

It has been found that one parents cells can be irradiated and mutated (damaged) so that the preferential segregation is reversed. Moderate doses of UV caused small loss of chromosome numbers while large UV doses caused large losses of chromosome numbers contributed by the irradiated parent. Treatment with chemical mutagens and drugs also can have a similar effect.

Chromosome loss rate is slow with cells of closely related species such as mouse and hamster cells. Segregation becomes much faster as the cells of the parents increase in phylogenetic difference from each other. Man and chick hybrids segregate very quickly for example. In a man and mouse hybrid line tested in 1967, all but 2-15 human chromosomes segregated out within 20 generations of the hybrid formation. After this time the human chromosome cell count stabilized at 1-3 chromosomes per cell line while all mouse chromosomes were present and intact. The numbers vary with particular cell types and strains but this pattern is generally followed in mixed species cell fusion.

It has been observed that the segregation also favors the media which best allows species growth. In the human and chick line, chick cells grow best at 40C and human cells at 37C. When hybrids are grown at 40C the chick chromosomes tend to be favored while growing them at 37C favors the human chromosomes. Insect cells require very special growth media and when insect cells are fused with human cells, the insect

chromosomes are favored in their own media while human chromosomes are favored in human tissue culture media.

Cell Fusion Weapons Principles

This last paragraph speaks to the heart of the application of bio-weapons via cell fusion (and all other types). Those cells that grow best inside of and infect a particular species are the best weapons. The best screening methods for single cells, combined cells and all daughter combinations is applied in the multiplier effects and bell curve weapons concept. All the cells are used in test animals to see which ones work. Those that are favored with superior growth and virulence by each particular exposure routes are the cells that make the best weapons.

The cultured cells can all be simultaneously tested by inhalation, dermal, and ingestion routes for effectiveness. Those that successfully infect will infect and/or kill the host will be found in large numbers inside the host tissues and can be recovered in this manner. These cells must have the virulence factors and toxin producing abilities to infect and grow in large numbers inside the host.

Cell fusion weapons were not reported to be developed by the US military although the Soviets apparently made some progress in the field. President Nixon ordered an end to US production and large scale experimentation of bio-weapons in the early 1970's. In addition, military weapons development usually requires the mass production of a single purified type of species, toxin, etc. that can be stored for long periods and then drawn on and used in time of war.

This combination has precluded development of concepts like multiplier effects weapons and bell curve weapons. These weapons are quite suitable for mini development by individuals and mass arming of desired populations with them. It is of course possible to produce master strains of any biological and retain it in a repository for later growth and use. In the authors concept, these strains can be developed, mass produced and used "on the run" by ordinary populations.

Multiplier effects weapons are those which use combinations of cultured organisms and selected carrier materials to produce multiple infections. Cell fusion lines have great promise in this regards since virulence genes, toxin producing genes, surface antigen genes and so on can be combined in single cells creating new potential super-weapons. These can also be used directly in combination with any suitable carrier and complementary organisms. This usually requires the culture of a hybrid with the desired factors and this specific strain is then added to the combination weapon.

Bell curve weapons can be made directly via cell fusion. The entire mass of fused cells can be introduced by the various routes of exposure into appropriate animal test models. Those that are most effective are the best candidates for future weapons use. In a serious condition in which weapons need to be produced in a hurry, they can be directly field tested. The fusion weapons will have an important psychological advantage in that

they will have new and unknown killing and infecting properties that have never been seen before. The early exposures to Aids and the Anthrax attacks gave indications of the ability of this category of weapons to induce panic into target populations. Since fusion weapons can be produced using any combination of bacteria, virus, animal, plant and insect cells, new and extraordinary weapons can be produced daily.

Fusion weapons do not need to be contagious to be effective. The fear of prions (mad cow disease) and various new virus outbreaks in the US is reported routinely by the press. This has the effect of magnifying, accelerating and amplifying the phsychological state of those affected to the general population. Any new illness brought on by an unknown or previously harmless organism is effective in this regard. These types of weapons will use parent cells which are not contagious but have toxin producing qualities (like anthrax). The weapon only affects people in the area it is released and does not spread from human to human.

Contagious weapons can be produced from any cell weapon in which one of the parents contribute genes for living in human tissues and living after being expelled as solid waste or breath in other media or humans. Once again this category is virtually unlimited in potential. Among these are the possible planet killers which can generally depopulate the planet of most or all human life and in some cases possible all upper life forms.

The most striking examples are the Clostridium species. They lack cellular machinery to grow in oxygen which kills them. This machinery (mitochondria, and other organelles) can be provided by other species in cell fusion resulting in progeny that can now live in oxygen. If these new hybrids also produce the toxins of the parent clostridium, they may be capable of turning all upper life into bacteria food. Botulinum, tetanus and the gas gangrene toxins all kill host animals. Even if human populations were to survive via vaccination and antibiotics, the entire animal populations of the planet may be left unprotected and eventually would die off. [Knowledge is Power translates directly in this blended field of biological and military sciences]

Combination chromosomes of hybrids also yield species with new and different surface antigens. This is a quick method of making vaccines ineffective. This also provides methods of exhausting the immune system. As the hybrid loses chromosomes which are less suitable in the host, it changes the antigens while retaining the favorable chromosomes for living in and infecting the host. This constant change should eventually overwhelm the host as each set of new generations becomes different from before. The disease appears to mutate inside the host while killing it. These are weapons that belong to a category by themselves.

One final aspect of these weapons is that a parent or both parents of hybrids can be mutated before hybridization. This allows for unique combined progeny from the start. The daughter cell lines can also be mutated to produce combined bell curve-multiplier effects fusion weapons. The future is unlimited in this field (If there is a future in teaching how to kill everyone on the planet). These types of weapons are useful in the

MAD (mutual assured destruction) concepts developed during the cold war. Their use obviously becomes evolutionary rather than revolutionary in nature.

Producing Plant Cell Hybrids

Plant cells can be fused with other species by stripping the cells of their cellulose wall. A difference in using plant cells is that entire organisms (a plant) have been grown from mixed species cell hybrids. This has not been possible with single cells of other mixed lines.

Plant cell fusion is accomplished by producing plant protoplasts (cells free of the cell wall). This is usually accomplished by using a mix of enzymes (cellulase, pectinase and hemicellulase) to digest the cell wall constituents. Sucrose is added to the digestion mix to aid in the recovery of the often damaged internal cell parts. Sometimes this process by itself produces multinucleate cells. Sodium nitrate (10-20 minute exposure) has been used to directly digest the cell walls in masses of aggregates which spontaneously fused. High pH and the use of calcium chloride increases yields of all types of plant fusion methods.

An obvious use of plant-bacteria hybrids is the ability of plants to yield some specific toxins that do not exist in the microbial world. Ricin toxin is one example. Bacteria and castor bean or plant fusion may yield progeny that can metabolically produce ricin. Since there is no vaccine, treatment or possible immunity to ricin, this makes a significant potential weapon. Ebola virus causes disease because it instructs cells to produce and enzyme which dissolves human blood vessels. Ebola virus may be hard to come by and work with but many plants yield comparable enzymes that cause identical injuries if ingested. All of these make considerable potential weapons when produced inside of virulent bacteria. [Bacteria do not need to be virulent to infect and kill a host. Many species live on and sometimes in human tissues consuming waste materials as food. These can be modified as well].

Chapter 8
Screening &Weaponizing Bacteria

[Authors Note-It had been my intention to include a chapter on genetic engineering at this point of the book but my resources, time and need to produce income have precluded this. The serious student of this art will find that genetic engineering skills are taught on all university campuses, many high schools and found in many published works. This method of modifying bacteria holds the greatest specific potential for effective weapons.]

Cell & Organism Choices

Bacteria can be prepared as weapons in a number of different ways. The most important of which is the modification choice itself. The following chart lists the main organisms and cells which have virulence factors or produce toxins that affect human and animal life. Many other combination possibilities exist and these will be discussed in general later in the chapter.

Bacteria	Toxin or Genetic Factor	Effect
Anthrax	Spores-Protective Shell for Hibernation & long term survival	Resistant to weather radiation chemicals & other environmental factors. Allows bacteria to survive for decades in alkali soil & storage
	Capsule	Protects the cell from attack by immune responses of humans and animals. Phagocytes ingest & carry anthrax throughout body which survive and spread into various tissues
	Metabolic Pathways	Allow anthrax to grow on blood, potato, organic materials in soil and many other materials
	Toxin-Protective antigen (PA) Lethal Factor (LF) Edema Factor (EF)	PA & LF required for lethal infection. PA & EF for local edema infection. PA acts on cells to open surface for entry by EF which causes edema.

		Toxin is only produced in live animal tissues. LF causes tetanic paralysis & respiratory failure via action on nervous system
Clostridium Botulinum	Spores	Cannot grow in air but allow cells to survive & spread. Resistant to heat, radiation Chemicals, etc.
	Botulinum Toxin of several types plus protective proteins as part of toxin	Causes food poisoning, deadliest substances known. .000000033 mg causes LD50 Acts on nervous system in manner similar to nerve gas Released on cell death
	C2 & C3 toxin	Increases vascular permeability & binds to cell receptors
	Grows in dead carcass & injured body tissues & decaying vegetation Can grow in colon but competing bacteria prevent colonization & lethal toxin production	Mass produces toxin in tissues & carcass
Clostridium tetani	Spores	Allows long life & spread Cannot grow in air Protects from heat & chems
	Tetanospasmin toxin	Causes lockjaw. Fatal in tiny Doses, released on cell death
	Tetanolysin toxin	Lyses red blood cells
Clostridium perfringens	Spores	Cannot grow in air but is most aerotolerant of all Clostridium species & may grow in colon Long lived in soil & resists all chemicals & heat
	Alpha toxin	Lethal lecithinase C enzyme Hemolyzes cells at cool Temperatures. It is the cause of classic gas gangrene & similar to snake venoms

	Theta toxin	Oxygen sensitive hemolysin that destroys erythrocytes
	Beta toxin	Lethal & necrotizing & causes blood pressure changes that can be fatal
	Epsilon toxin	Lethal, necrotizing, neuro-toxic, destroys kidneys, & & increases permeability of small intestines. Accumulates in brain & other tissues, produces edema & necrosis of nervous tissues.
	Iota toxin	Lethal & necrotizing, Drastically increases capillary permeability which increased diffusion & spread of all toxins
	Gamma toxin	Lethal
	Delta toxin	Hemolytic & lethal
	Eta toxin	Lethal
	Kappa toxin	Collagenase enzyme that dissolves muscle connective tissues
	Lambda toxin	Proteinase & gelatinase
	Mu toxin	Hyaluronidase which drastically increases spread of toxin & infection. Only most virulent strains produce Mu toxin
	Nu toxin	Deoxyribonuclease, necrotizing, destroys muscle & connective tissues, increases permeability of small blood vessels
	Aggresin	Bursting factor
	Neuramidase	Inhibits receptors of immune Cells
Clostridium Novyi	Spores	Long term survival, cannot grow in air
	Alpha toxin	Necrotizing & lethal Increases capillary permeability & gelatinizes muscle tissues
	Gamma toxin	Lecithinase C enzyme causes hemolysis of cells

	Beta toxin	Also a lecithinase C which is lethal, necrotizing & hemolytic
	Epsilon toxin	Lipase enzyme
	Theta toxin	Hemolysin
	Delta toxin	Oxygen sensitive hemolysin
	Zeta toxin	Hemolysin
	Eta toxin	Tropomyosinanse
Clostridium septicum	Spores	Long term survival, do not grow in air
	Flagella	Makes cells motile
	Alpha toxin	Lethal, necrotizing, hemo-Lytic & leukocidic
	Beta toxin	Deoxyribonuclease
	Gamma toxin	Hyaluronidase
	Delta toxin	Lyses erythrocytes
	Fibrinolysin	
	Neuramidase	
	Hemogluttinin	
Clostridium difficile	Spores	Long term survival, cannot grow in air
	Flagella	Makes cells motile
	Alpha toxin	
	Beta toxin	
Clostridium histolyticum	Spores	Long term survival, cannot grow in air
	Alpha toxin	Lethal & necrotizing
	Beta toxin	Collagenase
	Delta toxin	Elastase which is an oxygen sensitive hemolysin
	Epsilon toxin	“ “ :”
	Gamma toxin	Proteinase
	Peptidase	
	Gelatinase	
	One strain produces 9 different proteinases**	
Clostridium sordellii	Spores	Long term survival, cannot grow in air
	Lecithinase C	Lyses red blood cells
	Another toxin that combines to produce edema, muscle hemmorhage and proteolysis	
Corynebacterium diphtheria	Diphtheria toxin (A&B fragments) [A is lethal in tiny amounts B allows A to enter cells]	Requires virus (beta phage) to produce toxin. It is necrotic and diffuses throughout the body destroying many types of tissues. A & B fragments are

		both present for maximum effectiveness of toxin.
	Surface K antigen	Increases invasiveness
	Cord Factor	Increases virulence
	Neuraminidase enzyme	Allows invasion of throat tissues-breaks down mucus food
	Can infect via skin cuts, and dried cells are long lived	
Staphylococci	Live on human skin & in upper respiratory tract	
	Coagulase enzyme (2 forms which act differently)	Clots blood & separates infection site from immune response
	Enterotoxin	Vomiting, diarrhea
	Hemolytic toxins(many) forms called staphylolysins)	Skin necrosis & lyses red blood cells
	Fibrinolysin	Re-dissolves blood clots
	Capsule (some strains)	increases virulence
	Will grow at 45C and in 10% salt	
	Leukocidin	Attacks leukocytes
	Enterotoxins	Vomiting, Diarrhea
	Exfoliatins	Loosens & separates skin epidermis
	Pyogenic exotoxins	Lethal shock, heart & liver damage
	Hyaluronidase	Increases invasiveness
	Lipase	Dissolves fats & oils that accumulate on skin and allows survival there. Also causes skin boils
	Nuclease	Cleaves DNA & RNA
	Superantigens	Cause shock from immune response
	M surface proteins	Produces *fuzz* on staph cells that interferes with phagocytosis
	Protease	Attacks complement that attracts phagocytes to infection sites
Streptococcus	Survives in host respiratory fluids and dust making it contagious	

	Many strains and species producing many different toxins	
	Flagella (few strains)	Motility
	Hemolysins	Attack red blood cells
	DNAase	Attacks DNA
	Hyaluronidase	Increases invasiveness
	Proteases	Attacks proteins
	Neuraminidase	Allows throat infection
	Camp factor	Growth increased in presence of staphylococci by combined hemolysis
	Capsules of hyaluronic acid	Increase virulence
	Dextransucrase	Forms dental plaque
	Grows both aerobically & anaerobically	Can infect any tissue with different O2 levels
	Erythrogenic toxins	Produced by virus-phage, type C increases permeability of blood-brain barrier to toxins & bacteria
	Muralysin	Phage induced hemolysin
	M,T,R surface proteins	Protects cells from phagocytosis-evades immune detection
	Streptolysin O	Damages red cells & heart tissues & many other cells Pore forming toxin
	Streptokinase	Destroys blood clots
	Proteinase	
	NADase	
	ATPase	
	Phosphatase	
	Esterase	
	Amylase	
	N-acetylglucosaminidase	
	Lipoproteinase	
	Cardiohepatic toxin	
	Surface protein F	Binds to pharyngeal cells allowing colonization of respiratory tract
Pneumococci	Part of normal flora in respiratory tract flora, contagious	
	Grows both aerobically & anaerobically	
	Capsule	Different types with varied

		virulence, neutralizes antibody via free capsular material.
	Amidase	Self lysing enzyme
	Autolysins	Digest peptidoglycans
	Pneumolysin O (water soluble)	Anaerobic toxin produced in mucus & exudates, lethal dermonecrotic & dermotoxic Binds to cell cholesterol and forms pores in wall
	Neuraminidase	Breaks down mucus for food
	Hyaluronidase	
	Leukocidin	
	Necrotizing toxin	
	Protein adhesin	Binds to pharyngeal cells
	SIg A protease	Protects cell in mucus
	Hydrogen peroxide	Causes lung damage-food
	Inflammatory shock response triggered by various factors such as peptidoglycan fragments released at lysis. Protects bacteria from immune response clearing action.	
	Host with spleen dysfunction are very susceptible	
	A few strains with "bile resistance" are more virulent	
	Naturally *transformable* to capsular form Some have genetic resistance to classes of antibiotics	
E-coli	Normal inhabitant of intestines	
	Capsule	Protects from immune system
	Flagella	Motile strains
	Surface component	Masks surface antigens
	Colicin	Bacteriocide
	Enterotoxins	Cause diarrhea, ataxia
	Cytotoxic hemolysins	Kills leukocytes, inhibits phagocytosis & chemataxis
	Neurotoxins	Causes paralysis & neuro damage
	Adhesins (pili)	Binds to intestinal surfaces for colonization-many types
Salmonella	Lives in soil, sewage, manure for months Pass through epithelial lining-by engulfment into surface cells and multiplication & release (Invasin genes)	
	Surface component	Masks surface antigens
	Adhesive fimbriae	Colonize tissues, linings

	Flagella	Motile strains
	Long O antigens	Resist phagocytosis & inhibit MAC
	Endotoxins (LPS)	Causes fever & shock
	Entereotoxins	
	Enterochelin	Iron scavenging increases fatality of infections with iron present
	Neurotoxin	Nerve damage & paralysis
	Antibiotic & Dye resistance	
	Virulence plasmids	Survival in macrophages Increased growth in liver and spleen
	Vi antigen	Capsular polysaccharide in *S. typhi*
Shigella	Lives in intestines, survives in sewage, water, etc.	
	Surface K antigens	Mask O antigens
	O antigens	Induce inflammation
	Endotoxins (LPS)	Severe diarrhea
	Exotoxins-Shiga toxin	Neurotoxic
	Invasins	Invades epithelial cells Invade & grow in and kills macrophages
	Inducers & adhesins	Causes other cells to engulf them for food & transport
	Trigger apostosis	Causes programmed cell death of macrophages
Klebsiella Pneumonia	Capsule	Protects cell. Especially In respiratory tract
	Fimbriae adhesins-pili	Bind to cell surfaces Inhibit phagocytosis, impairs intraphagocytic killing
	Sidephores	Scavenge iron
	Enterotoxins	Disintegrate alveolar wall and necrotize lung parynchema
Proteus	Live in soil, water, sewage, decaying matter & in humans	
	Urease activity	Destroys kidneys
	Flagella	Motile & swarming
	Surface antigens	Resist immune response
	Endotoxin (LPS)	Causes diarrhea
Yersinia (Black Death) (Plague)	Multiplies in stomach of fleas causing disgorging during bites-transmitted via the bites Lives longer at 50F than 80F Grows at freezing temp's slowly As few as 10 cells produce fatal infections	

	Can survive and grow in unactivated macrophages which allows proliferation in lymph nodes & eventually lungs	
	Capsule	Antiphagocytic
	Endotoxin (LPS)	Lethal-Septic shock
	Murine toxins	Lethal edema & necrosis
	Fibrinolysin	
	Pesticin	
	Coagulase	Blood clotting-Blackish appearance of tissues
	Multiple sidephores	Scavenge iron & increase virulence (a lot) directly with iron added to infection site
	Uptake of entire hemin molecule, not just cleaved iron, and can use it as stored hemin directly	
	Virulence genes coded on plasmids for all Yersinia and easily exchanged with E-coli to which it is closely related Plasminogen activator protease (Pla) is a gene on a plasmid that aids in systemic spread through the body	
	Invasins	Allows invasion to interior of host cells
	[E-coli have had the plague gene for the invasins cloned and inserted via transformation-they subsequently produced the ability to be engulfed and then internally devour the host cells]	
	Pilus adhesins	Protects yersinia from digestion by phagocytes
	Excreted Outer Membrane Proteins (Yops)-11 types which interfere with immune response signals and attack host cell cytoskeleton	
	Serum resistance	Survival in blood in fleas
Francisella tularensis (Tularemia)	Infects and kills at 14 cells or less	
	Invades through eyes, mucus & abraded skin	
	Endotoxin (LPS)	
	Heat-labile toxin	
	Ability to invade cells mimics the factors listed above for plague-invades most cell types	
Vibrio cholerae	Endotoxins (LPS)	Cause profuse diarrhea & vomiting
	Lives in and spreads via contaminated water	
	Hemolysins	Dissolve blood cells and are

		cardiotixic & lethal
	Flagella	Motilityto swim to surfaces
	Exotoxin (Cholera toxin)	Similar action to diphtheria toxin which attaches to cells and causes them to discharge internal fluids
	Mucinase	Attacks lining cells
	Adhesins-pili	Adheres to intestinal lining
	Hemagglutinin	Surface protein that agglutinates erythrocytes
	Enterotoxin (Zot toxin)	Disrupts binding together of mucosal cells
	Enterotoxin (Ace toxin)	Causes diarrhea
	Sidephores	Iron sequestering
	Surface protein	Binds hemin & hemoglobin
Mycobacterium Tuberculosis	Intracellular parasite that can spread to bone marrow, lymph nodes & liver of host. Grows inside monocytes	
	Virulence is attenuated in subcultures on media	
	Contagious via air droplets	
	Cell wall very resistant to strong acid & alkali	
	Has 3 layer cell wall & 3 layer plasma membrane	
	L form of TB has no cell walls	
	Virulent strains form cords in liquid media	
	(cord factor-mycolic acids) triggers inflammatory response	
	Colonizes lymph nodes of trachea & bronchi	
	Grows inside of cells & kills host cell in 3-4 days	
	Usually grows inside of unactivated macrophages and is killed by activated macrophages	
	Serum resistant	
	Causes caseous necrosis & self lyses	
	Macrophages that do not kill TB may wall off site forming tubercle lesions	
	TB cells can survive for decades inside of lesions	
	Spreads if colony is near a blood vessel with high fatality	
	Inhibits fusion of lysosomes in macrophages	
	Exochelin	Robs iron from cells
	Mycobactin	Iron chelating substance in cell envelope
	Invasins?	Stimulate uptake by phagocytes
	Acidification inhibitor	Prevents acid formation in phagocyte vesicles
	Lipoarabinomannan	Suppresses T-cells
	Antigen 85A	Protein that binds fibronectin-suppresses recruitment of immune cells

Mycobacterium leprae (Leprosy)	Intracellular parasite Contagious via skin contact-lesions which form granules that eventually spread throughout host Can invade every type of tissue & cell Live in cytoplasm but do not invade nucleus Has 3 layer cell wall & membranes Capsule — Protects from host defenses Many of the same cell traits for TB are present in M. leprae ** Mycobacterium species found in soil & water also infect humans with suppressed immune systems via similar biology ** Mycobacteria cell walls are very tough and do not respond to chemical attacks as well (calcium chloride, etc) making them harder to modify	
Haemophilus	Microbial parasites that colonize the upper respiratory tract, as well as cause venereal disease Contagious via air droplets Requires hemin and/or NAD to grow Capsules — Protects from host defenses Pili — Adheres to pharyngeal tissues Hemagglutination of human type O eryhtrocytes Endotoxin (LPS) — Epithelial cell damage & ciliostasis	
Listeria	Found in many animal species & lives in plant-soils Grows slowly in refrigerated temperatures Grows at high pH and in high salt environments One of few organisms that can pass though placenta Invades intestinal cells & macrophages Uses host cell actin to form long tails to move within & between host cells Adherence & invasive factors that bind to cells and allows Listeria to move through mucosa & reach underlying tissue Up to 4 flagella — Highly motile MPA — Causes monocytosis MEF — Mortality enhancing factor-cell disruption product Endotoxin (LPS) Cardiotoxin — Also contributes to	

		monocytosis
	Hemolysin-Phospholipases	Invasive factor leukocidic, disrupts lysosomes and is cardiotoxic
	Lecithinase	Attacks lecithin, causes major tissue damage
	Listeriolysin O	Antiphagocytic action-allows Listeria to escape vesicles & multiply in cell cytoplasm
Brucella	Contagious via ingestion, inhalation and skin contact Long term intracellular parasites Uses lymph system to spread to blood & many organs Only a few cells required to initiate infections	
	Capsule	Protects from host defenses
	Cytotoxin	Heat sensitive protein that also acts as a mild neurotoxin
	Endotoxin (LPS)	
Bordetella Pertussis (Whooping cough)	Human pathogen spread by air droplets or skin contact Virulent mutants arise from non-virulent strains at 10(-6) Binds to cilia in airway allowing colonization via various adhesins (pili, proteins) -also adheres to phagocytes and survives phagocytosis. Toxin kills cilia, stopping "wafting" to remove debris	
	Pertussis toxin	Similar to cholera toxin, increases mucus & respiratory secretions
	Adenylate cyclase	Binds to & lyse erythrocytes from inside the cell
	Dermonecrotic toxin	Causes necrosis, is lethal
	Tracheal cytotoxin	Peptidoglycan fragment that kills cilia when Bordetella lyse
	Endotoxin (2-LPS)	Cause inflammation
Neisseria gonorrhoea	Fragile cell which does not live long outside a human host Normally transmitted by sex but can also colonize the throat, and rectum. Constantly changes its surface antigens (especially pili antigens) to avoid immune responses. N. gonorrhoea is naturally transformable, it takes up DNA without chemical shock or electroporation, but discriminates against foreign DNA. Adheres to and invades tissues via actin rearrangement, numerous pili (which change allowing adherence to different cell receptors), proteins, receptors, etc.	

	LOS	Triggers intense inflammatory response
	Serum resistance	
	Antibody blocking surface arrangements	
	Bacteria killed in phagocytes also kill phagocytes resulting in discharge seen in VD.	
	Iron binding proteins	
Pseudomonas aeruginosa	Occurs in all people with cystic fibrosis due to increased mucus which impedes phagocytes and airway clearing	
	Grows in huge range of media, soil, water, mild disinfectants, etc.	
	Can grow in mucus but does not overcome host defenses	
	Alginate	Coating that resists phagocytes
	Pili & other adhesins	
	Neuraminidase	
	Exoenzymes S	Inhibits phagocytes
	Exotoxin A	Damages tissue
	Elastolytic activity	Damages lung tissues, blood vessel walls, immune response injuries
	Proteases	Damage lung, tissues
	Endotoxin (LPS)	Septic shock
	Pyocyanin pigment	Damages endothelial cells
	[**Elastolytic activity is similar to the enzyme produced by the Ebola virus that dissolves the lining of blood vessels and causes the bleeding into tissues-blue color like bruises]	

Important Plants	**Toxic Factors**	**Effect**
<u>Water insoluble alkaloids producers</u>		
Tansy Ragwort	Pyrrolizidine alkaloids	Irreversible liver damage
Common Groundsel	" "	" " "
Tarweed or Fiddleneck	" "	Hard liver disease
Comfrey	" " (8+)	Liver cancer, fatal
<u>Piperadine alkaloids</u>		
Poison Hemlock	Coniine & others	Nervous system, fatal & teratogenic
<u>Pyridine alkaloids</u>		
Tobacco	Nicotine	Descending paralysis of nervous system
	Anabasine	Teratogenic

Indole alkaloids		
Tall fescue	Ergot alkaloids	Pulmonary emphysema
Cereal grains (fungus)	Ergot alkaloids (many)	Vasoconstriction Elevated blood pressure Hallucinogenic (LSD)
Strychnos ignatii	Strychnine	Nervous system convulsant
Quinolizidine alkaloids		
Scotch broom	Various toxins	Birth defects, fatal
Golden chain	" "	& pharmacological effects
Steroid alkaloids		
Nightshade, green potatoes	Solanum	Nerve agents
Jerusalem cherry, tomatoes	"	(cholinesterase inhibitors)
Veratrum species	Veratrum alkaloids	Teratogenic, vasoconstrictors
Death camas	Steroid alkaloids	Lethal
Polycyclic diterpene alkaloids		
Larkspurs, aconite, monkshood		Neuromuscular blocking, paralysis
Indolizidine alkaloids		
Locoweed	Cumulative toxins	Addictive, teratogenic, Attacks immune system Congestive heart failure
Red or white clover	Slaframine	Nerve agent
Tryptamine alkaloids		
Reed canary grass		Heart failure, nerve agents
Tropane alkaloids		
Jimson weed Belladonna, Henbane	Atropine, etc	Nerve system-psychotic effects, dilate pupils, Lethal
Fescue alkaloids		
Tall fescue	Perloline, perlolidine	Respiratory effects, & gangrene in extremities
Quaternary ammonium compounds		
Strychnos, erythrina	Curare, etc	Paralyzing, used in arrow & dart tips
Cyanogens		
Cherries, peaches, apple, etc Apricot, bitter almonds Bracken fern tapioca	Cyanide yielding	Produces cyanide with water (hydrolyze)

Glucosinolates		
Cabbage, mustard, turnip	Enzymes	Damages thyroid, goiters
Coumarin		
Sweet clover	Inhibits blood clotting	Internal hemorrhaging
Steroids & triterpenoids		
Garden foxglove	Cardiac glycosides	Heart acting, fatal
Oleander	" "	Lethal
Milkweed	Cardenolides	Lung congestion Pupil dilation, depression, staggering, collapse
Nitrogen containing glycosides		
Milk vetch	Causes locoism	Weakness, convulsions Coma & death
Fava bean (Racial groups highly susceptible-see V6-B)	Visine	Hemolytic anemia Vomiting, fatal
Cycads	Azoglycosides	Carcinogenic, hepatic & GI disease
Weed Cocklebur	Carboxyatractyloside Hydroquinone	Depression, nausea vomiting, dyspnea, hypoglycemia, liver necrosis, gallbladder edema, coma & death
Buttercups	Protoanemonin	Blistering agent like mustard gas & lethal
Isoflavones & Coumestans		
Subterranean clover	Phytoestrogens	Inhibits ovulation, Decreases fertility
Oxalates		
Diffenbachia	Oxalic acid	Burns tissues, toxic
Halogeton	Oxalic crystals	Burns tissues, toxic
Hemaglutinins		
Castor Bean	Ricin	Clumps red blood cells, phytotoxic, one of most lethal substances known-No antidote

Rosary bean	Abrin	Vomiting, coma & death
Black locust	Robin	Anorexia, nausea, paralysis diarrhea & death
<u>Enzymes</u>		
Bracken fern, horestail	Thiaminase	Bone marrow damage, convulsions & death
Kao haole	Mimosine	Teratogenic, hair loss & eye cataracts
Purple mint	Tryptophan metabolites	Pulmonary emphysema
Chick pea	Lathyrogens	Skeletal deformities, Aortic rupture, paralysis
Flax	Linatine	Anorexia, convulsions
Creeping indigo	Indospecine	Liver damage
Jack beans, alfalfa sprouts	Canavanine	Severe lupus
Brassica	Glucosinolates	Liver, kidney & spleen damage, anemia
Blighia sapida	Hypoglycin	Hypoglycemia, vomiting, convulsions, coma & death
Pineapples, bananas, etc	Biogenic amines	Vasoconstrictors, heart disease
<u>Glycolipids</u>		
Annual ryegrass	Corynetoxins	Convulsions, spasms, death
St. Johns Wort	Hypericin	Skin necrosis, skin cancer
<u>Various toxic compounds</u>		
Sleepy grass	Diacetone alcohol	Catatonia
Sneezeweed	Sesquiterpene	Vomiting, pulmonary Damage, tear gas =
Water hemlock	Cicutoxin	Violent convulsant, Fatal

White snakeroot	Tremetol	Nausea, ketosis, delirium coma & death
Pine needles	Diterpene resin acids	Embryonic mortality
Acacia	Sodium monofluoroacetate	Cellular respiration stopped, lethal
Bracken fern fronds	30 substances	Induce cancers
Blind grass	Unknown	Destroys optic nerves
Blue green algae	Cyclopeptides	Liver damage
Poison ivy	Dermotoxic substances	Itching, severe rash

Mycotoxins from mushrooms & molds

Amanita mushrooms	Ammatoxins & phallotoxins	Liver damage, death
Cortinarius mushrooms	Orellanine	Destroys kidneys
Gyromitra mushrooms	Monoethylhydrazine	Hemolytic & nuerological damage, fatal
Inocybe mushrooms	Muscarine	Slowed pulse, asthma Vomiting, diarrhea
Fly agaric & Panther mushrooms	Ibotenic acid	Hallucinogenic effects
Psilocybe mushrooms	Psilocybin	Strong hallucinogen
Asperigillus	Aflatoxins	Potent carcinogens, Damages immune system Cause mutations
Fusarium species	Trichothecenes	"Yellow rain", dermotoxic, hemorrhage, nervous disorders, death (Numerous effects)
Fusarium roseum	Zearalonone	Estrogenic toxin
Stachybotrys	Stachybotrytoxins Saratoxins	Damages entire digestive tract, blood cells

Claviceps purpurea	Ergot alkaloids	Blood vessel constrictor Causes gangrene Hallucinations, convulsions (many effects)
Penicillium	Citrin, various toxins	Toxic effects, lethal Ascending paralysis

Combination Concepts

It should be obvious by now that there is virtually no limit to the number of possible ways that bacteria genes can be combined with each other and with other species. Almost all bacteria produce enzymes to live in their current environment. Any of these can be modified to yield potential weapons with new and useful properties. Genes for virulence and other factors have been cloned in many cases already and can be purchased outright from various laboratories and institutions for insertion via genetic engineering.

Bacteria x Bacteria

The ATCC catalogue lists many tens of thousands of bacteria and the enzymes they produce as well as journal articles pertinent to the bacteria. This is a good source of information and "safe" starter organisms. Some of these safe bacteria produce enzymes that dissolve blood vessel linings like Ebola but are harmless to people because they have not evolved virulence factors and are incapable of infection in their current form. The human body can recognize these and destroy them immediately, before they cause harm. Modifying any of these with the necessary protective (capsules) and virulence factors is possible.

The most interesting bacteria combinations for genetic exchange the author looked at includes the following –

Anthrax plus just about any other toxin producer. The reason for this is that Anthrax produces a spore that makes it long lived and storable. The spore coat permits its release into any environment where it will persist for long periods. It also has a capsule that is generally not immunogenic in vaccine preparations. The toxin it currently produces is the focus of vaccine work today. Almost any toxin can be added to this core organism in place of the current toxin or in addition to it. This greatly increases the killing power and the simultaneous ability for the anthrax to make more food in its surroundings.

A combination with Corynebacterium species to provide machinery to live in mucus would likely make anthrax highly contagious as would comparable combinations

with Staph and Strep. The ability to form dental plaque (from Strep) would be a definite infectious quality because the anthrax would easily live on the food present in the mouth. Since it doesn't produce its current toxin in that kind of environment, it would have to spread to the lungs and bloodstream or tissues before killing its host. This may make its spread harder to stop. If it doesn't kill its host it can turn the host into a lifetime carrier and spread via airborne routes.

A combination with Klebsiella genes appears synergistic because Klebsiella destroys the alveolar walls that Anthrax first infects. This would give it immediate access to the blood stream where it can spread. It would also give it another food supply upon entering the respiratory tract and likely increase the effective germination rate (number of foci of infection).

A mix of genes with Cholera to give the anthrax spores the ability to live in aquatic conditions (including salt water) so it can feed off of algae and other organics without the need for high CO2 is promising.

A combination with E-coli permitting growth in solid wastes, marginal soils and colonizing the human intestinal tract with the appropriate cellular machinery is another possibility.

Clostridium perfringens & other Clostridium

The family of spore formers lack the machinery to survive in oxygen. Any modifications that permit it to do so yields a new class of organisms. These organisms would be able to infect any human tissues directly to turn them into food. They cannot do so now because of the high O2 content of tissues and atmosphere. A mix with the genes of Staphylococci to provide O2 tolerance and live directly on or in skin and tissues like the normal bacteria populations makes this class almost unique in its ability to transform the planet (planet killers). The staph also confers the ability to clot blood which may be helpful in those strains lacking aero-tolerance after modification.

This type of change is considered both revolutionary and evolutionary since it will likely displace most other life forms on the planet by turning them into food. The perfringens species is most likely to achieve this since its toxin mix spreads out ahead of it destroying tissues and cutting off possible immune responses. A combination with Klebsiella could yield the gas gangrene producer a new food supply in the lungs causing damage to the alveoli and permitting spread of the Clostridia to all parts of the body shortly after infection.

Corynebacteria

This organism infects people worldwide even without its toxin and many humans are long term carriers via dermal and respiratory routes for it. It causes disease when a phage infects it and turns on its toxin production. The addition of an alternative toxin will

yield a new and effective species. (Combining it with many species in combination bell curve weapons could yield many new types in a single event).

Staphylococcus

Since this organism already inhabits the human body, modifying with genes that interfere with the immune response is an obvious approach. Staph increases toxin production with increased CO2 levels (in which oxygen is cut off). Those organisms with machinery and toxins that injure surrounding tissues immediately and cut off blood flow, or supply fresh blood that the staph can clot (such as C. perfringens) would work well in this concept.

Streptococcus

The species that forms bio-films (dental plaque) holds a unique niche and can serve as a platform for a staggering array of possible weapons. They can be modified to produce toxins that make better food for the bacteria in the mouth environment. These toxins can also be specific for the persons genetic type as an individual, a family, an ethnic group or an entire race. This is also a communicable platform that would easily spread and is tough to eliminate. (Scientists have been trying to get rid of dental plaque for centuries).

Strep produces a huge array of toxins and disease. Adding various virulence factors and new toxins will expand these capabilities, in some cases putting them beyond the reach of antibiotics. [This idea is that organisms can infect an area, cut off the blood supply to the area and then the antibiotics and immune processes can no longer reach the infection sites making them useless.]

Adding strep genes for M proteins that allow colonizing of the pharynx will endow many bacteria species with the ability to become contagious as well as colonize and live away from many of the immune defenses that they are susceptible to.

Cholera

Cholera does not invade the body beyond the intestinal tract. Modifying it with invasiveness genes and/or additional toxins would create new super weapons that would persist in environments that cholera is endemic in now and make it a strong depopulation weapon.

Neisseria gonorrhoea

Combined with any other infective organism yields new possibilities. This species changes its external antigens regularly which makes it an exceptional organism. It is largely confined to transmission sexually or in close physical contact. By adding virulence, invasiveness and toxic producing genes, it will yield new weapons that the

bodies immune system cannot identify and that vaccines will simply not work on. This is a wild card that may produce many unexpected new properties. A combination with anthrax would be interesting and may yield a much more effective organism even without its toxin. Anthrax can already invade but without a toxin it eventually is defeated by the host immune system. By enabling it to change its surface antigens, its infection may persist indefinitely. This will hold true of all organisms that are modified with these genes. The reverse of simply adding anthrax (or other) toxin to this form of VD would make it nearly universally fatal effecting the sexually active segments of society.

Mycoplasma

These intracellular parasites are obvious candidates for any cellular machinery that speeds their growth. Contagious and systemic toxin genes would make these weapons more effective. They are an ideal platform for long term disease because they persist inside of cells and spread inside the host slow enough to insure effective spread through the local populations.

By transferring its own genes for intracellular invasion to other organisms like E-coli (which has already been done in tissue culture medical experiments), new infective mycoplasma equivalents can be produced. Imagine tuberculosis that can live in the intestinal tract or has the increased ability of anthrax to invade beyond the alveoli. The use of a slower toxin such as one that causes cancer would be most effective in these designs (so the host doesn't expire before spreading the infection).

Bacteria x Plant cells

Plant cells have the ability to grow entire new organisms (plants) with new genes inserted into them. This permits the creation of new crops that can be harvested which produce new substances. This is already widely practiced in the United States and other modern countries. In this case, the plant cells are modified with toxin genes from bacteria which permit their mass production as a crop. These can be harvested and stored for use like other crops. The obvious weapons applications is the mass production of specific toxin and then harvesting and purifying it for weapons use.

An alternative is the modification of a plant that can grow in targeted areas like lakes and streams and effectively poison them as they grow and release their enzyme products. Plants that only grow in target areas are the best candidates for this type of modification. They poison the area with the toxin and its degradation products destroying other crops and the ecosystem. In some instances they would make the area uninhabitable. An example would be the botulinum toxin mass produced by a common weed or grass that poisons any animals that nibble on the tiniest amount of its leaves. As the plant dies off (in winter), it releases the deadly toxin in massive amounts to the wind. Eventually deadly levels may accumulate in the soil killing off other intolerant plants and the areas inhabitants. The use of aquatic vegetation to produce botulinum toxin is an obvious strategy for the mass long term poisoning of water supplies (lakes and streams) or targeted populations.

Transferring plant genes to a desired bacteria is also an effective possibility. The ricin toxin produced in castor beans has no antigens or anti-toxin and there is no cure. It is an ideal toxin for custom made bacteria to produce and deliver inside targeted hosts. The specific gene for ricin can easily be transplanted into any bacteria of choice. The plant kingdom has a huge array of possibilities as you can see from the short list presented earlier in this chapter. Many toxins target specific groups of people, cause cancer, attack body tissues in unique ways and cause other effects that may be desired.

The insertion of psychotropic drug yielding genes is among the most interesting and has been the subject of military and CIA study since the 1960's. The most obvious weapons concept is to be able to make a targeted army or area "crazy" or incapable of defending itself without killing them so that the army can move in and take over with little or no resistance. Bacteria that yield LSD and various ergot pharmaceuticals could accomplish this. The bacteria would infect the targets, making them helpless until their bodies fought off the infection, In several days the effects wear off and they wake up with a new government and are effectively disarmed (unless they know how to do this as well).

An interesting possibility is the inclusion of the poison ivy rash causing molecule into a persistent skin colonizer like staph. The host would never be able to evade continuous exposure to the toxin and would slowly become debilitated by his own immune response. This effect may be lethal over time.

Bacteria x Molds

Molds toxins are among the deadliest known. The cancer causing toxins such as aflatoxin B1 can be combined in a bacteria like avirulent anthrax causing cancer via long term exposure without quickly killing the host. The spores can be distributed throughout the targeted area without causing immediate effects. No one knows that they have been attacked until months later when they die of cancer by the thousands (or millions). The target area then becomes uninhabitable until the spores die off or a new cure is found.

By using bacteria that infect and grow without causing obvious disease is the best choice. Even a mild disease or infection is satisfactory because the damage can be done in a matter of hours or days via the initial infection, growth and spread of the new toxin into the tissues. It may be completely unnoticed. The example is a mass outbreak of staph ot strep in the middle of winter. By summer, they all begin die off of the cancer causing effects (all needing liver transplants in the case of B1). This allows covert attack on enemy military bases without anyone knowing they have been attacked for months.

Some molds produce infective and contagious spores themselves (coccidioides immitis-page 128 V-6C) or yield persistent infection like athletes foot. These can be modified with various bacteria genes for increased virulence or dermotoxic compounds. In the case of athletes foot, instead of itching, the modified strain destroys the skin areas as it feeds on the cells via dermal necrosis. This clears the way for a variety of other

infections. If the mold can use the underlying tissues as well, it creates a new category of disease.

The ideal weapon of this type is one in which fusion has combined a deadly mold and bacteria. The new organism is released on a target population destroying it. The genes for the fused combination slowly eliminate one of the donors chromosomes and lose their new virulence. The weapons cell line becomes less effective and "dies off" over time. The area can then be inhabited safely.

Bacteria x Human cell lines

This has already been mentioned and can yield weapons currently seen only in science fiction. A donor cell from an individual can be modified so that the new cell has a human coating which protects it from that individuals immune response. The bacteria machinery inside reproduces itself and produces a lethal toxin that kills the host.

There are thousands of cell types. Some have genes and antigens unique only to the individual donor. Some have antigens which apply to the targets close relatives. Others will have antigens that are not recognized inside their own ethnic group and even entire races have specific surface proteins that their bodies will collectively not fight back against.

This branch of weaponry is an entire field unto itself. It permits the specific targeting of individuals and groups using their common surface antigens for protection.

Bacteria x Virus

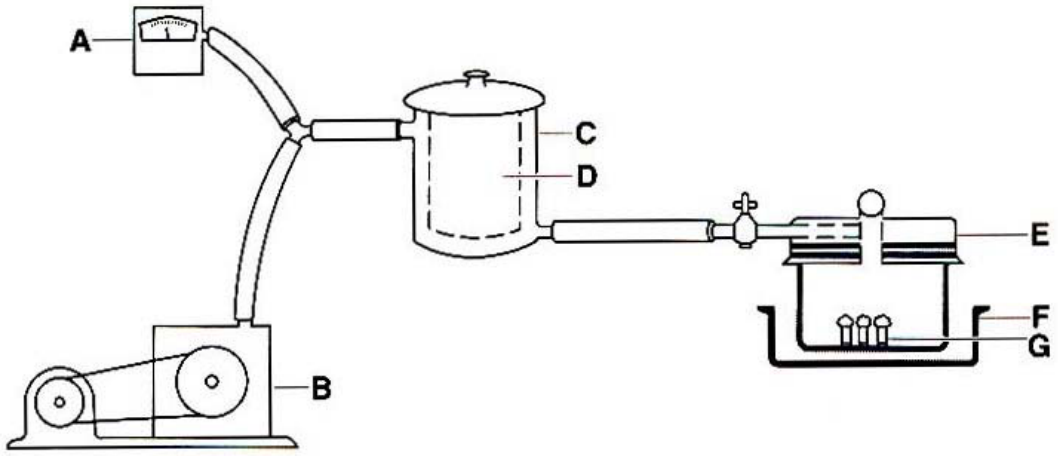
Virus weapons have many advantages over bacteria. They are universally contagious and antibiotics do not work against them. The author plans a book on virus weapons but the main concepts here involve the modification of viruses with new bacteria toxins inserted in them. Flu, cold and other mild virus infections can be modified with toxin producing instructions. Once the virus multiplies in a host, the virus copies also have the new toxic enzyme property that injure the host tissues. Ebola is deadly only because it has an enzymatic effect on blood vessel linings. Any virus can be modified in a similar manner.

Certain populations are more susceptible to specific viruses than others. These can be selectively targeted in a manner similar to that described for human cell lines.

Physical Weaponizing Methods

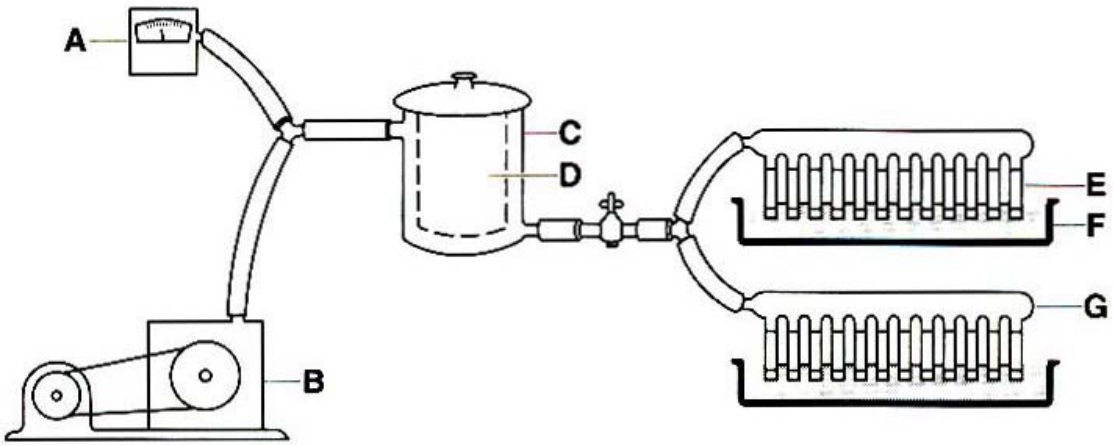
Bacteria must be storable (at least master samples to grow from) in some form in order to be useful. This is accomplished in military weapons via the use of spores in the case of anthrax. If the organism you wish to use does not have spore forming machinery, it can be given the spore forming genes from anthrax, clostridium or coccidioides mold.

Most bacteria are *freeze dried* for storage and later use. This is done not only in the military, but in laboratories all over the country as well. The drawing below illustrates a typical freeze drying chamber. The bacteria are placed in a container in a pan. The air is pumped out while the bacteria are chilled by dry ice. The result is freeze dried cells. This method often eliminates clumping together of cells so that they disperse as tiny individual cells which is used in weaponizing anthrax spore weapons. If the cells still bind together, they grind much more easily in the freeze dried form.



Double-vial Method of Freeze-drying (*A*) Vacuum gauge; (*B*) vacuum pump; (*C*) condenser; (*D*) reservoir filled with dry ice and Cellosolve; (*E*) plastic plate; (*F*) stainless-steel pan filled with crushed dry ice and Cellosolve; (*G*) specimen.

A similar method using pans filled with dry ice is also commonly used and is illustrated below.



Manifold Method of Freeze-drying (*A*) Vacuum gauge; (*B*) vacuum pump; (*C*) condenser; (*D*) reservoir filled with dry ice and Cellosolve; (*E*) specimen vial; (*F*) stainless-steel pan filled with crushed dry ice and Cellosolve; (*G*) manifold.

Bell Curve Weapons Concepts

We earlier described the concept of bell curve weapons. The general idea is that a wide variation of cell types are produced by mutation or other methods. These have many different properties and those with the best growth and infective characteristics are those that successfully do so in a target population. The idea is to produce the many variants and then release them letting the targets act as the screening method for the most effective cell lines. These can be recovered from the infected individuals (or cadavers).

Ordinary bacteria can be made deadly by simply mass producing them and letting nature yield the effective mutants as the army did off the west coast of San Francisco in the 1970's. Some of the bacteria will fit into the "highly effective" end of various bell curves and these bell curves overlap a corresponding bell curve of human susceptibility for the target population. Wherever these bell curves meet in the distribution area, you have successful infection. In the case of anthrax where the capsule gives it nearly 100% protection against host defenses, the bell curve will be approximately equal to the

germination rate in all affected individuals. In less effective bacteria, the bell curves overlap less and the weapon is less effective.

By using combinations of virulence genes, effective contagious weapons can be developed. In this case it does not matter if the bell curves have a large overlap. Only one bacteria cell that is successful inside a host is necessary. The host becomes the manufacturing plant as the new bacteria species or strain spreads affecting all others that fit its properties. Its own new bell curve of infectibility and susceptibility is the only one which applies here.

Some bacteria grow poorly in artificial culture media or the soil may find its biological niche in humans. If a mutation takes place in the soil that causes this, the bacteria often die in the soil without reproducing because it is outside its niche (or soil bell curve). In a human host, the biology changes everything. This is often seen in mutated strains that become obviously superior in animal tests but quickly die off lacking a living animal host. These are the useful parts of the bell curve weapons and are often missed in laboratories that examine them only for a specific change rather than potential effective combinations of changes.

All forms of modification can be used to yield true bell curve weapons. Mutation was already described in this context but it has the limitations of only being able to modify genetic machinery that the bacteria already have inside them.

Fusion, conjugation, transformation and the other methods described will permit bacteria to acquire new genes and the corresponding biological machinery. Individual cloned genes can be obtained from various institutions for insertion directly. These can simply be added to the mix of bacteria under the appropriate conditions.

A process for home-made bell curve weapons can be described as follows –

1. Obtain the bacteria to be modified. If the operator is unable to learn the basic biology taught in all these manuals then unknown bacteria with potential weapons properties can be obtained from manure samples, hospitals, ill individuals and selected soil samples.
2. The samples can be grown on plates or in soups with calcium chloride added at levels of .01 to .5% (experimentation is necessary).
   The calcium chloride causes the cell membranes to become more porous and permits the exchange of genes from its surroundings.
3. Some cells will pick up the desired genes and incorporate them into their own chromosome.
3. The entire mixed sample can be used in direct release weapons,

It is obvious that the above procedure can be improved upon by using selected strains, professional media, specific lab procedures and so on. These have already been described. In the field, many of these are not available to the soldier so improvisation

becomes necessary. The weapons can be delivered as multiplier effects weapons in forms that can be safe for the handlers (see V-6D). The ideal concept is to have an already developed and tested strain that can be delivered to an entire army of bio-warriors overnight.

To yield selected genes from one organism and transfer them to another, you take the first bacteria with the desired genes, grow large numbers of it and then kill the cells and cause them to lyse releasing their genes into the surrounding pool. This solution is then mixed into a grown bacteria culture that you wish to insert them into. The bacteria will take in the new genes in the various combinations and some cells may have the ideal mix. The likelihood of this happening increases with increased sample sizes. These can then be tested in animals for the desired properties and then recovered for weapons production and use.

In genetic engineering, the desired genes are cloned and mass produced. Instead of a mix of genes from one the bacteria, you have only the desired one. This also increases the numbers in your favor.

In 2001, a team of Canadian scientists tested a non-lethal powder similar to the anthrax that was used to attack the US senators. At a dose of $1/10^{th}$ of a gram released in a 10 x18 foot room, occupants inhaled 480 times the lethal dose for anthrax in 10 minutes. It a gram dosage, the occupants received 3,080 times the lethal dose. This illustrates how easy it is to produce massive doses necessary for effective weapons.

In the case of the senate attacks, the senators knew they were exposed and everyone received massive doses of antibiotics immediately to ward off infection. Had the doses been distributed in tiny amounts and mass delivered at levels below the threshold of attack, their would have been no immediate treatment and the origins may have never been identified (thrown away and taken to a landfill before discovery). This also commends the preparations of mutated or modified strains that have various antibiotic resistance. This insures that one susceptible strain is not crucial. Other resistant strains will take its place once the antibiotic regimen takes effect. The use of combined organisms, one of which causes damage that cuts off the blood flow and immune responses, is also synergistic.

Surprise attack always confers special benefits to the attacker (like Pearl Harbor). These types of bio-weapons will always confer surprise because there is no limit to the possible types of combinations that can produced. They can be delivered anywhere easily disguised and are invisible. Had the powder used in the senate attack been distributed by letting it drain into a car exhaust (via a funnel) while driving around the senate building, the effect would have been far greater and the source would have been unknown.

In conclusion, the author wishes to finish with a simple anecdote. At a gun show in January 2002 in Atlanta, three kids came up and wanted to buy my CD. They were not interested in bio-weapons. They were mainly interested in pipe bombs and related explosives so I talked to them about it. Three years earlier, I had worked on the concept

of using gasoline as a fuel air explosive. Getting it mixed into the air via atomization or evaporation in the right amounts for detonation was a problem. The initiator in this system was electrical and in the simplest case is accomplished by using a spark plug and wire running from a coil for an automobile. In the middle of my conversation, I realized that the ultrasonics used in humidifiers to atomize a fog of water could be used here. I had published its use in dispersing biological weapons as part of the humidifier vapor. In this case, I suddenly realized that it would aerosolize and evaporate gasoline. This solves a major material handling problem and puts large scale fuel air explosives based on gasoline into the hands of everyone.

The lesson is that you never know what new ideas can be combined and used in the future. That is why understanding basic sciences is so important. Even useless knowledge can sometimes be converted to potentially great power. In this context, all knowledge is power and this power can be used to balance the power held between governments, their armies and police, and ordinary citizens like you and I.

Enjoy!