Laboratory Exercises for Microbiology Biology 552

Fall Quarter 2002

Department of Biological Sciences
University of Cincinnati

OVERVIEW

Biol 552 has a challenging goal, which is to give students practical experience and training in the fundamentals of microbiology. Practical experience is especially important because microbiology is one of the most experimental branches of biology. With the proper techniques, huge numbers of individuals of a microbial species can be produced, manipulated, and analyzed in ways that are not practical for plants or animals. As a result, many advances in biology have come out of research on micro-organisms. Although this course concentrates on bacteria, many of the skills and experimental methods practiced here relate to other areas, as well, including cell biology, biochemistry, and molecular biology.

The course will challenge you to learn at different levels. The most basic level is simply assimilating the facts and carrying out the procedures as described. This requires you to read the labs ahead of time and come prepared. We also expect you to understand why certain things are done, and what cellular or chemical processes lead to the experimental results that you see. This middle level requires you to ask questions of yourself and the instructors and to think about the underlying principles of the phenomena we study. At the third level, we believe that experimentation on micro-organisms provides an excellent context for demonstrating the scientific method. The course will challenge you to understand this approach and begin to use it. In particular, the last two weeks will give you the chance to formulate experimental questions, design experiments, interpret the results, and communicate them to others.

In this context, we should admit that the course itself is something of an experiment. During the summer of 2002 Zachary Blount (with some input from me) extensively revised the former 552 syllabus, developed several new exercises, and wrote this lab manual. The goal was to produce a course that offers a more rigorous and informative experience working with micro-organisms. Both of us are interested in your feedback as to what aspects of this succeed, and what needs more work for the future.

Dennis Grogan September 2002

Dedication

I owe a great deal to those who first introduced me to microbiology back in my undergraduate days at the Georgia Institute of Technology. As the ones who set in motion the mental events that led to my coming to UC and eventually writing this manual, it is dedicated to them: Dr. Paul Edmonds, Dr. Patricia Sobecky, Dr. Thomas DiChristina, and, of course and most of all, Dr. Thomas Tornabene. I can only dream that I might one day have the effect on students that they had on me.

Zachary Blount

Class Overview:

Session	Date	New Exercises and Material	Continuations	Due
1	Thursday 9/26/02	Administrative Overview Microscope Familiarization Mixed Bacteria Slides Tooth scraping Slide Open plate exercise		
2	Tuesday 10/01/02	Aseptic technique (Effect of technique on broth inoculation) Staining: Simple vs. Gram Staining (Mixed culture and tooth scraping) Capsule stain demo Inoculation of potato slices	Examine open plate	
3	Thursday 10/03/02	Gram staining of broths from last time Practice of Gram Staining with mixed cultures Isolation streaking In groups: Acid fast staining Endospore staining TTC motility medium inoculation Assignment of Purcell paper	Examine aseptic technique broths Examine potato slices	Report 1
4	Tuesday 10/08/02	New Streak plates Hay infusion slides Flagellar stain demo Spreading of dilution series to determine cell density; spectrophotometer readings In groups: FTM medium inoculation TGYA shake inoculation	Examination of plates streaked for isolation Examination of TTC tubes and discussion of results	Report 2
5	Thursday 10/10/02	Anaerobic organisms discussion *E. coli* phage plate preparation Streak plates for GasPak Jars Winogradsky column construction	Examination of streak plates Plate counts for cell density determination Examination of GasPak plates, FTM, and TGYA tubes and discussion	
6	Tuesday 10/15/02	In groups: Growth curve and phage-induced lysis exercise	Titer determination of phage suspension dilutions from last time	Report 3

7	Thursday 10/17/02	In groups: Environmental factors preparation Temperature PH Salinity Alone: Kirby-Bauer test for antibiotic sensitivity UV lethality	Plate counts for growth curve exercise and collection of class data Winogradsky column examination	Report 4
8	Tuesday 10/22/02	Assignment of known cultures: Inoculations for cultural characteristics and metabolic properties I MIC testing of various antibiotics Environmental sampling	Examination of Kirby-Bauer plates (RIS etc.) Plate counts for temperature, pH, and salinity - discussion Data collection for UV exercise	Report 5
9	Thursday 10/24/02	Examination of colonies on environmental plates Gram staining Isolation streaking of selected 4 Inoculation of media with knowns for metabolic properties II	MIC data collection Examination of media for cultural characteristics and metabolic properties Examination of environmental plates Winogradsky column examination	Report 6
10	Tuesday 10/29/02	Criteria for identification of *B. anthracis* Use of Bergey's manual for bacterial identification Gram staining of colonies from environmental streaks Choosing of unknown Inoculation of working and storage stocks for unknown ID Miniaturized multitests	Examination of media for metabolic properties II and Vogues-Proskauer test Examination of environmental streaks	
11	Thursday 10/31/02	Official Gram stain of unknown from working stock Inoculation of media for cultural characteristics and metabolic properties I for unknowns Agglutination exercise Sequence-based identification methods Assignment of 16S sequence exercise	Winogradsky column examination	Report 7

12	Tuesday 11/05/02	Inoculation of media for metabolic properties II of unknowns Introduction to bacterial genetics section UV mutation experiment Intro to group projects	Examination of media for cultural characteristics and metabolic properties I for unknown	
13	Thursday 11/07/02	Use of copies of Bergey's manual to attempt to ID unknowns (Determination of any further tests that might be needed, with orders given to Julie) Conjugation experiment Koch's postulates I Group project sign-ups	Examination of media for unknowns' metabolic properties II Colony counts for UV experiment Winogradsky column examination	Report 8
14	Tuesday 11/12/02	Inoculation of any further tests needed for ID of unknowns Koch's postulates II Group project planning and evaluation	Colony counts for conjugation experiment	
15	Thursday 11/14/02	Koch's postulates III Group projects I	Winogradsky column examination	Report 9
16	Tuesday 11/19/02	Koch's postulates IV Group projects II		**Unknown Report**
17	Thursday 11/21/02	Group projects III Winogradsky column interpretation?	Winogradsky column examination	Koch's Postulates Report
18	Tuesday 11/26/02	Group projects IV Synthetic epidemic?		**Winogradsky** Column Report
19	Tuesday 11/03/02	Presentations		Group Project Reports
20	Thursday 11/05/02	Final and practical		

Laboratory Rules

1. **The organisms used in this lab may be pathogenic. For safety's sake, treat ALL cultures as though they are pathogenic. ALL cultures are to be handled with the utmost care and respect. Aseptic technique is to be used AT ALL TIMES.**
2. **No cultures are to be left in or on the benches. No slides are test tube racks are to be left in the drawers of the benches. At some points during the quarter, however, it will be permissible to have test tube racks in the bench cabinets in which you will incubate most of your media.**
3. **If cultures containing live organism are broken or spilled, notify Dr. Grogan, Julie, or a TA IMMEDIATELY. Do not attempt to clean up the spills without special instructions.**
4. **Do not pour anything containing live organisms into sinks, and never place a used pipette on the bench top. Receptacles containing Lysol are available at the front ends of the benches for the disposal of slides, swabs, and pipettes.**
5. **All work areas must be scrubbed with Lysol BEFORE beginning the day's exercises AND at the end of each lab period. Also, wash your hands carefully with soap and water both before starting work, and before leaving the lab at any time. This is not only for your own protection, but is also to prevent contamination of your experiments that might result in the obtaining of invalid results.**
6. **Do not lick any labels, rub your eyes, or introduce ANYTHING into your mouth while in lab. While in lab, it is best to assume that anything can be contaminated with pathogenic bacteria that can make you very sick. So nothing is to enter your body save for the air you breathe while in class. This means absolutely NO smoking, eating, or drinking in the lab.**
7. **Do not wear anything to lab that you would not mind getting ruined. In addition to working with live bacterial cultures, we will also be using flammable materials and staining solutions that will discolor clothing permanently. It is suggested that you <u>wear old clothes</u>, and preferably a lab coat while in lab.**
8. **You should have at your bench only those materials that you will need over the course of a lab period. All other personal possessions, book bags, coats, and the like should be kept at the coat racks just inside the entrance to the lab.**
9. **Waste paper, towels, tissues, matches, and any garbage generated during the course of class is to be placed in the garbage cans. Do not leave any of this in your desks, on the floor, or in the sinks. Keep your area clean…or else it may become necessary to dock the entire class points on their assignments.**
10. **All inoculated tubes and plates should be clearly labeled with your name, the organism, and the date. This is both for purposes of identification and safety. However, <u>DO NOT WRITE ON THE TEST TUBE CAPS</u>. The writing does not come off easily, if at all. Wax crayons are provided, but SHARPIES work better and it is advisable that you buy yourself a couple at the UC bookstore (Please only red, blue, green, or purple Sharpies only – NO black ones).**
11. **Remove ANY and ALL labels and markings from all tubes, caps, and bacterial culture tubes BEFORE discarding them in the proper receptacles. There are bottles of 95% ethanol in the back of the lab for this purpose. <u>If it is found that markings are not being removed, the entire class may have points docked.</u>**
12. **ANYTHING opened over the course of a lab period – stains, reagents, and cultures, ANYTHING.**
13. **Any supplies that are set out for use during a lab period that are not normally found in the staining boxes or in your benches are to be put back where you got them from when you are finished working with them.**
14. **The inoculating loops and needles used in making aseptic transfers should always be sterilized BEFORE and IMMEDIATELY AFTER use, BEFORE they are laid down. If the loop is covered with infectious material, it should be held at the side of the flame until dry before being sterilized. If this is not done, it is possible for the bacteria to become aerosolized and spread through the air of the lab. To sterilize loops, hold the full length of the wire in the flue flame of a Bunsen burner in as nearly a vertical position as possible, until the wire is red hot. Then pass ½ of the length of the metal handle through the flame, rotating it so that the opposite side will be heated as you withdraw it from the flame. Without setting it down, let the loop cool slightly before use, or about four to five seconds. At the end of the period, you should place your loop and inoculating needle back in the drawer of your bench, being certain that it is sterile before doing so.**
15. **You are responsible for the health and safety of not only yourself, but also of your classmates. Failure to follow the above rules will put yourself and them in danger, and thus result in your dismissal from the lab, and potentially expulsion from the course.**

Directions for Laboratory Work

This laboratory course has been carefully planned to provide you with the opportunity to operationally learn about various aspects of the biology of microorganisms. Please, please, please, view this course as a great opportunity and privilege, and not as a necessary evil to be gotten out of the way (If you do choose to see it as merely an inconvenience, you should ask yourself if you really want to be in college.). If should not be slighted or underestimated. The benefits you will derive from the work you will do in here will be limited only by your own motivation and efforts. Faithful observation of the following suggestions will increase your understanding of the exercises and your efficiency in carrying out the procedures. This is an advanced, upper level course, and dedication is required in order to successfully complete it. Let it be said right here and now that you are not guaranteed an A in this course. You are not even guaranteed passage of this course. Your performance will determine what grade you will receive in this course.

1. **CAREFULLY read the materials for the assigned exercises of a lab session BEFORE you come to class.**
2. **PLAN YOUR WORK. Assemble the materials you will need to carry out the instructions in an orderly and precise manner, to better economize your time. Often items need to be shared – obtain, use, and return them promptly so that others may use them as well.**
3. **Laboratory work begins at the beginning of the period, and continues until the exercises are completed.**
4. **Be in class when class begins. In addition to potentially missing out on important instructions, quizzes are given at the beginning of class. If you miss a quiz because you are late to class, you will not be allowed to make it up.**
5. **LISTEN CAREFULLY to the instructions and lectures given by Dr. Grogan and the TAs.**
6. **Complete notes from the lab are not required for inspection, but the final exam is cumulative and will assume that you know the material gone over in detail, so it is recommended that you take notes for your own study purposes.**
7. **Strive for good technique. Correct technique is essential for safety and accurate results. Procedure is as important as results.**
8. **Be analytical. Observe carefully. A glance through a microscope, or at a plate is never enough.**
9. **Cleanliness and exactness are the keynotes to success in this lab course.**
10. **Save for those times when you are instructed to work in groups, you should do all your own work. Those times when you are to work in groups, you are still expected to know all the ins and outs of the work to be done.**
11. **All lab reports are to be done individually. No teamwork is tolerated. Any evidence of collaboration on lab reports will result in all parties involved receiving zeroes for the assignment.**
12. **There is to be no copying in the completion of your lab reports. All answers to all questions must be in your own words. There are no exceptions. Any evidence of plagiarism will be punished by a loss of all credit for the assignment. If the offense is especially flagrant, you may be reported to the dean, or even be subjected to prosecution (Plagiarism is a felony.).**
13. **Please allocate sufficient time for the completion of your lab reports. They are not that long. They are not that hard. Many of the questions on the lab reports are either thought questions or require library research, so you will not be able to do them right before class. Please also take time to read the questions before answering them so that you may be certain that you are answering the question asked.**
14. **Lab reports will be examined for proper spelling and grammar. Egregious abuse of the English language will be penalized. You have plenty of time outside of class to work on them, so if you are uncertain as to the spelling of a word, consult a dictionary! Please also take care to make your writing legible. If we can not read it, we can not grade it.**
15. **If you have any problems understanding anything or need some extra help, please talk to Dr. Grogan or the TA's. They are happy to help. They do not bite. They are nerds, but they are not vicious demonic creatures that will eat your souls.**

16. **Always remember:**

"…beginning students can reel off the words they have heard, but they do not yet know the subject. The subject must grow to be part of them, and that takes time." -- Aristotle

Common Terminology Problems

There are many terms you will encounter in this course that will be unfamiliar to you, and it is possible that many of the terms you will encounter are improperly used in the "real world". The following is aimed at helping your with a few of these.

Singular and plural

It is quite common for plural forms of a term to be used when the singular is appropriate. The most common ones are listed below. If you are ever confused by them, please consult the list.

Singular	Plural	Definition (Singular)
bacterium	**bacteria**	**A microorganism belonging to the domain Bacteria**
archaeon	**archaea**	**A microorganism belonging to the domain Archaea**
coccus	**cocci**	**A microorganism with a roughly spherical shape**
bacillus	**bacilli**	**A microorganism with a rod-like shape**
spirillum	**spirilla**	**A microorganism with a roughly cork-screw shape**
medium	**media**	**A substance, liquid or solid, used to grow microorganisms**

Diseases vs. Organisms

It is quite common in popular media for a disease to be confused with the organism that causes it. In reality, a disease is a condition or illness that may be the result of the interaction of an organism with the body of the person suffering from the disease; in short it may be caused by an organism. Thus, plague is a disease caused by the bacterium *Yersinia pestis*. *Yersinia pestis* alone is not a disease. Plague is an effect, and *Yersinia pestis* is its cause.

Bacterial Names

Bacteria and other prokaryotic organisms, along with most other microorganisms, often have no common names, so they are referred to by their Linnaean, or binomial names. These names are made up of their genus and species names.

Examples:

Genus	Species
Eschericia	***coli***
Bacillus	***subtilis***
Proteus	***vulgaris***
Sulfolobus	***solfataricus***
Mycobacterium	***tuberculosis***
Staphylococcus	***aureus***

A species is a group of organisms that share most all of the same characteristics and share a close common ancestor (i.e. A close evolutionary relationship.). A genus (plural form: genera) is a larger group of organisms that share a smaller set of similar characteristics. It may be considered a group of species.

Please note from the table that the genus name is ALWAYS capitalized, while the species name is NEVER capitalized. Whenever the binomial name of an organism typed, it is italicized. When it is handwritten, it is best to underline in.

Lab Session 1

Background

I. Introduction

(See Brock, Chapter 2)

If you were to think of a "typical" living thing, you would be likely to think of some sort of plant or animal, or perhaps even a fungus. In any case, it is quite likely that the organism you would have in mind would be visible to the naked eye. The fact of the matter is, though, that, because we are very restricted in our views of the world to things that are within only a few orders of magnitude of our own size, we miss much of the world. Most life is very small; microscopic in fact.

Figure 1.1 is a depiction of one of the most important scientific achievements of all time: the **universal tree of life** that displays the evolutionary relationships between all known cellular life on Earth (See Brock pp. 26 – 27). In the figure, you will notice that the whole tree is contained within a box, save for the twigs at the very top of the branch labeled **Eucarya**. These twigs, the animals, plants, and fungi, represent the macroscopic, multicellular organisms of the world – those you were most likely to thin of a moment ago. Everything in the box is microscopic. This means that most of the diversity of life, both past and present is microscopic. This realization can be a bit humbling.

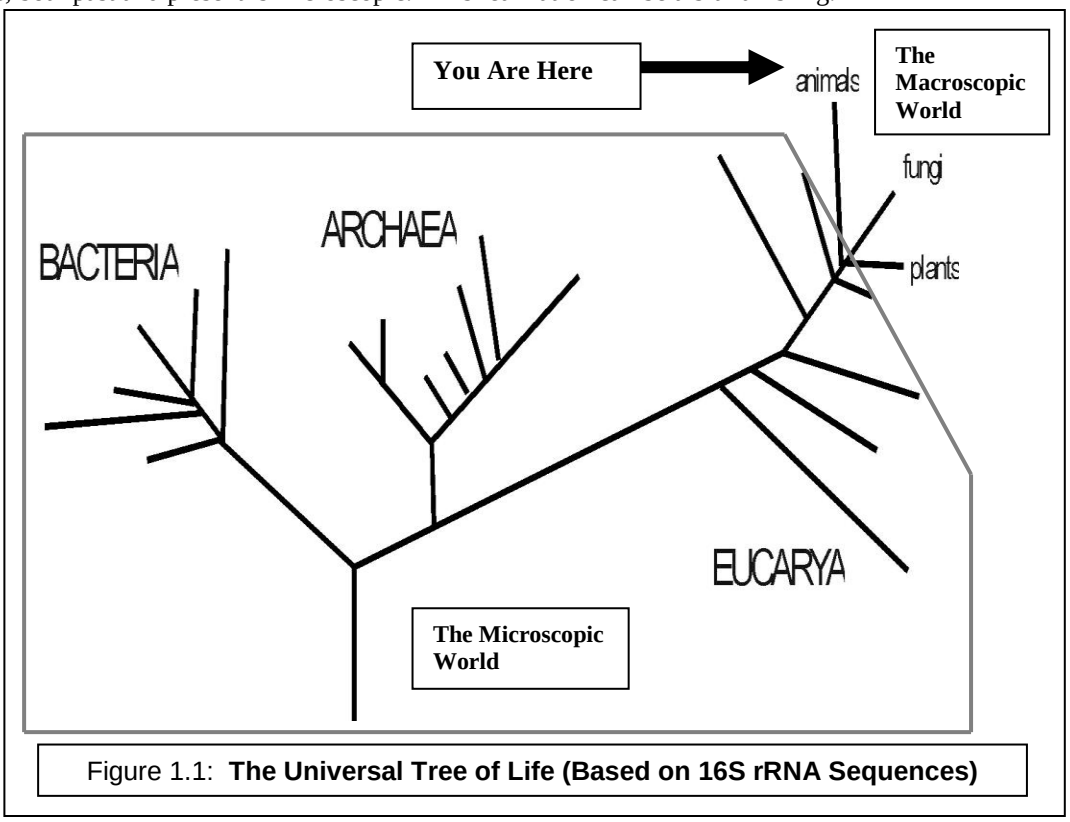


Figure 1.1: **The Universal Tree of Life (Based on 16S rRNA Sequences)**

Within the box you will notice that there are two other major branches to the tree. One is labeled as **Bacteria**, a group of organisms you know best for its disease causing members, while the other is labeled **Archaea**, a group of organisms of which you are likely to have heard little, if at all. These are the **prokaryotic** domains, meaning that they are large groups of organisms that lack nuclei or membrane-bound organelles, and that are most all unicellular. These domains are full of wonders. There are prokaryotes that can eat gasoline and breathe uranium. They have been found living in such unimaginable conditions as boiling volcanic pools, burning coal piles, Antarctic ice, deep sea chunks of frozen methane; in lakes saturated with salt, in distilled water, acid so concentrated it would eat the flesh off your bones in mere seconds, even in tiny cracks in rock miles below the surface of the Earth. There are even some that have survived years in the airless cold of the surface of the moon. One type of bacterium is even claimed to have been revived after being dormant for 270 million years.

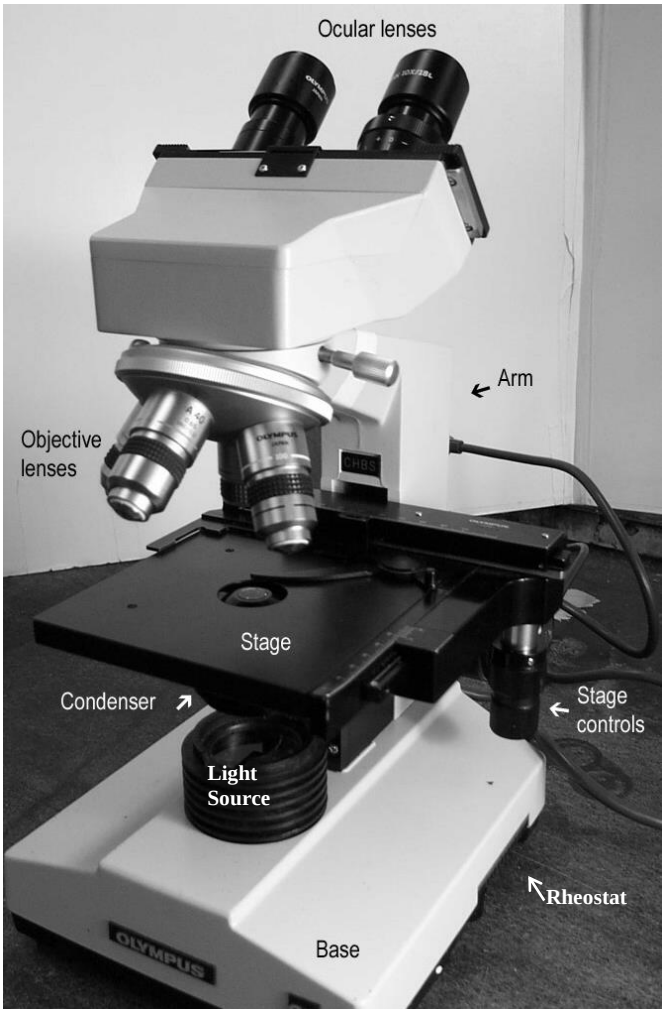
Prokaryotes are spoken of rather little in most biology classes, as though their small sizes were proportionate to their importance in the world. Even the scant attention paid them in other classes is much more than that given them by the popular media. It is likely that the only times you hear of prokaryotes in the news is when some bacterial disease crops up somewhere. While it is true that there are some bacteria that can cause disease (No archaea that we know of do, however.), these are very few in number, being only a few dozen species amidst perhaps millions. The fact of the matter is that not only are prokaryotes such as bacteria important, but they are essential. The world would not work in

such a way as to allow life to exist if they did not exist. Most biogeochemical cycles regulating the flow of important chemicals through the planet are driven by the activity of prokaryotes – including oxygen and carbon. They are also intimately bound to humans as well. It has been estimated that 10% of a human's mass is made up of prokaryotes residing on and in the body. They cover your skin and coat the inside of your mouth. They are in the food you eat, the air you breath, and the water you drink. This is usually for the best, for it allows your resident **microflora** to be constantly replenished, and this is good, because you need them. You would be unable to digest most food properly were it not for the organisms present in your intestines. To put not too fine a point on the matter, were all eukaryotes, yourself included, to disappear from the Earth, the prokaryotes would not care a bit. They would carry on very well. Were they to all disappear, all the eukaryotes in the world, yourself included, would die rather painful deaths. Now that is humbling.

II. Microscopy

(See Brock, Chapter 4, Part I)

It is strange to imagine, if we consider how important prokaryotes really are, that we had only the vaguest idea they existed for most of our history. Indeed, it was not until 1676, when an ill-educated Dutch fabric merchant with an uncanny knack for grinding glass lenses named Leeuwenhoek discovered what he called "animacules" with a homemade microscope. Based on the careful drawings he made during his studies over the course of nearly sixty years, many of the "animacules" were definitely bacteria. While the existence of invisible organisms that did such things as cause diseases and sour milk had been suspected by various Greek and Renaissance philosophers, it was not until Leeuwenhoek that their existence was taken seriously.



Picture 1.1: **A Standard Compound Microscope Used in this Class**

If nothing else, this makes clear just how important the microscope really is to microbiology, for how would anyone get the idea to study the microbial world if they had no way of knowing it was there to begin with? In short, microbiology began with the microscope, and it should come as no surprise that microbiology lab begins in the same place.

The microscopes we will be using this quarter are called **compound brightfield microscopes** (See picture 1.1). They use multiple lenses to generate a magnified image of the object under observation against a bright background. This is as opposed to a simple microscope with a single lense. Compound microscopes are complicated pieces of equipment, with many components that contribute to their function, as you can see in picture 1.1. The basic structural frame is composed of the **base** and the **arm** rising from it. Housed in the base is the **light source** that may be turned on and off by the on/off switch on the side of the base. Also on the side of the base is the **rheostat** that controls the flow of power to the light source. The light source shines upward into a **condenser** under the **stage** that holds the slide under observation. The condenser focuses the light impinging upon it through a small hole in the stage and then through the sample on the slide. The stage may be moved back and forth and from side to side to change the portion of the slide through which the light passes. This light passes upward first through one of the **objective lenses**, through the head to the **ocular**, or eyepiece lenses. The microscopes we will use this quarter have four objective lenses (See table 1.1) mounted on a rotatable **nosepiece** that allows each to be swung into operational position above the slide. The object on the slide is magnified by a total factor equal to the product of the magnification of the

objective lens times that of the ocular lens (See table 1.1). The object on the slide is brought into focus by altering the distance between the slide and the objective lens by raising and lowering the stage. This is done by rotating the **coarse and fine adjustment knobs** on the side of the microscope.

Objective Lens Name	Objective Lens Magnification	Ocular Magnification	Total Magnification
Scanning	4X	10X	40X
Low Power	10X		100X
High Dry	40X		400X
Oil Immersion	100X		1000X

Table 1.1: **Objective Lenses and Total Magnifications of a Standard Microscope for this Class**

The magnification of a microscope may be increased indefinitely, but beyond a certain point, simply increasing the magnification does little to improve the images that may be obtained. This is because the **resolving power** of a microscope is just as important as its magnification. Resolving power refers to the minimum distance that must separate two adjacent objects before they can be seen to be separate objects, and is determined in part by the wavelength of the light used in observation. Because of this, the limit of resolution of most visible light microscopes is 0.2 μm (Electron microscopes, which, of course, use electrons rather than the visible spectrum to generate images of specimens, are capable of reaching resolutions as great as 0.5 nm, permitting the degree of useful magnification to be as high as 100,000X.).

You have likely noticed that the most powerful objective lens on the microscopes we will be using is referred to as the **oil immersion lens**. This comes from the necessity of placing a drop of oil on the slide before rotating the lens into place. The oil is a special kind used in microscopy because its index of refraction is the same as that of the glass used in the lens. This permits light passing through the object on the slide to be better guided into the lens. This helps to increase the resolution at higher magnification. If immersion oil were not used, an image would still be obtained, but it would be blurry to the point of not yielding any useful information.

The exercises of today's lab session are focused on getting you familiarized with basic microscopy in general and the microscopic observation of prokaryotes in particular. This will first involve the microscopic observation of a slide of mixed bacteria. There are a number of things that you should get out of this exercise. For one, though bacteria and other prokaryotes come in sizes ranging in diameter from 0.3 μm (*Mycoplasma species*) to up to 750 μm (*Thiomargarita namibiensis*), they tend to be uniformly tiny with an average diameter between 1 and 2 μm, a bare fraction the size of the typical human cell, as you can see in figure 1.2. Indeed, one thing you will notice as you observe them under the microscope is that, at lower magnifications, they appear as little more than grains of dirt, and at higher ones little can be discerned aside from their shapes, or **morphologies**. There is a great diversity of prokaryotic morphologies, but the ones you will encounter most frequently are **bacilli**, or rods, **cocci**, or spheres, and **spirilla**, or corkscrews (See figure 1.3 and Brock, pp 64 – 66). The slide you will examine today will include a mixture of bacteria displaying these three basic shapes. While you are examining it, think also about the arrangement of the cells. There are four basic cell arrangements: **diplo**, in which you usually see two cells joined together, **staph**, in which the cells are in bunches and clusters, **strep**, in which cells are in a chain, or random, in which there is no discernable order.

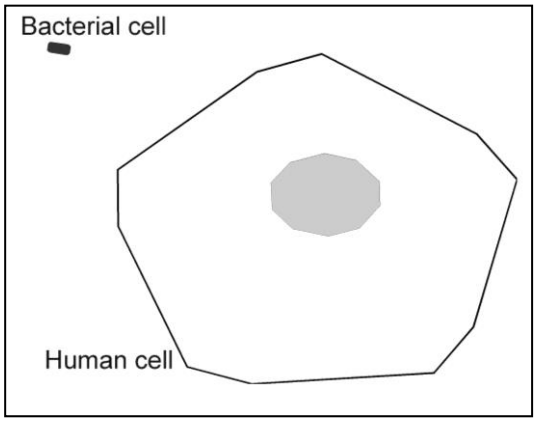


Figure 1.2: **A Typical Bacterial Cell Compared to a Typical Human Cell**

You will likely also notice on this first slide the brilliant colors of the cells. It is important that you realize that this coloration is not natural. Most prokaryotes, and indeed, most microbes in general tend to lack any coloration. This, combined with their small size, makes them difficult to observe even with excellent optics. This problem was dealt with early on in the history of microbiology through the development of many special dyes, or **stains** with which to color the cells and make them easier to observe. While there are now available special microscopes called phase contrast microscopes that make it easy to observe cells in their natural states without the aid of stains, staining is still a crucial technique for the microbiologist.

A typical stain is composed of a solution of a charged **chromophore**, or color-bearing molecule, and an ion such as choride^{-} or sodium^{+}**.** Bacteria and most other microbes have a slight negative charge to them, so most stains used

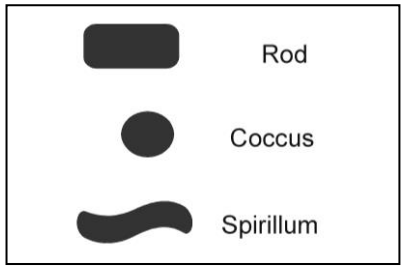


Figure 1.3: **The Three Basic Bacterial Morphologies**

with them are basic, meaning that the chromophore is positive in charge, thus permitting it to stick to the cells. Such basic stains are also referred to as **direct stains**, as they stain the cell directly, permitting it to be directly observed on the basis of the contrast of their coloration with the bright, white background. However, there are instances in which acidic stains with negative chromophores are useful. In these cases, referred to as **negative stains**, the role of the stain is to color the background, allowing the cells to be seen as clearings on a colored background. For the most part, you will only be doing direct stains this quarter.

Today you will be performing a direct stain on a smear prepared from the bacteria-laden material that develops between your teeth, what is called a toothscraping. Toothscrapings were a favorite subject for observation by Leeuwenhoek. There is a story of how overjoyed he was to meet an old man who claimed to have never cleaned his teeth in his life, as he had noticed that his animacules were especially abundant in the mouths of those not given to good dental hygiene. Keep this in mind as you view your stained toothscraping and see the diversity of organisms in your own mouth.

Microscope Rules

You are to use the microscope you sign up for this entire quarter. Do not switch microscopes without permission or first consulting a TA.

The microscopes we use in here cost roughly $1,500 a piece. If you do not carefully follow the instructions and rules regarding their use, you might well break one, and if you do, you will be paying for it. In other words: we mean it with these rules. Ignore them at your peril.

Microscope Rules I: Getting Your Microscope and Preparing it for Use

1. **When you need to use your microscope, you can retrieve it from its cubby in the microscope cabinet on the side wall of the lab.**
2. **When you are moving your microscope from its cubby to the bench, vice versa, or any other time, you must hold it with both hands. One hand should support it under the base, while the other holds it by the arm.**
3. **Once at your bench, remove the dust cover and place it in the large drawer of your bench to make sure that it does not get close to any open flames from the Bunsen burners.**
4. **Make sure the rheostat on the side of the microscope is set to zero, and plug it in.**
5. **Turn on the power switch, and slowly increase the rheostat to between 4 and 6. NEVER increase the rheostat to above level 6. Doing so feeds too much power into the bulb, shortens its life, and bulbs are expensive. If you need more light on the sample, do this by adjusting the iris diaphragm below the stage.**
6. **If it is not already, lower the stage as far as you can. Make sure the 4X objective lens is in place. Do this every time you get ready to put a slide on the microscope for examination.**

Procedures

Exercise 1: Microbes are Everywhere

As you have been told already (And you are likely to hear it again.), microorganisms are to be found just about everywhere on Earth. This exercise will very easily demonstrate this, though you will not find out the results until next time.

1. Go to the front of the bench, get an NA plate, and take it back to your workstation. "NA" stands for "Nutrient Agar", a substance that contains all the minerals and nutrients required for a wide variety of microorganisms to grow, as well as a solidifying agent called agar.
2. Take the top off the plate and place it to the side. Leave the plate open like this until the end of the period. If you like, press one finger onto the surface of the plate just hard enough to leave a fingerprint.
3. At the end of the period, place the top back on the plate, turn it over, and use a wax crayon from one of the drawers of your workstation to write your name and the date on the bottom. Place the plate upside down in the cabinet of your workstation (This cabinet will be the place that you will incubate most of the cultures you inoculate this quarter. You can think of it as a room temperature incubator.).

Exercise 2: Microscopic Examination of a Slide of Mixed Bacteria

In this exercise, you will use the microscope you have been assigned to examine a slide of multiple types of bacteria. These represent the three different basic bacterial morphologies of rod, coccus, and spirillum. As you examine the slide, try to find a field of view in which you can see all three. Keep in mind that the helical bacterial on the slide do not look so much like corkscrews as they do commas.

1. Go to the front of the bench, where you will find a wooden box holding the prepared slides. Choose one (They are all the same.), and take it back to your work area.
2. Make sure that the rheostat on your microscope is turned to zero, turn the microscope on, and then move the rheostat to between 6 and 7. Make certain that the stage is as far down as it will go, and that the 4X, or scanning lens is in place. Take the slide, and place it on the stage, making sure that it is securely held by the slide holder.
3. Once the slide is in place, move the stage as far up as it will go. Adjust the ocular lenses so that they are comfortable for you. As you look through the oculars, use the course adjustment knob to slowly lower the stage. With the slide you are using for this exercise, when you get it in focus, the image you will find at this magnification will likely appear as just grit with a bit of color to it. This can present some problems. As you lower the objective, you might slowly see an image that could look as you expect coming into "focus". Unfortunately, it is common for dust on the optics to be the first thing you see, so you must check to make sure that the image you have found is that of what is on the slide. To do this, you must move the slide using the slide adjustment knobs. If what you are seeing is on the slide, the image should move as you move the slide. If it does not, then keep moving the stage downward until you find a good image. When you do so, check to make sure it is on the slide. Once you have gotten the proper image in focus, find a decent field of view and draw it in the space provided in the report page, being certain to provide a description of what you see (Include any colors or details that are apparent.), as well as the total magnification.
4. Once you find the image with the 4X objective lens, swing the 10X, or low power objective lens into place. Do not raise or lower the stage before you do this! Remember that this is a parfocal microscope, so you should only need to use the fine adjustment knob to bring the image into focus. You should be able to see a bit more detail than you did before. Find a good field of view and draw it, providing a description of what you see along with the total magnification.
5. Once you are done with the 10X view, swing the 40X, or high-dry objective lens into place. Do not raise or lower the stage before you do this! You will likely find when you look through the oculars and try to focus with the fine adjustment knobs that you really cannot find a nice, crisp image. This is not unusual. You are running to the problem of light being scattered between the slide and the lens. Just try to get it as focused as you can. You should be able to see a good bit more than you could with the earlier two lenses, and it should now be possible to make out individual cells fairly clearly. If they seem to be a bit dark, try opening the iris diaphragm until it looks better. Find a good field of view, draw it, and provide a description inclusive of the total magnification used. Try to make this field as good a one as possible so that you will not have to search much for an appropriate view while using the oil immersion lens (It is a little trickier to use, as you will find out, and this step may well save you some time.)
6. It is now time to use the 100X, or oil immersion objective lens. This lens is a bit different than the others. Before you swing it around, take out a piece of lens paper, and carefully clean the lens. Now swing the lens into place without moving the stage. It might appear as though the lens will not have room to swing around without touching the slide, but if you look closely, you should be able to see a small distance between the lens and the slide.
7. Now that you have been reassured that the lens is not going to hit the slide, swing away the 100X objective so that you do not have a lens in place above the slide. Get out the small vial of immersion oil that is in the wooden box at your workstation. Unscrew the top. You will notice that there is a small, glass rod attached to the bottom of the lid. As you draw this rod out, rub it against the edge of the bottle top to remove excess oil. You will then use this to place a small drop of oil on the slide, right under where the lens is when it is swung into place. Put the top back on the vial of oil and swing the 100X objective lens into place. If you look closely, you will see that the drop of oil now bridges the gap between the slide and the lens. This funnels more light into the lens, and permits a sharper image.
8. When you look through the oculars, you might be disappointed to find that an image is not immediately apparent. This is to be expected. You need to first open the iris diaphragm all the way. To bring the image into focus, you will need to fiddle with the fine adjustment a bit. Use the slide adjustment knobs to move the

slide a bit as you look through the oculars. The unfocused image often looks like a shadow or such, and is unrecognizable. By watching for what moves when you move the slide, you can figure out better what in your field of view is what you are looking for, and what you need to work on focusing with the fine adjustment. Often times, slowly turning the fine adjustment knob toward you works best in getting the proper focus. (Note: You have to be careful in doing this. Turning the fine adjustment knob in this direction lowers the objective lens, and if you are not careful, you can press the lens into the slide so hard that the slide actually breaks. You really do no want to do this. In addition to being something that is frightening, it can do real damage to the microscope.). It is usually hard at first to get a good image with the oil immersion lens, so do not get discouraged if you have a hard time at first. Feel free to call for a TA to help you.

9. Once you have gotten an image in focus, and have found a good field of view with a variety of bacterial shapes apparent, draw it and provide a written description inclusive of the total magnification in the report section.

Exercise 3: Microscopic Examination of a Toothscraping

You have likely noticed that scraping your teeth with a fingernail sometime after having brushed produces a whitish, pasty substance. This substance is made up of residues of food, as well as bacteria that are growing on this residue (As these bacteria produce acids that can eat away at enamel as a byproduct of their metabolic processes, this is why it is a good idea to brush after meals.). There are a great many bacteria that inhabit the mouth, and, indeed, the human mouth is among the most septic in the animal world (This is why a human bite is more dangerous than a dog bite.), making it a good place to find abundant bacteria for observation. As mentioned in the introduction, in this exercise, you will be taking a sample of the bacteria in your mouth, preparing it for microscopic examination, and then observing it. Preparation of a sample requires that you obtain the sample, make a **smear** of it, **heat fix** it, and finally stain it.

1. Go to the front of the bench and obtain a sterile toothpick, a fresh microscope slide, and a Gram stain pen. Take these back to your bench.
2. Using the Gram stain pen to draw a large oval perhaps two inches long (5 cm if you do not like English measurements.) in the center of the fresh microscope slide.
3. Slowly turn on the faucet at your workstation, and adjust to a very slow drip (Be very careful not to turn the water on too much.). Once you have this dripping going, pick up your microscope slide and hold it under the drip so that you get a single, small drop on the slide in the middle of the circle you drew for step 2.
4. With the toothpick (The flat end works best.), scrape between a couple of your teeth until you see a bit of that whitish paste on the end. This is your sample. Touch the end of the toothpick with the sample to the drop of water on the slide, and swish the drop around, trying to cover the area of the slide inside the circle you drew (It is not necessary to cover the entire area, but you need to spread the sample out as much as possible.). See A in figure 1.4. Congratulations! You have just made your first smear!
5. Set the slide aside until the water on it dries, as depicted in B in figure 1.4. While it dries, do step 6.
6. At this point you will need to turn on your **Bunsen burner**. If you will open the large drawer at your workstation, you will find the burner you will be working with. If you do not find a burner in your drawer, tell a TA, and one will be obtained for you. Take this out, along with the box of matches that should either be in the same drawer, or in the smaller one beside it. Plug the rubber hose attached to the burner into the gas port next to the faucet. Light a match, and then immediately open the gas valve. Hold the match above the burner

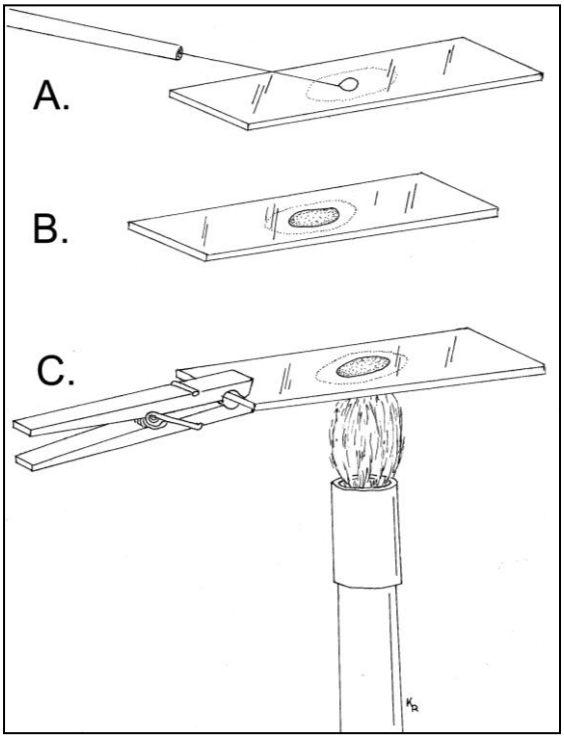


Figure 1.4: **Preparation and Heat-Fixation of a Smear for Staining**

while you do this. You should quickly have a small, blue flame perhaps two inches high. If you do not, adjust the gas flow until you do. If you have any problems, please ask a TA for help. Once you have the burner going, place the slide you just prepared next to the burner. It will be a bit warmer here, so it will dry faster.

Be very careful with the burner while it is lit. Be aware of where it is in relation to yourself at all times. Be very careful not to lean over the flame (If you need an object lesson about this, ask Dr. Kinkle about the time he did it.) or allow your clothes or hair to get near it!

7. Once the slide is dry, you will have to heat fix it. Heat fixing is important for two reasons. First, it will kill the bacteria on the slide, and, second, it will make the cells stick to the slide. To heat fix the smear, slowly pass the part of the slide with the smear on it over the flame of your burner three or four times as shown in C of figure 1.4 (Not too slowly, or you will end up heating the smear too much, and thus burning it. Not too fast, either, or you will not completely heat fix it. You know you are doing it properly if you pass the slide over the flame just fast enough to see a bit of condensation appear and then disappear from the bottom.).
8. Get a clothes pin out of one of the drawers of your workstation and attach it to the end of the slide. With this pin attached to the slide, place the slide on the slide holder (It looks like two glass rods held together by rubber hoses on the ends.) that straddles the sink at your station (Always try to keep a slide over the sink while you are staining it, as it is generally better for stain to spill into the sink than on you or the bench top.). You will now stain the smear. Find the bottle marked "Methylene Blue" in the wooden box from which you earlier got the vial of immersion oil. Use the dropper attached to the top of the bottle to drip the stain onto the slide, completely covering the part with the smear. Allow the stain to stay on the slide for 1 minute.
9. While you are waiting, get out the pad of blotting paper (It might be labeled "Bibulous Paper".) that you will find in one of the drawers of your workstation, and adjust the faucet, so that the drip is a bit faster. Once the minute is over, you will need rinse the stain off the slide. Hold the slide under the drip at an angle so that the water hits it above the smear and then flows over it. Rinse the slide until the water running off it is clear.
10. Blot the slide dry by placing it between a few pages of the blotting paper in the pad, and gently pressing on it (Do not press too hard, or you will lose the smear.). Never wipe a slide to dry it, as this will wipe the slide clean as well. Blot the slide until it is mostly dry.
11. You now need to examine the slide as you did earlier with the prepared slide, following the same procedure. As before, when you have found a good field of view under each of the objective lenses, you need to draw it and provide a written description and commentary. By the time you get to the oil immersion objective, you will likely be able to see a good variety of small, bacterial cells of a number of different shapes, as well as very large cells. The bacteria you can not really know the identity of, but those other, large cells you can identify very accurately. Be sure to note what they are.
12. When you are finished, dispose of the used slides by placing them in one of the Lysol bins in the front.

Note: If you notice large numbers of long, corkscrew-shaped bacteria, this is a problem. These bacteria are the origin of some forms of gingivitis, and you likely need to work on brushing and flossing better.

Preparation for Next Time:

There is a lot to cover next period, so be sure that you read ahead and know very well what will be expected of you. It would help to make out a flow sheet

Microscope Rules II: Storing Your Microscope

1. **Turn you rheostat to zero and turn off the power.**
2. **Lower the stage as far as it will go and turn the nosepiece so that the 4X objective lens is in place.**
3. **Use a piece of lens paper (NEVER, NEVER, NEVER use a KimWipe!) to clean any remaining oil from the oil immersion lens.**
4. **If it is still there, remove the slide from the slide holder, and move the slide holder to the right so that there is no part of it sticking out on the left side.**
5. **Wind the power cord around the microscope, and replace the dust cover.**
6. **Carry the microscope to the correctly numbered cubby using both hands.**

The above storage procedure is to be used every time you store your microscope. Microscopes will be periodically checked through the quarter to ensure that they are being properly stored. Points will be deducted from those who are found to not be properly storing their microscopes.

Lab Session 2

Background

I. Aseptic Technique

(See Brock, pp 109 – 110)

As was mentioned last time, microorganisms are to be found everywhere, a principle that should be apparent from examining the plate you left exposed to air last session. This can present major problems with contamination. Microbes are much too small to study a single cell at a time as an ornithologist might study a single bird at a time. It is thus necessary to study large populations of them in what are called **cultures**. Cultures are grown in **media** that contain all the nutrients required by the microbe to live and reproduce, and that can be sterilized to permit it to be seeded, or inoculated, with only the microbe of interest. Unfortunately, due to the ubiquity of microorganisms, it is very easy for sterile media, and even grown cultures themselves to be contaminated by microbes from the environment. This can result in very inconsistent results and can utterly ruin experiments. Indeed, this problem with contamination was one of the reasons why so many thought microbes were spontaneously generated for such a long period of time. One of the most important advances in microbiology during the nineteenth century was a series of procedures designed to prevent contamination and collectively known as **aseptic technique**.

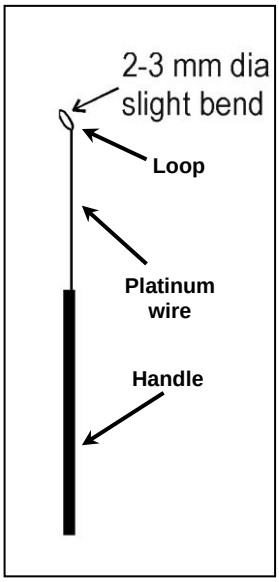


Figure 2.1: **An Inoculating Loop**

It cannot be stressed enough that aseptic technique is of the most crucial parts of microbiology to learn and understand. You will not do well in this course if you do not learn aseptic technique. You will learn the cornerstone of aseptic technique, **sterile transfer**, today in exercise 1, which is designed to show you just how crucial it in fact is. Sterile transfer involves using an **inoculating loop** (See figure 2.1) to either transfer a sample of a microbe of interest to a medium for its growth (In which case it would be called an **inoculum**.), or to a slide for the making of a smear. There are a number of steps involved in these procedures, as you will find in exercise 1. The entire point is to prevent contamination of the sample or culture that is being transferred by outside organisms, and then to prevent contamination of the new medium being inoculated by these same outside organisms. Keep this in mind as you perform exercise 1, and try to understand how the steps involved relate to these ideas.

II. Differential Staining

Last session you learned how to do a direct stain. What you did is more precisely termed a "**simple" direct stain**. It is called simple because it involves only a single stain that turns every type of cell in the smear the same color. Simple stains can be of great value when one is dealing with only one single kind of cell. There is another class of staining techniques that are referred to as **complex stains**. These permit one to use multiple stains to differentiate between different kinds of cells; for this reason, they are more commonly called **differential stains**.

The most important and historically useful differential stain is that developed by and named after the Danish pathologist Christian Gram in 1884. This stain divides bacteria into **Gram positives** and **Gram negatives**. **Gram staining** had been in use for a long time before it was finally discovered that it works by differentiating bacteria on the basis of the architecture of their cell walls. As you can see in figure 2.2, Gram positive bacteria have a thick cell wall of peptidoglycan surrounding their cell membrane, while Gram negative bacteria have a thin peptidoglycan cell wall between their cell and outer membranes (See Brock, pp 74 – 81).

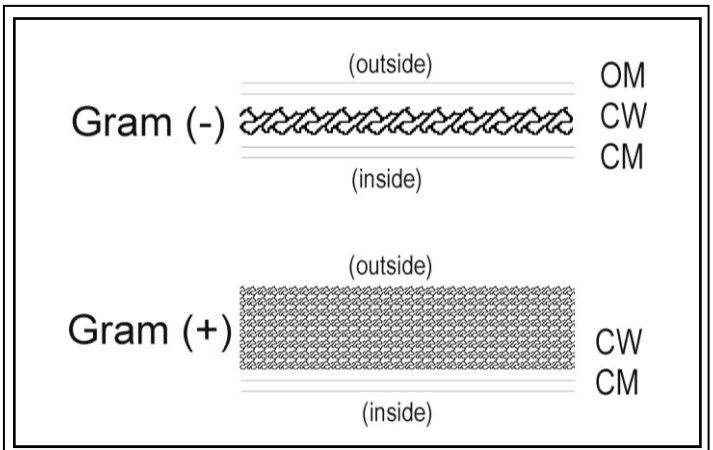


Figure 2.2: **Molecular Architecture of Bacterial Cell Envelopes**

In the Gram stain procedure, there is an initial

staining of cells with crystal violet, the **primary stain**, followed by the application of a **mordant**, Gram's iodine. The iodine complexes with the chromophores of the crystal violet, causing them to bind to the cell walls of Gram positive cells. In the next stage, the cells are **destained** with ethanol. Provided this destaining is brief enough, the result is the loss of the crystal violet from the cell walls of Gram negatives, while it is retained by those of the Gram positives. Safranin is then used to as a **counterstain** that turns the decolorized Gram negatives red, permitting them to be clearly contrasted from the purple Gram positives.

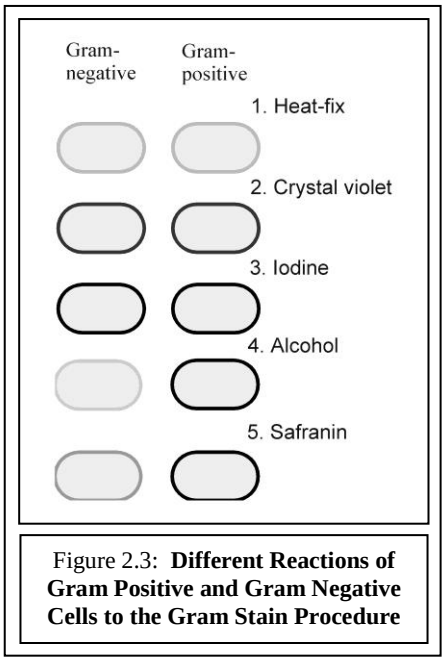


Figure 2.3: **Different Reactions of Gram Positive and Gram Negative Cells to the Gram Stain Procedure**

Gram staining can be a bit tricky, but it is not generally hard to get the hang of. Today you will be practicing on a mixed culture of Gram positive and negative bacteria. To allow you to see how a differential stain can highlight differences not perceptible with a simple stain, you will first simple stain this same culture. You will also be Gram staining a toothscraping such as the one you simple stained last session. This is an even more mixed culture, of course, and it will give you a chance to determine the Gram stain reaction of the bulk of the bacteria in your mouth.

There are a great many types of specialized differential staining techniques. Many of these are designed to specifically allow the observation of cell structures present in only some bacteria. One of these is the **capsule stain**. A **capsule** is a gelatinous substance composed primarily of carbohydrates and proteins that is secreted by and surrounds many prokaryotes. Capsules have a number of functions for those organisms that produce them, ranging from food storage to protection, along with a great many more (There is a question about this in the report section. I am not going to give you the whole answer. There would be no point in asking the question otherwise.). For humans, capsules are important because they are linked to an increase in the **virulence** (Disease-causing potential.) of various pathogenic bacteria, and because of their industrial value. Xanthan gum, for instance, is a solidifying and emulsifying agent commonly used in a great many foodstuffs derived from capsules produced commercially from large batch cultures of *Xanthomonas campestris*, a Gram negative rod. The problem with observing the capsule microscopically is that it is very fragile, being easily destroyed by heat fixation, and it does not retain stains well. The capsule stain thus relies on a combination of negative and direct stains. You will today examine a prepared slide of a capsule-stained smear. There are more details on the procedures involved in this staining process in the instructions for the exercise.

III. The Original Solid Growth Medium

Any substance used to grow microbes is referred to as a **growth medium**. Growth media, of which there are an innumerable variety, can be either liquids, such as the broths that you have already used this session, or solids, such as the NA plate that you used last session. The problem with broth media is that organisms are not localized in them, making it difficult to ensure that one has a pure culture of only a single type of organism. However, solid media provide a large surface area on which organisms may be localized (We will get into this aspect a great deal more in the future.), and thus give rise to discrete populations of those organisms. This then allows one to produce a culture one knows to be at least initially pure.

Media are today usually made to exacting specifications, and can be solidified by an agent called "agar", a substance derived from algae and originally used to make jams and jellies. Prior to their advent, various concoctions of unknown constitution such as beef broth were used for culturing. The original solid medium culture was the humble potato, sterilized and sliced (Imagine bags of potatoes as standard lab equipment! This seems like a good place for a Dan Quayle joke, but I will refrain.). While providing a large, firm surface area for microbial growth, potatoes had their problems. Today, you will inoculate a potato slice medium with a bacterial culture, and it is hoped that some of these problems will become clear to you.

Procedures

Part 1: Follow Up from Session 1

Get the plate with which you worked last time out of your bench cabinet. Examine the plate, providing a description, drawing, and commentary in the space provided in the data section for it in report 1. Be sure to answer all the questions. When you are finished with the plate, you may dispose of it by putting it in one of the biohazard bags in the back.

Part 2: Today's Exercises:

Exercise 1: Demonstration of the Necessity of Aseptic Technique

Materials needed: 3 NB (broth) tubes
1 Tube of *E. coli* broth culture
Test tube rack

1. Collect the materials listed above, and take them to your work station. Using a wax crayon from one of your bench drawers, label the NB (broth) tubes with the numbers 1 through 3, in addition to your name and the date. **Do not label the caps of the tubes. If you do so, something horrifying will happen and you will not like it (I promise.).** Then look through the drawers of your workstation to find your inoculating loop. Get your Bunsen burner going.
2. Without flaming anything, remove the cap of the tube labeled "1", and stick the end of the loop into the broth in the tube (Never stick any part of the handle into a medium. NEVER. Only the platinum wire should ever enter any medium.). Recap the tube and place it in your test tube rack. Flame your loop by holding it so that the full length of the wire is in the blue flame of your burner (The upper parts of the flame tend to be the hottest. If you hold it right at the mouth of the burner, the wire will likely never get hot enough to glow.) in as nearly a vertical position as possible until the wire is red hot. Then, pass ½ of the length of the metal handle through the flame, rotating it so that the opposite side will be heated as you withdraw it from the flame. Then put your loop down.
3. Take the tube labeled as "2". As you hold it, flame your loop until it glows orange. Hold it for a moment before going on to give it a chance to cool. Two or three seconds is good (Do not wave it around no matter how tempted you might be to do so.). As you hold the loop in your right hand, take up tube 2 with your left. With the loop still in your right hand, remove the cap from the tube by gripping it with your right pinky; do not put the cap down. Pass the mouth of the tube through the flame of your burner, carefully dip the end of the loop into the broth in the tube, pass the top through the flame again, replace the cap, and then flame the loop again.
4. Take the tube of *E. coli* broth culture. As you hold it, flame your loop until it glows orange. As before, hold it for two or three seconds to allow it to cool. Holding the loop in your right hand, take up the *E. coli* culture in your left hand. As before, remove the cap of the tube by gripping it with your right pinky, and do not put it down. Pass the mouth of the tube through the flame of your burner and carefully dip the end of the loop into the broth culture in the tube. Pass the mouth of the tube though the flame again, replace the cap, and place the tube back in the tube rack. Do not flame the loop. Take up tube 3, flame the mouth of the tube, and carefully dip the end of the loop into the broth in the tube. Flame the mouth of the tube again, replace the cap, put it in the rack, and flame the loop.
5. When you are finished your work for this session, place the rack of tubes in the cabinet of your bench. The *E. coli* culture tube should be returned to the rack in the front from which you obtained it (Others might be waiting for it.).

Exercise 2: Gram Staining vs. Simple Staining

Materials: Tube of mixed broth culture
Four clean microscope slides

1. Gather the above materials from the front, and take them to your workstation.
2. Using the Gram stain pen you used for the last exercise, draw ovals in the centers of the four fresh slides. Label one of them with "SS", and then label the others GS1, 2, and 3.
3. Arrange the four slides in front of you, and then flame your loop. While holding the loop in your right hand, take the tube of mixed broth culture in your left hand. Remove the cap from the tube by gripping it from with your right pinkie, and flame the top of the tube. Carefully dip the loop into the broth in the

tube. While holding the loop in your right hand, flame the top of the tube, recap it, and place it back in your rack. Swish the loop around on the surface of the slide inside the oval you have drawn on it. Set the slide aside to dry.

4. Repeat step three for the three remaining slides (Do not, under any circumstances, leave the tube of culture uncapped for longer than it takes to flame it and get a loop of sample out! Do not, under any circumstances, insert the loop into a tube of culture without first flaming it!).
5. When the slides are dry, heat fix the four slides.

Stain each of the slides separately according to the following directions. If you do them all together, you will regret it.

6. Stain the first slide, the one marked "SS", with Methylene Blue as you have learned to do previously.
7. The remaining slides you will perform Gram stains on. To do this, follow the Gram stain procedure as shown in table 2.1. Pay close attention to the staining and decolorization steps, as these are critical. The thing you really have to keep in mind while doing a Gram stain is that **tidiness does not count, but timing does!** Once again, stain each slide separately. Go on to step 8 each time you finish staining a slide so that you may have a chance to improve your technique.
8. Observe the stained slide under the microscope. Draw what you see under the oil immersion lens, and give commentary where requested in report 1. Answer the questions on the report sheet. While you have that field of view in focus, ask a TA to comment on the stain so that you can get advice for the next slide that you stain.
9. When you are finished, the used slides should be disposed of by placing them in a Lysol bin in the front.

Stage		Reagent	Duration of Stage	What this Step Does	Color of Gram Positive Cells at End of Stage	Color of Gram Negative Cells at End of Stage
1	Primary Staining	Crystal Violet	1 minute	Stains the cells purple	Purple	Purple
2	Rinse	Water	Until runs off clear	Removes excess stain	Purple	Purple
3	Mordant	Gram's Iodine	1 minute	Cause crystal violet to bind to G+ cell walls	Purple	Purple
4	Rinse	Water	Until runs off clear	Removes Gram's iodine	Purple	Purple
5	Decolorization	Ethyl Alcohol	10 – 15 seconds	Removes crystal violet from Gram- cell walls	Purple	"Clear" (Very light purple, actually)
6	Rinse	Water	20 seconds	Removes alcohol	Purple	"Clear"
7	Counterstain	Safranin	1 minute	Stains decolorized G- cells so they can be seen more clearly	Purple	Red
8	Rinse	Water	Until runs off clear	Removes excess stain	Purple	Red

Table 2.1: **Stages in the Gram Stain Procedure in Detail**

Exercise 3: Gram Staining a Tooth Scraping

Materials: Sterile Toothpick
Clean microscope slide

1. Gather the materials listed, and prepare a smear of a tooth scraping as you learned to do last time.
2. Gram stain the smear according to the Gram stain procedure shown in the figure.
3. Observe the slide under the microscope. When you find a good field of view under oil immersion, draw it in the space provided, give a commentary, and answer the questions in the appropriate part of lab report 1.
4. When you are finished with it, your slide should go in a Lysol bin in the front.

Exercise 4: Capsule Stain Demonstration Slide

The capsule staining procedure involves first performing a negative stain, which stains the background, and not the cells, followed by a positive staining procedure that stains the cells. These procedures leave the background colored, the cells colored. Because the capsules interfered in the initial negative stain, and because they did not retain the stain during the positive stain, the result of a capsule stain is seen as cells with what look like halos surrounding them. These halos are where the capsules originally were.

Demonstration slides of smears that have been subjected to capsular stains are set up on the microscopes on the side bench. At some point during the period you should observe one of them (They should already be set to the oil objective lens and in focus.). For your own benefit, you should sketch what a field of view of one looks like. For the report section, however, you merely have to provide a good, detailed description of what you see.

Exercise 5: Propagation of Microorganisms on Potato Slices

Materials: Petri plate with a sterile potato slice
E. coli broth culture

1. Flame your loop. While holding the loop in your right hand, take the tube of mixed broth culture in your left hand. Remove the cap from the tube by gripping it from with your right pinkie, and flame the top of the tube. Carefully dip the loop into the broth in the tube. Pass the top of the culture tube through your flame and recap it.
2. Carefully remove the top of the plate with the potato slice. Draw the loop over the surface of the potato slice, being careful not to gouge it. A single line should be good.
3. Flame your loop again and set it down.
4. Replace the top of the plate, label the top, and place it in the cabinet of your workstation.

Lab Session 3

Background

I. Gram Staining Problems

There tend to be a small number of problems that people make early on when learning how to properly Gram stain, and it really just takes time and practice to work these out. Today's session will give you and opportunity to do this while getting advice from the TA's. The most common problems are with the decolorization step. Go over the chart for exercise 1 and see if the advice in it does not help you with any problems you are having. It might be a good idea to go over this chart before you work on the follow up to the aseptic technique exercise yesterday.

II. Specialized Staining Techniques

Last time you learned how to do the Gram stain procedure, and you learned about and saw a demo slide of the capsule stain. As mentioned previously, though, there a great many specialized staining techniques designed to either differentiate specific kinds of organisms or to improve observation of certain cellular structures. Today you will learn two fairly easy and important specialized staining procedures that represent these two functions of differential stains:al stains: the **endospore stain** and the **acid-fast stain**.

A. Acid-Fast Staining

(See Brock 414 – 416)

Three bacterial genera, *Mycobacterium*, *Nocardia*, and *Rhodococcus* contain species that produce cell walls with high concentrations of a large, waxy lipid called **mycolic acid**. The presence of this lipid gives their cell walls a very hydrophobic character, and thus makes them hard to stain. Despite this, the acid-fast stain specific for them was among the first developed due the medical importance of a number of the bacteria in these genera. While *Rhodococcus* includes few pathogens, and several *Nocardia* species are significant opportunistic pathogens, the genus *Mycobacterium* includes some of the most significant, feared, and destructive of human pathogens. *Mycobacterium tuberculosis*, the causative agent of most forms of **tuberculosis**, infects one out of every three humans on Earth (Roughly 2 billion people), kills more children than anything else, and has many strains that resistant to multiple antibiotics. *Mycobacterium leprae*, a close relative, causes leprosy, and *M. bovis* can produce a type of tuberculosis transmitted by cow milk. The value of a stain that permits the rapid identification of an organism as being amongst these should be clear.

The acid-fast stain involves first staining the smear of cells in question with carbolfuchsin. Due to the mycolic acid in the cell walls, it is necessary to do this staining under prolonged heating of the smear. The heat works to drive the stain into the waxy cell walls. Once the cells are stained, they are not easily destained, and the procedure gets its name because, unlike other bacteria, bacteria with mycolic acid in their cell walls will retain the carbolfuchsin after exposure to acid-alcohol (They hold it "fast" against the acid.). The smear is then counterstained with methylene blue so that non-acid-fast bacteria will be visible. In the end, the acid-fast bacteria will appear pink, and the non-acid-fast bacteria will appear blue. Despite the heating, acid-fast staining is really very easy, and you should have no problems with this very crucial staining procedure.

B. Endospore Staining

(See Brock, pp 95 – 100)

In late 2001, the whole country was abuzz over not only the events of September 11, but also the mailings of Anthrax letters to Senate Majority Leader Thomas Daschle and others. These letters did not contain active, or **vegetative cells** of *Bacillus anthracis*, the bacterium that causes Anthrax. Instead, they contained cells in a special dormant form called an **endospore**. An endospore is a survival structure formed by a few genera of bacteria, most notably *Bacillus* and *Clostridium*, when the cell is stressed by such factors as dehydration and lack of sufficient nutrients. An endospore may be described as a small body containing a

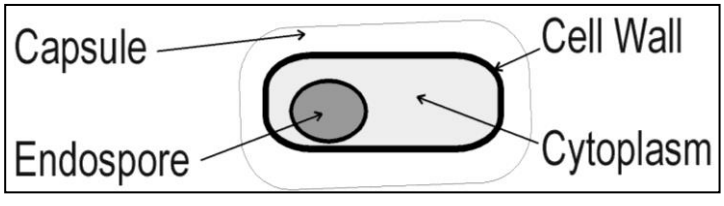


Figure 3.1: **Appearance of a Typical Encapsulated *Bacillus* with an Endospore**

copy of a cell's DNA and inactive ribosomes, surrounded by a **spore cell wall**, a thick layer of peptidoglycan called the **cortex**, a thick, though layer of protein called the **spore coat**, and, in many cases, all this is surrounded by a thin material called the **exosporium**. When a cell forms an endospore, the structure at first looks like a large seed containing in the cell, as shown in figure 3.1. At some point after the formation of the endospore, the cell lysis, releasing the endospore into the medium.

Spores can survive conditions that would ordinarily kill an active cell many times over. They are metabolically inactive, so they cannot be starved. Their cytoplasm is already dehydrated, so desiccation does not harm them. Their spore coats protect them against chemicals and radiation, and their DNA is bound by special binding proteins and **calcium-dipicolinic acid complexes**, preventing damage from heat. You can think of an endospore as a cell sleeping in a suit of armor. This is an important thing to remember. Unlike the spores of fungi, bacterial endospores are not reproductive in nature. A single cell produces a single endospore, and when conditions arise that permit growth, the endospore germinates, giving rise to the same cell that produced it.

Endospores present a number of major problems because of their tough nature. A number of pathogens, including *B. anthracis*, of course, produce endospores, meaning that they can be dormant on objects for years before causing a new infection. Because the spores are chemical-resistant, they are also difficult to kill by use of disinfectants. In the food industry, measures must be taken to ensure that spores are killed in the process of canning or bottling foods for long-term storage. *Clostridium botulinum*, an anaerobic spore former can grow in oxygen-depleted cans and jars of low acid foods, producing one of the deadliest toxins known to man, and causing **botulism** (Basically your muscles stop working and you suffocate from flaccid paralysis of the diaphragm.) when ingested. In media preparation, sterilization is primarily aimed at destroying contaminating endospores, and is the reason why pressurized steam, or autoclaving, is the preferred method.

Spores are thus very much important, and it is useful to know if a bacterium is capable of **sporulation**. This can be done microscopically, but only by using a specialized staining procedure necessitated by the impermeable nature of the endospore that makes staining them difficult under ordinary conditions. The procedure we will use today, the **Schaeffer-Fulton method**, involves driving a stain, malachite green, into the endospore using prolonged heat, followed by a counter stain with safranin that allows one to see the vegetative cells.

III. The Many Uses of Solid Media

(See Brock pp 108 – 109)

Last time we briefly discussed solid media and why they are important. One of the reasons given was that it permits one to localize cells that can then give rise to discrete populations purely of a single type of organism. There are number of techniques for achieving this localization, but among the most useful and basic is the **streak plate method**. This technique involves the use of an inoculating loop to streak a sample on an agar plate, followed by a number of sterilizations and subsequent streaks from the first one. There are many variations on this, and you will learn two of the more common ones, but they all share the same basic principle. This principle should be quite clear if one really thinks about the procedure itself (What does each step result in?), and will not be gone into today, though it will be discussed next time, when you can see the results of your work. Consider this as you do exercise 4 today.

Solid media can also permit for the observation of certain types of microbial growth not apparent with broth media, and can be dispensed into test tubes, permitting them to be used for a variety of tests not otherwise possible. This is gone into in a bit more detail in the introduction to exercise 3.

Procedures

Part I: Session 2 Follow-up
Aseptic Technique:

The exercise you did yesterday was designed to help you see the true importance of developing good aseptic technique for your work in lab this quarter. This is especially true when working with broth cultures, as it is often very, very difficult to tell if a broth culture is contaminated without viewing it microscopically (And there is no guarantee with that, either.). To highlight potential differences you might observe, you will be Gram staining smears made from the three tubes you inoculated last time. If you wish to improve on your Gram staining skills before doing this, please wait to do this portion until after you have done exercise 1.

Materials needed: 3 microscope slides
Gram stain pen

1. Remove the rack of tubes you worked with last time from the cabinet at your workstation, and then go to the front of the bench to get the clean slides you need.
2. Use the Gram stain pen to draw a circle in the center of each of the slides. Label them 1, 2, and 3, just like the tubes.
3. Vortex each tube. The vortex is an apparatus for mixing the contents of a test tube, and is needed to evenly distribute the cells throughout the broth. There should be a vortex close to your station. To vortex a tube, securely hold the tube near the top between your thumb and middle finger. Your index finger should be pressing on the top of the tube, holding the cap in place. Touch the bottom of the tube to the tube rest of the vortex. The tube should start shaking, with the fluid inside rapidly forming a tornado or vortex-like shape (Hence the term "vortexing".). If it does not, turn the vortex on. If this does not work, make sure that it is plugged in. If it is plugged in, and still is not working, then let a TA know. You want to vortex the tube until it looks like the cells are pretty evenly distributed. Whenever you vortex the tube, be sure that you are securing the cap with your index finger. If the cap comes off, there is the possibility of the culture being flung out of the tube, and onto either you or your neighbors, and this would not be a good thing (To make matters worse, this could also aerosolize some of the culture, meaning that tiny little, bacteria-laden droplets would be floating around the lab, just waiting to land on people and surfaces, or to be breathed in. You really do not want the bacteria we work with in your lungs.).
4. Aseptically make smears of samples from each of the three tubes on their corresponding slides (Yes, you have to follow the procedures that you learned last time. It may seem tedious, and perhaps it is, but it is necessary for getting decent results. If you cut corners on your aseptic technique, all you end up doing is shooting yourself in the foot.). Allow the smears to dry, heat fix them, and then perform Gram stains on each. Do not stain them all at the same time. Do them individually.
5. When you are finished staining the slides, observe them under the microscope. Draw a representative field of view for each smear under 1000X total magnification in the appropriate spaces in lab report 2, and provide commentary. Be sure to answer the questions.
6. When you are finished, take the tubes to the back window, remove their labels, and place them in the waste-tub racks. The used slides should go into one of the Lysol bins in the front.

Potato Slices:

Get the petri plate containing the potato slice out your cabinet. Make a rough sketch of what you see on the surface of the potato slice and give some commentary about it in the appropriate section of lab report 2. Be sure to answer the questions. When you are finished, the plate and the potato slice may be simply dumped into one of the biohazard bags in the back.

Part II: Today's Exercises:

Exercise 1: Gram Staining Practice

Gram staining is one of the most fundamental tests in traditional microbiology, and you are going to be using it a lot this quarter. This will be your last official chance to practice the procedure this quarter, and you should try to get as much out of it as you can today. You should, however, practice it on the side whenever you have a spare moment in class. If you are still having trouble after today, please ask a TA for help and advice. One would likely be very happy to help you outside of class to correct your problems.

Materials: Two clean microscope slides
Gram stain pen
Tube of mixed broth culture

Problem	Likely Cause	Solution
Cells black, with few recognizable shapes	Cells were heat-disrupted	Do not heat fix for so long
No cells visible	Cells likely washed off slide	Heat fix a little longer
Gram positive cells stain red	1. Insufficient mordant application (Unlikely)	1. Keep Gram's iodine on slide longer (20 seconds or so)
	2. Over-decolorization of Gram positive cells (Likely)	2. Reduce decolorization time by a few seconds
Gram negative cells stain purple	Insufficient decolorization	Increase decolorization time by a few seconds

Table 3.1**: Gram Stain Troubleshooting**

1. Collect the materials listed above and take them to your workstation.
2. Use the Gram stain pen to draw ovals in the centers of the two slides.
3. Aseptically make smears on each of the two slides using a loopfull of the mixed broth culture for each. Follow the aseptic procedure as you learned last time, being sure to not skip any steps. Allow the smears to dry.
4. Gram stain one of the smears, using table 3.1 to help to avoid any problems you might have had before. Do not bother with the other smear for the time being. When you are finished, get an image in focus under oil immersion (Note: It is unnecessary to go through all the objective lenses, however. Once you get an image in focus using the 4X objective, just go right to the oil immersion objective, skipping the 10X and 40X objectives altogether. Use this procedure from now on.) . When you have found a representative field of view, call for one of the TAs to look at it. The TA will evaluate your stain. If the stain is satisfactory, the TA will initial where requested in lab report 2, and you are finished with this exercise. If the stain is not satisfactory, the TA will give you advice on how to improve your technique. Once the TA has done so, stain the second smear taking this advice into consideration. When you have finished it, and found a representative field of view under oil immersion, call for a TA to look at it again. You do not have to draw anything for this exercise.

Exercise 2: Specialized Staining Techniques: The Acid-Fast and Endospore Stains

Note: This exercise is to be done in groups of two. Please work with one of your neighbors (If your neighbors will not work with you, please find a new neighbor. You are better than they are, anyway.).

So far as technique and the procedures involved are concerned, the acid-fast and endospore stains are very much alike. Because of this, you will do only one of the two, and your partner will do the other. You will, however, be responsible for knowing the principles behind both procedures, how they are performed, their importance, and how to interpret their results. You will also have to record data from each. In other words, there is to be no loafing despite the split workload.

Steam Bath Set Up: Open the large drawer at your workstation. You should find a tripod, a square wire mesh, and a badly stained soup can. Take these out and put them on the bench. Fill the can to about a third to half full with water. Be careful with turning on the water. There is a lot of dried stain in these cans that can be splattered very badly by these faucets if you turn it on too high. Place the wire mesh on top of the tripod, and place the can of water on the mesh. Light your Bunsen burner, get a small to medium flame, and slide the burner under the mesh. The bath is ready when the water is producing a steady steam, or when it is gently boiling. If you are getting a rolling boil, you need to turn your flame down.

Exercise 2a: The Acid-Fast Stain

Materials: Steam bath
Tube of *Mycobacterium smegmatis* culture
Tube of *E. coli* broth culture
2 clean microscope slides
Dropper bottle of carbolfuchsin
Dropper bottle of Acid Alcohol

1. Obtain the materials listed above and take them to your bench.

As always, use aseptic technique when making the smears! Use all parts of the procedures you have been previously taught!

2. Make a smear of *E. coli* on both slides. After this is done, make a smear of the *Mycobacterium* culture on top of it. This is a culture from a solid medium, meaning that the bacterial growth is very concentrated, so very little will be needed. This also means that it is not easy to smear evenly over the slide. This is normally solved by placing a drop of water on the slide before making the smear. However, the fluid from the smearing of the *E. coli* broth culture will achieve the same end. The result will be a mixed smear containing both organisms. It is unnecessary to draw a circle on the slides if you do not want to. At this point, you should have no problem with finding where on the slide you need to be looking.
3. Set the slides near the burner and permit them dry. When they are finished drying, heat fix them as usual.
4. When the smears are ready, get a clothes pin out of one of the drawers of your workstation, and attach it to the end of one of the slides (Only work with one smear at a time.). With this on there, place the slide over the mouth of the can as shown in the diagram.
5. Tear a piece of paper towel large enough to cover the smear, and place it on the smear.
6. Drip carbolfuchsin on the piece of paper towel covering the smear until it is completely saturated. Allow the smear to stain for **5 minutes.** If the stain starts to dry-up, you will need to add more, so be very watchful of the paper towel. Add more carbolfuchsin whenever you feel it is necessary.
7. After 5 minutes, remove the slide from the top of the can, and allow it to cool a for a minute or two, and then remove the paper towel carefully, being sure to pull it up, and not wipe it off (Doing so will remove the cells, too.). Dispose of the paper towel in either the garbage or a Lysol bin. **Do not put it in the sink!** Do not be afraid to touch the paper towel – no matter how careful you are, you are going to get stain on your hands while doing this staining procedure, so it is best to not stress over it.
8. Decolorize by rinsing the smear with acid alcohol for 15 to 20 seconds.
9. Rinse the slide of the alcohol by allowing a moderate drip of water to flow over it for 10 seconds or so.
10. Cover the smear with methylene blue and allow it to stain for 1 minute.
11. Rinse the slide of the methylene blue with water until it runs off the end of the slide without a blue tint, and blot dry.
12. Observe the slide under the microscope. Find a representative field of view under oil immersion, and ask a TA to take a look. If the TA approves, draw the field of view and provide commentary in the appropriate part of lab report 2. If the TA says finds the stain to be improper, you will need to go ahead and stain the second smear you made. Allow your partner to view the slide before you move on (While your partner is observing the slide you stained, you should observe the slide that he/she stained. Draw a representative field of view of it and provide commentary.). Answer the questions in the report section.

When you and your partner are finished, dispose of the slides in one of the Lysol bins at the front.

Exercise 2b: The Endospore Stain:

Materials: Steam bath
2 Clean microscope slides
Slant of *Bacillus megaterium* culture

1. Obtain the materials listed above and take them to your bench.
2. Make a smear of the *Bacillus megaterium* culture on both slides. This smear will be made from a culture on a solid medium, meaning that the growth will be very concentrated, and difficult to spread evenly on the slide. To reduce this problem, you should place a drop of water on the slide before you smear your

sample of the culture. It is unnecessary to draw a circle on the slides if you do not want to. At this point, you should have no problem with finding where on the slide you need to be looking.

As always, use aseptic technique when making the smears! Use all parts of the procedures you have been previously taught!

3. Set the slides near the burner and permit them dry. When they are finished drying, heat fix them as usual.
4. When the smears are prepared, get a clothespin out of one of the drawers of your workstation, and attach it to the end of one of the slides (Only work with one smear at a time.). With the clothespin attached, place the slide over the mouth of the can as shown in the diagram.
5. Tear a piece of paper towel large enough to cover the smear, and place it on the smear.
6. Drip malachite green onto the paper towel until it is soaked. Stain the slide for 5 minutes over the steam bath. If the paper towel begins to dry out, add more
7. Drip malachite green on the piece of paper towel covering the smear until it is completely saturated. Allow the smear to stain for **5 minutes.** If the stain starts to dry-up, you will need to add more, so be very watchful of the paper towel. Add more malachite green whenever you feel it is necessary.
8. After 5 minutes, remove the slide from the top of the can, and allow it to cool for a minute or two, and then carefully remove the paper towel, being sure to pull it up, and not wipe it off (Doing so will remove the cells, too.). Dispose of the paper towel in either the garbage or a Lysol bin. **Do not put it in the sink!** Do not be afraid to touch the paper towel – no matter how careful you are, you are going to get stain on your hands while doing this staining procedure, so it is best to not stress over it.
9. Rinse the slide by allowing a moderate drip to flow over it for 30 seconds or until the water runs off the slide without a noticeable green tint.
10. Cover the smear with safranin and allow it to stain for 1 minute.
11. Rinse the slide of the safranin until the water runs off the slide without any red tint, and blot dry.
12. Observe the slide under the microscope. Find a representative field of view under oil immersion, and ask a TA to take a look. If the TA approves, draw the field of view and provide commentary. If the TA says finds the stain to be improper, you will need to go ahead and stain the second smear you made. Allow your partner to view the slide before you move on (While your partner is observing the slide you stained, you should observe the slide that he/she stained. Draw a representative field of view of it and provide commentary.). Answer the questions in the report section.

When you and your partner are finished, dispose of the slides in a Lysol bin in the front.

Exercise 3: Stab Inoculation of a Solid Medium Test Tube Culture

While working with the solid cultures of *Bacillus megaterium* and *Mycobacterium smegmatis* in exercise two, you likely noticed two things. For one, the bacteria were only growing on the top of the medium, and for another, the media was slanted. These were cases where the desire was simply to propagate the cultures on the media for later use, and it is much easier to get at a culture that is on the surface of a solid medium than it is to get at it when it is under the surface. In such cases, the surface area of the medium is increased by leaning the tubes before the medium solidifies so that it hardens at a "slant" (Indeed, these types of tubes are referred to as slants for this reason.).

However, there are also times when it is desirable to have the organism under study growing under the surface. The problem, however, is that it is not desirable to greatly damage the medium when it is inoculated. In such cases, the loop is simply too clumsy an instrument. Luckily, a wire loop may be modified into an instrument of more finesse called an inoculating needle that has the benefit of not greatly disrupting a medium it is used to inoculate, so long as one is careful. You should find one in the same drawer as your loop. If you do not, use the following procedure for modifying your loop (Ask a TA before using this procedure):

1. Flame your loop.
2. Allow the loop to cool (Very important.).
3. Untwist the loop and straighten the wire as much as possible.

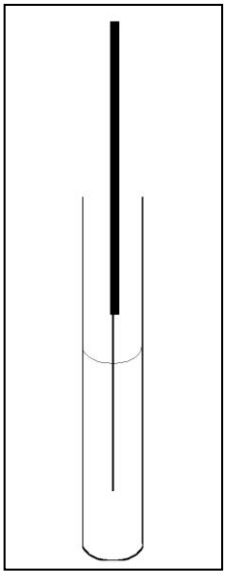


Figure 3.2: **Stab Inoculation of a Solid Medium**

In this exercise you and your partner will each be inoculating one such medium, the purpose of which you will learn of in the next session.

Materials: Tube of Culture "A"
Tube of Culture "B"
Two tubes of TTC media

1. Choose one of the two cultures, leaving the other for your partner, and get out your inoculating needle (If you do not have one, please inform one of the TAs before you modify your loop.
2. Take one of the two tubes of TTC media and label it with the letter of the culture you have chosen, your name, and the date. Use a wax crayon from your workstation to do this.
3. Flame your needle, and allow it a moment or two to cool. While holding the needle in your right hand, take up the tube of culture you have chosen, and remove the cap by gripping it with your right pinkie. Pass the top of the tube through the flame of your Bunsen burner, and dip the needle into the broth (Gently touch the tip of the needle to the bottom of the tube to get as much culture on the needle as possible.). Before doing anything else, pass the top of the tube through the flame again, place the cap back on it, and put it back in your test tube rack.
4. While continuing to hold the needle in your left hand, take up a tube of TTC media, and remove the cap by gripping it with your pinkie. Pass the top of the tube through the flame of your Bunsen burner, and then gently, with a steady hand, stab the needle into the medium in the tube until the tip touches the bottom of it (See figure 3.2). DO NOT ALLOW ANYTHING OTHER THAN THE PLATINUM WIRE TO TOUCH THE MEDIUM WHILE YOU DO THIS! Then withdraw the needle, being careful to pull it out along the path it entered. Pass the top of the tube through the flame again, replace the cap, and place the tube back in the rack. Flame your needle.
5. When you are finished today, the tube you just stabbed should be kept in the cabinet of your bench to incubate until next time.

Exercise 4: Streaking Plates

This exercise is to be done individually. Do not work in groups on this exercise.

In this exercise, you will be using your loop to spread bacterial culture over the surface of a plate of artificial medium such as the one with which you worked during session 1. When a culture is spread on a plate using a loop, the procedure is known as "streaking" (Hence the microbiologists' joke: What did one microbe say to the other microbe? A: Let's get looped and go streaking.). There is a bit of skill and technique to streaking in a proper way, so do not worry if you do not succeed this first time. Today's work is meant primarily to familiarize you with the procedure, to emphasize the need for certain parts of the technique, and to allow you to see the results of certain variations on the streaking procedure. In this exercise, you will have three plates. On one you will use a type of streaking procedure known as quadrant streak, on the second, you will use the T-streak procedure, and on the third, you will choose to do either of these techniques, though you will this time skip a very important step to allow to you see just how important it is.

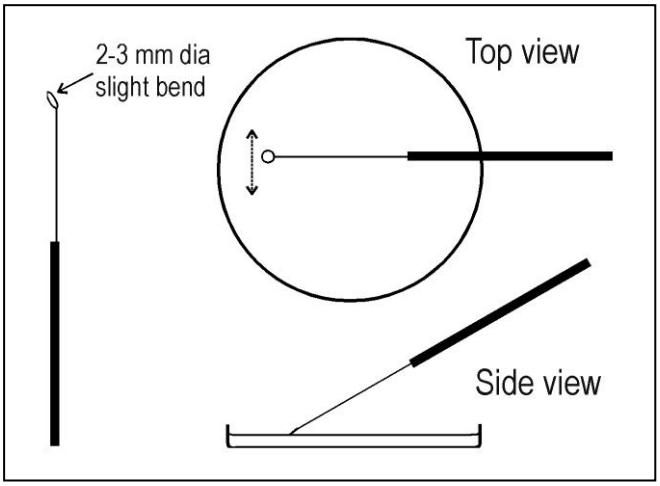


Figure 3.2: **Proper Loop-Plate Orientation When Streaking**

Materials: Inoculating loop
3 NA plates
Streaking broth culture (*S. aureus, E. coli, S. marcescens*)

Once you have gathered your materials, use your wax crayon to label the bottoms of the three plates with "T-streak", "Quadrant Streak", and "No Flame".

4 A: Quadrant Streaking:

1. Take the plate labeled "Quadrant Streak", and draw a cross on the bottom of the plate with your wax crayon so as to divide the plate into four sections as equal to each other as possible.
2. Flame your loop and hold it for a moment to allow it to cool. While holding the loop in your right hand, take the tube of streaking culture in your left, and remove the cap by gripping it with your pinkie. Pass the top of the tube through the flame of your Bunsen burner and dip the loop into the broth culture. Remove the loop, pass the top of the tube through the flame, and replace the cap. You now have on the loop the sample of the culture that you are going to streak on the plate. **Do not dip your loop back in the culture in between the quadrants. You will regret it if you do so.**
3. Take the top off the Quadrant Streak plate and set it down next to the plate. Gently touch the loop to the surface of the media in the plate at the left edge of quadrant 1. Hold your loop at the angle indicated by figure 3.3. Very carefully draw the loop across quadrant 1 in a zigzag pattern as shown in the figure. Be sure that the line of the zigzag does not stray into any of the other quadrants, and make sure that the line does not cross over any of the earlier ones. While you are streaking out the zigzags, do not take your loop off the surface of the plate. **Do not gouge the surface of the medium!**

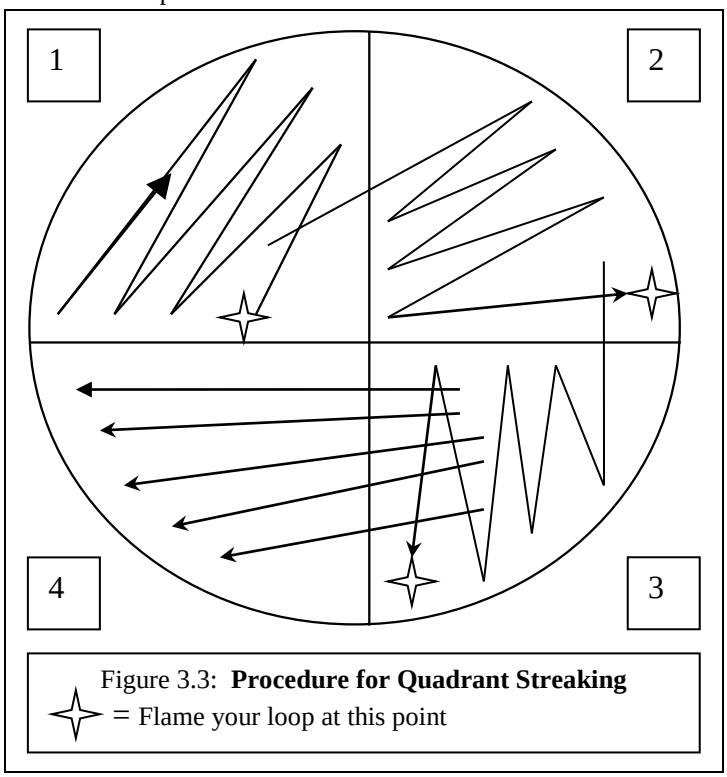


Figure 3.3: **Procedure for Quadrant Streaking**
= Flame your loop at this point

4. When you have finished the zigzag for quadrant 1, place the top back on the plate and flame your loop. Hold the loop for a moment to permit it cool.
5. Remove the top from the plate, and carefully draw your loop through the final line of quadrant one, and streak out another zigzag through quadrant 2, again being sure not to cross any of the new lines and to not go outside of quadrant 2.
6. Replace the top of the plate, flame your loop again, and permit it to cool.
7. Remove the top of the plate, and carefully draw your loop across the final line of quadrant 2 (It is okay to go through two lines with this one, but try to avoid crossing more than this.), and streak a new zigzag through quadrant 3, as before being careful not to cross any of the new lines and to not go outside of quadrant 3.
8. Replace the top of the plate, flame your loop again, and permit it to cool.
9. Remove the top of the plate, and gently draw your loop in a straight line through quadrant 3 into quadrant 4 as shown in the figure. Without flaming in between, draw out several such lines from quadrant 3 into quadrant 4 as shown in the figure.
10. Replace the top of the plate, turn it upside down, and place it in your bench cabinet to incubate. Flame your loop and move on to the T-streak.

4B: T-Streaking:

1. Take the plate you have labeled as "T-streak" and place it in front of you right side up.
2. Flame your loop and hold it for a moment to allow it to cool. While holding the loop in your right hand, take the tube of streaking culture in your left, and remove the cap by gripping it with your pinkie. Pass the top of the tube through the flame of your Bunsen burner and dip the loop into the broth culture. Withdraw the loop, pass the top of the tube through the flame, and replace the cap. You now have on your loop a sample of the culture that you are going to streak on the plate**. At no point in this procedure should you dip your loop back in the culture. You will regret it if you do so.**
3. Remove the top from the top of the T-streak plate and set it beside the plate. Gently touch the loop to the surface of the medium, and draw out a single line across the top of the plate as shown in the figure.
4. Replace the top of the plate, flame your loop, and give it a moment to cool.
5. Remove the top of the plate and gently touch the loop to the surface of the medium at a point just above the middle of the line you streaked across the top of the plate. Draw your loop in a line across the first line to just below the middle of the plate.
6. Replace the top of the plate, flame your loop, and give it a moment to cool.
7. Remove the top of the plate and, as shown in figure 3.4, gently touch your loop to the surface of the medium, drawing out a line parallel to the first one, and crossing the second line. Without taking your loop from the surface of the medium, continue this line out in a wide zigzag to the bottom of the plate as shown in the figure.
8. Replace the top of plate, flame your loop, and set it down. Invert the plate and place it in your bench cabinet to incubate.

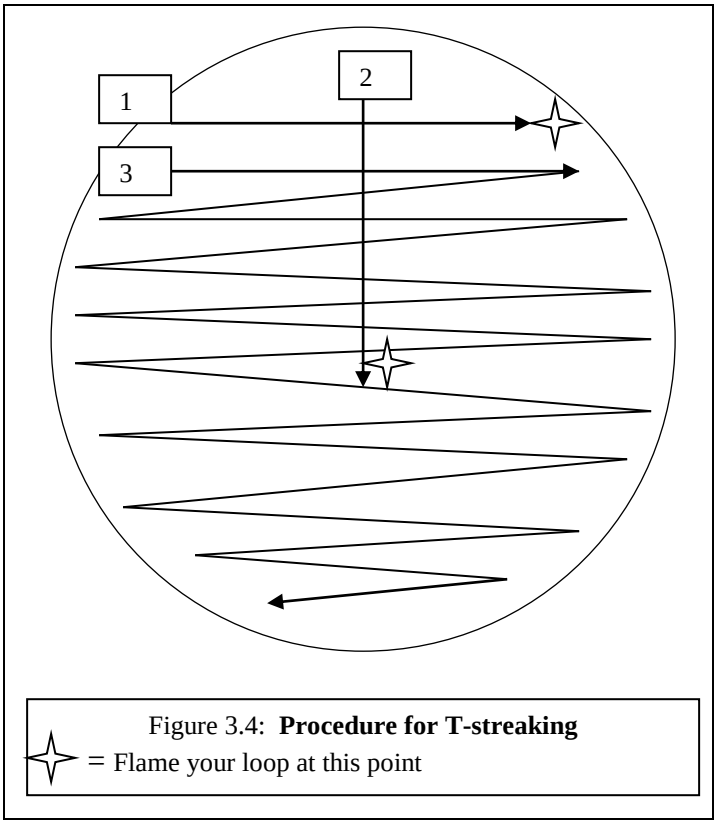


Figure 3.4: **Procedure for T-streaking**
= Flame your loop at this point

4C: The Horrible Mistake of Streaking Without Flaming Your Loop

The most common mistake made in making streak plates is to fail to sterilize the loop at the appropriate places. There is a good reason why one flames the loop in between the quadrants of the quadrant streaking method, and between the streaks of the t-streaking method. If you think about it, the reason is clear, though we will discuss the method behind the madness in the next session. In any case, this exercise is designed to allow you to see just how important the loop sterilization is to the streak plate technique.

1. Take the plate you have labeled as "No-flame" and place it in front of you right side up.
2. Flame your loop and hold it for a moment to allow it to cool. While holding the loop in your right hand, take the tube of streaking culture in your left, and remove the cap by gripping it with your pinkie. Pass the top of the tube through the flame of your Bunsen burner and dip the loop into the broth culture. Remove the loop, pass the top of the tube through the flame, and replace the cap.
3. Now that you have a sample on your loop, go through the procedure for T-streaking the plate, but do not flame your loop in between the different streaks**. Do not flame your loop until you are finished streaking the plate.**

4. When you are finished streaking the plate, replace the top of the place, turn it upside down, and place it in the cabinet of your bench to incubate until next time.

Assigned Reading for Next Session:

Before you leave, please be sure to pick up a copy of "Life at Low Reynolds Number" by E. Purcell as well as the homework sheet that accompanies it. The paper should be read, and the homework questions completed in time for the next session.

Lab Session 4

Background

I. Microbial Motility

(See Brock pp 82 – 87)

You should have read through the paper assigned last time about the physics of microorganisms. If you did (And you had better have.), you should have a good idea as to what movement is like for a bacterium, and what forces they face that we do not usually encounter in our day-to-day lives. You know, for instance, that bacterial **flagella** do not whip about like those of eukaryotes, but instead behave more like the propellers of boats. Since you now know about these things in theory, lab today will be focused more on practical aspects of microbial motility. How does one determine if an organism so small is motile? How do you know when it is really moving itself? What does a flagellum look like? Lets look at these one by one.

There is an indirect method and a direct method of assessing a microbe's motility. In the indirect method, you watch a culture for evidence of the organism in question moving through a soft gel medium. You and your partner from last time actually prepared such a medium to examine for evidence of motility, when you stab-inoculated what is called a **TTC** tube, or motility test tube. If your organism were motile, you will see growth in the shape of an upside down Christmas tree centered on the line of inoculation. This result comes from motile cells having moved out through the medium from the line of inoculation. If, however, your organism were not motile, then you will see that it has grown only along the line of inoculation, with no spreading evident. While there are other indirect ways of looking for motility, often based on watching for evidence of bacteria moving toward or away from a chemical introduced on a plate, the TTC motility medium test that you have used is the most common.

In the direct method, on the other hand, one watches living cells under a microscope and looks for evidence of **true motility**. This can be made a little difficulty by certain factors involved in the microscopic observation of living cells. One of these is that, as you have to look at living cells, you cannot stain them. Further, cells must be kept wet in order to continue living and moving if they are capable of movement. This requires the use of either a **wet mount**, in which a fluid sample is trapped between a microscope slide and a coverslip, or a **hanging drop slide**, in which a drop of a fluid sample is suspended from the bottom of a coverslip over a depression in a special type of microscope slide. A problem develops here for the simple reason that the light going through the sample from the light source is hot. This not only kills the cells under observation after a time, but it also causes the fluid to evaporate. Often, this happens on one side of the coverslip or hanging drop faster than the other, causing water currents to develop on the slide. This can cause one to see a type of movement called "**flow**" caused by this bulk movement of water from one place to another, and which can confuse one looking for motile cells. Flow, however, can be recognized easily, as it causes everything in the field of view to move in the same direction at the same speed. Aside from flow, another type of movement that can confuse one searching for motile cells is **Brownian motion**. Brownian motion results from the bombardment of small cells (And anything else that is really small.) by water molecules. Because the cells are so small, they actually move when water molecules slam into them. This, too, is easily recognized because it presents as a totally random jiggling. True motility, then, may be recognized when a cell under observation moves several times its own length in a single direction independent of the other objects in the field of view.

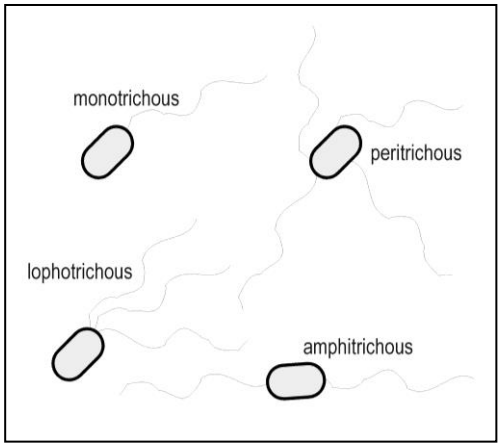


Figure 4.1: **Bacterial Flagellar Patterns**

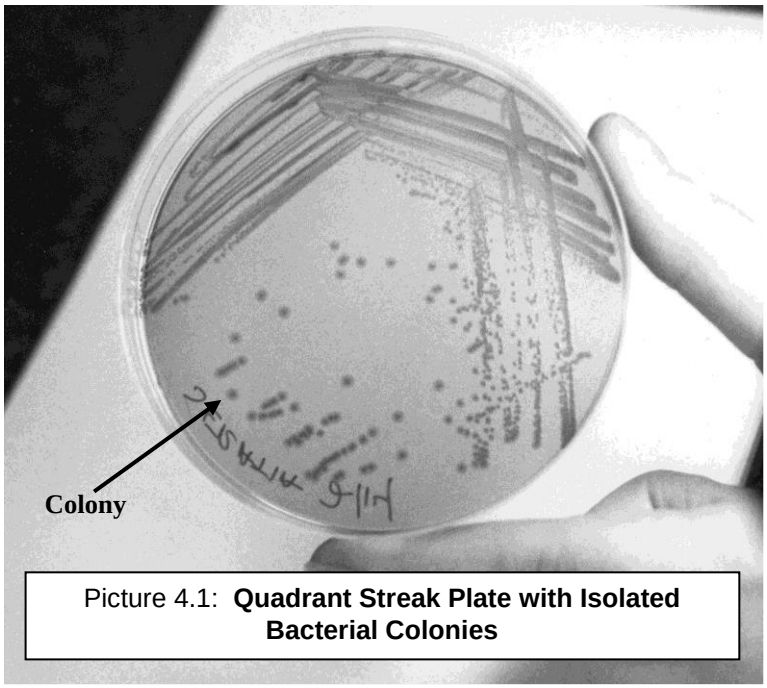
Today you will directly observe the motility of live microorganisms in a wet mount of a **hay infusion** (Pond water in which hay and grass have been soaking for a long period of time.). It should contain a wide assortment of microbes, from paramecia and algae, to microinvertebrate animals and bacteria. You should pay close attention to the different means of true motility that you observe amongst these organisms. It is unlikely that you will actually observe any bacterial flagella in action here, as they are typically much to small to see even under oil immersion. Because of this, you will also have the opportunity to observe a prepared slide of a bacterial smear that has been stained specifically to make the flagella of the cells stand out. Some of the varieties of **flagellar arrangements** seen in bacteria are shown in Figure 4.1.

III. Bacterial Enumeration

(See Brock 146 –149)

As has been mentioned before, one of the greatest benefits of solid media is that they permit one to localize individual organisms on the surface, resulting in discrete, isolated populations of a single type of cell. This was demonstrated (Hopefully) with the streak plates. What has not really been discussed, however, is how this is so.

Bacteria use **clonal reproduction**, meaning that they simply grow to a certain point and divide into two identical cells. Provided there is nothing to make them stop, they keep doing this. If a single cell ends up on a solid medium and well separated from any other cell, it reproduces until eventually a population of cells identical to that single starting cell forms around it. After a time, this **clonal population** grows large enough to be visible to the naked eye. This is what is called a **colony**. As you might have guessed, the entire point of streaking a plate is to get individual cells spread out enough on the surface of a plate to form separate and distinct colonies. See picture 4.1 for an example.



Picture 4.1: **Quadrant Streak Plate with Isolated Bacterial Colonies**

It should be obvious that, if each colony forms from a single cell, then, by counting the colonies, one can also determine the number of individual cells that were spread out, giving one a means of determining the concentration of cells in a given volume. This, of course, cannot be done with a streak plate, because there are still the early streaks where the cells are so close together as to not form separate colonies (There is also the problem of not knowing the volume of a loopfull of culture.). Instead, one must spread a sample of known volume evenly over a plate. This is done by first dispensing the volume onto a plate, and then spreading it over the surface using a sterile glass rod bent so as to resemble a hockey stick. This is referred to as **spread plating**. The only problem with this is that a typical broth culture contains billions of cells per milliliter, so that it is impractical to dispense and spread small enough of a volume to get countable, isolated colonies.

To solve this problem, **dilution series** are made. A dilution series first involves taking a small volume of the original culture of interest, and dispensing it into a large, known volume of either sterile water or a saline solution. If, for instance, you dispense 1 mL of the original culture in 9 mL of water, the result is a suspension of cells one tenth as concentrated at the original culture. This means that each milliliter of the dilution contains the number of cells originally present in a tenth of a milliliter of the original. Typically, a single such dilution of a broth culture is still insufficient to allow one to effectively obtain isolated colonies. To get reduce the concentration of cells far enough; one must repeat this dilution procedure over and over again, each time diluting the previous dilution. Usually one must dilute a culture out to a point where the cells have been diluted by a factor of a million or more before spreading a volume.

Dilution series can be a bit confusing, and if you still do not understand it fully, that is not unusual. Appendix one in the back of the manual is aimed at trying to simplify this further. If, after reading it, you still do not understand, please seek out one of the TAs, and he/she will be happy to help you out.

The ability to determine the concentration of cells in a liquid sample is of great importance. Indeed, as you will find later this quarter, cell concentration per unit volume is a very important thing to know for a variety of situations in microbiology. It allows one, for instance, to determine the concentration of bacteria in drinking water to determine if it is potable, as well as to do a number of important experiments. Today, however, you will simply be making and plating a number of dilutions to determine the concentration of cells in a typical bacterial broth culture. The enumeration of cells in this way is called a **viable cell count**, as it results in one only counting the number of living or active cells in a culture that can grow on the medium in the plate upon which they are spread. Alternately, there is the **turbidimetric method of cell enumeration**. This method is based on the fact that the cells in a broth impact the amount of light that can pass through the broth. As the number of cells in a culture increases, the amount of light that can pass through it is reduced, meaning that the **optical density**, or turbidity of the broth increases. This permits one to

easily and rapidly determine the number of cells present per unit volume of a culture by determining the optical density using a **spectrophotometer**. The problem with this is that inactive cells, dead cells, and cell debris can increase turbidity just as well as active, living cells. This makes it necessary to calibrate a turbidimetric measure with a viable cell count so that one can know what the turbidity measures mean in terms of living cells. This will be done today as well.

Procedures

Part 1: Session 3 Follow-Ups:

Examination of TTC Tubes:

Retrieve the tubes that you and your partner stab inoculated last time. Please note if there was any difference between the two cultures that you used to inoculate the separate tubes. After the class discussion of the results, sketch what you see in the space provided in lab report 3. Provide commentary and answer the questions.

Examination of Streak Plates:

Retrieve the three plates you streaked last time from your bench cabinet. Examine these and sketch them in the space provided in report 3 after the class has discussed the results. Provide commentary and answer the questions.

Part 2: Today's Exercises:

To prevent back-ups, everyone should do today's exercises according to the following schedule:

Bench 1: Exercise 1, 2, 3, 4, 5
Bench 2: Exercise 2, 3, 4, 5, 1
Bench 3: Exercise 4, 1, 5, 2, 3

Exercise 1: New Streak Plates

Materials needed: 2 NA plates
Streaking broth culture (*E. coli* and *S. marcescens*)

1. Before you start, show the streak plates you made last time to a TA so he/she can comment on them and give you any advice before you make new ones.
2. Once you have been advised and/or praised on your technique, incorporate what you have been told in the making of a new quadrant streak plate and a new T-streak plate. Follow the procedure given in the last section for each, being sure to observe proper aseptic technique at all times.
3. When you have finished with the new streak plates, turn them upside down and place them in the cabinet of your bench to incubate until next time.

Exercise 2: Microbial Motility: Microscopic Observation of a Hay Infusion Slide

Materials needed: 1 Fresh microscope slide
Cover slip
Phase contrast microscope

1. Go to the front of the bench where the hay infusion slide station has been set up. Use the plastic pipette to transfer one drop of hay infusion from the beaker onto your slide. Once you have gotten your sample on the slide, go back to your workstation.

2. Take the cover slip you have obtained and touch one edge to slide just to the side of the drop of hay infusion. Gently tilt the cover slip until you bring it down flat on the drop of hay infusion, which should now be uniformly spread out underneath with few if any bubbles evident. A slide such as this, where a cover slip covers a liquid sample for observation is called a "wet mount".
3. Go to the side or back bench were a number of phase contrast microscopes have been set up. These microscopes use a type of light interference to allow cells to be observed without staining. Make certain that you understand how to properly use these microscopes before using them. Carefully view the slide under the 10X and 40X objective lenses (You can try to use the oil immersion objective if you wish, but this can be difficult because the cover slip ends up sliding around as you move the slide.). You don't have to draw what you see, but you do need to provide detailed descriptions of what you see (This does not mean something along the lines of "The squiggly thing moved." Your description should make it very much clear as to what you saw and that you thought about it.). Be sure to carefully describe the different means by which the different organisms moved around, and to take note of which of the three types of microbial motion we discussed you observed.

Exercise 3: Observation of a Demonstration Slide of a Flagellar Stain

The flagella that prokaryotes such as bacteria use to move around their environments are very fragile, and it is difficult to properly stain them so that they can be seen under a microscope. On the microscopes on the side bench are demonstration slides with smears that have been stained using a method that is designed to enlarge the apparent size of flagella, making them readily visible at higher magnifications. At some point this period, observe one of these slides. The microscopes should already be in focus under oil immersion. You do not have to draw a field of view for the report, but you should do so for your own benefit. For the report, you should provide a detailed description of what you see, and answer the questions (It would be a good idea to read these questions before examining one of the slides.).

Work in Groups of Two for the Remainder of the Period

Exercise 4: Determination of the Cell Population Density of a Broth Culture

In this exercise, you and your partner will be using both the viable cell count and turbidimetric methods of determining the concentration of cells in a broth culture of E. coli. In the viable cell count method, dilutions of a fluid sample in which you are interested are spread onto a plate in an aseptic manner. At higher dilutions (See appendix concerning dilutions), it is possible for this spreading procedure to result in single cells being deposited onto the medium surface that are far enough apart that isolated colonies form. If it is assumed that each colony arose from a single cell, it is then possible to use a count of the number of colonies and the dilution factor plated to calculate the number of cells per milliliter of sample. On the other hand, the turbidimetric method involves using a **spectrophotometer** to measure the degree to which the cells in the sample interfere with the passage of a beam of light through the sample. It is necessary to calibrate a turbidimetric measure by combining it with a viable cell count so one can figure out just what a turbidimetric reading means in terms of living cells present in the sample.

Exercise 4A: The Viable Cell Count:

Materials:
- E. coli cell suspensions at various dilutions
- NA plates (3)
- 9.9 mL tubes of sterile saline (2)
- 9 mL tubes of sterile saline (2)
- Blue pipump
- 1 mL sterile pipettes (7)
- Hockey stick with beaker of ethanol and a lid

1. Gather the materials listed. You only have to work with a single E. coli suspension dilution, so choose whichever you or your partner like best and record its dilution factor. Arrange the materials for this exercise so that they are within easy reach. Label the two 9.9 mL tubes of sterile saline as 1 and 2, and the three 9 mL tubes as 3, 4, and 4.
2. Open the top of one of the 1 mL pipettes. Without removing the pipette from its protective cover, insert the top of the pipette into the end of the blue pipump.
3. Vortex the E. coli culture to disperse the cells. As though you were going to get a loop of culture from it, take the tube of broth culture, uncap it, and pass the top through your flame.
4. Remove the pipette you inserted into the blue pipump from its wrapper. Insert it into the tube of broth culture, and use the wheel on the side of the pipump to suck up 0.1 mL of the culture. The pipettes are numbered from the top, so you will be drawing the broth up to the 0.9 ML mark, which is the first tenth of a mL mark on the pipette. Once you have gotten the sample, Flame and recap the culture tube.

5. Aseptically transfer the broth sample to the saline tube you labeled as "1" (Note: This means that you need to flame the saline tube just as you would a tube of broth that you were inoculating and did not want to be contaminated.). After the aseptic transfer is complete, vortex this tube. Remove the pipette from the blue pipump and discard it in the special Lysol beaker set up in the back for them.
6. Insert a fresh 1 mL pipette into the blue pipump as you did earlier. Aseptically transfer 0.1 mL from saline tube 1 to saline tube 2. Vortex tube 2, and then discard the pipette.
7. Using a fresh 1 mL pipette, aseptically transfer 1 mL from saline tube 2 to tube 3. Vortex tube 3, and then discard the pipette.
8. Repeat step seven for transfers from tube 3 to 4 according to figure 4.2.
9. Now that your dilution tubes are ready, take your three NA plates, and label them 10^{-5}, 10^{-6}, and 10^{-7}, respectively.
10. Using a fresh 1 mL pipette, aseptically transfer 0.1 mL of fluid from saline tube 2 to the plate labeled 10^{-5}.
11. Remove the top from the beaker of ethanol. Dip your hockey stick into the ethanol, and pass it through the flame of your Bunsen burner. Hold the hockey stick at a downward angle so that no alcohol can drip onto your hand, and allow the alcohol on it to burn off. Immediately remove the top of the 10^{-5} plate on to which you just dispensed 0.1 mL from saline tube 2, and use the now sterile hockey stick to spread the fluid evenly around the plate. It helps to turn the plate as you do this. When you have finished spreading the plate, place the hockey stick back in the Ethanol.

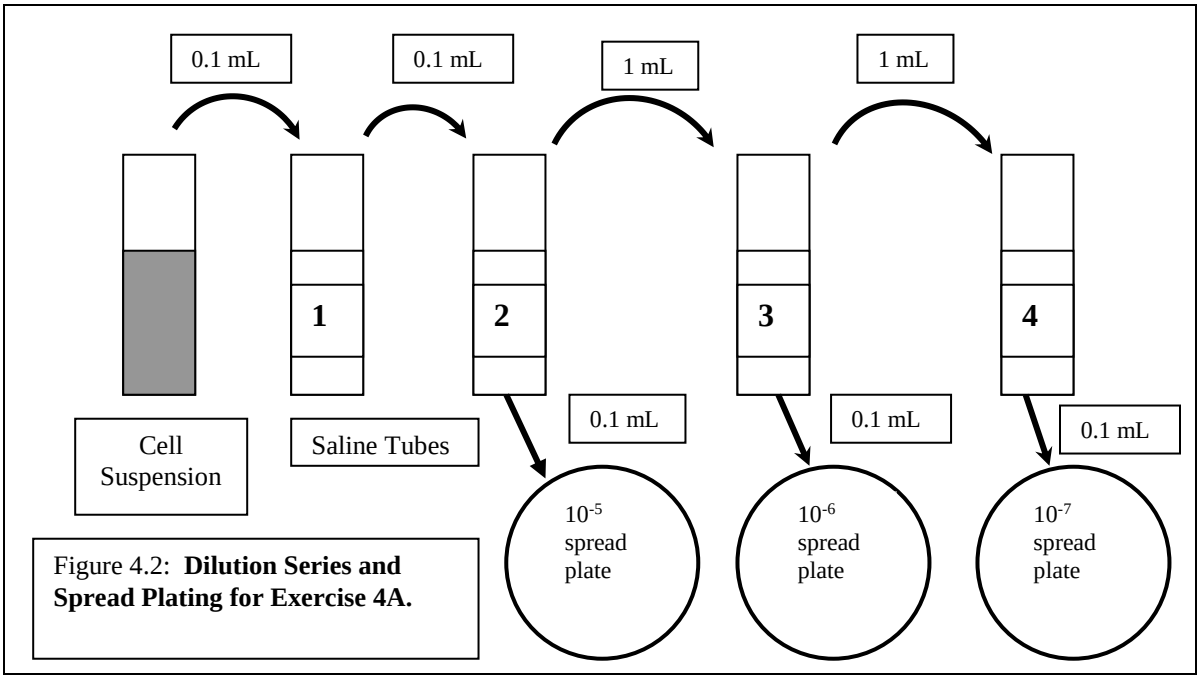


Figure 4.2: **Dilution Series and Spread Plating for Exercise 4A.**

12. Using a fresh 1 mL pipette for each, aseptically transfer 0.1 mL from saline tube 3 to the plate labeled as 10^{-6}, and 0.1 mL from saline tube 4 to the plate marked as 10^{-7}, and spread each over the respective plate using the hockey stick, being sure to sterilize it before each spreading procedure.
13. When you are finished, set the plates you have spread to one side to allow them to dry. At the end of the period, invert them and set them in your bench cabinet to incubate.

4B: The Turbidimetric Method:

Materials: *E. coli* cell suspension (Same as for exercise 4A.)
Green pipump
13 X 100 mm test tubes (2)
Sterile 5 mL pipette (2)
Bottle of saline
Small test tube rack

1. Label one of your 13 X 100 mm tubes as "blank", and the other as "dilution".

2. Insert one of the sterile 5 mL pipettes into the green pipump. Pipette 4 mL of saline into the tube marked "blank". After you have done this, dispose of the pipette in one of the beakers of Lysol.
3. Vortex your *E. coli* cell suspension again. Insert a new sterile 5 mL pipette into the green pipump, and transfer 4 mL of culture to tube marked as "dilution". Dispose of the pipette in one of the beakers of Lysol.
4. Take the two tubes you have just prepared to one of the Spec20's set up at the ends of the benches.
5. You will now need to calibrate your Spec20. The spectrophotometers were turned on earlier, so they should be nice and warmed up by now, so no need to worry about that. Calibration for this kind of spectrophotometer is a three-step process:
    a. Make sure that the machine is set to 600 nm, which is the wavelength of the beam of light in the machine. The indicator for wavelength should be on the top of the machine, with the knob to control it next to it. Rotate the knob until the indicator line and the 600 nm wavelength line line up.
    b. With the sample chamber empty and its lid closed, turn the knob on the left hand of the base of the machine until the indicator needle reads zero.
    c. Use a KimWipe to clean the clear sides of the tube marked as "blank", being careful to avoid subsequently touching the now clean tube (Do not, under any circumstances, touch the clear sides of the tube.). Place the blank tube in the sample chamber so that the label faces forward and close the lid. Rotate the right hand knob on the base of the machine until the absorbance indicator reads zero. Once the machine is calibrated, remove the blank tube.
6. Clean the cell suspension tube with a KimWipe, insert it into the Spec20, close the lid with the label facing forward, and record the absorbance indicated for the data sheet in the report section. When you have taken the absorbance reading, remove the tube from the sample chamber.
7. When you are finished, dispose of the tubes you have used in the racks in back.
8.

Exercise 5: Preparation of Media for Next Session:

As before, you should be working with a partner on this exercise. One person in each group should inoculate half of the FTM and half of the TGYA tubes, and the other person the other half.

Exercise 5A: Inoculation of a Semi-solid Deep:

Materials: 6 FTM tubes
Test Cultures A, B, C, D, E, and F
Test tube rack

1. Get the inoculating needle out of its drawer.
2. Label the six FTM tubes with A through F, as well as with your name and the date.
3. **Aseptically** get a sample of culture A, and then aseptically stab inoculate the FTM tube labeled as A as you did for the TTC tube during the last session. As before, be careful to draw your needle out along the same path as it went in. **Do not shake the tube! The medium in it is semi-solid only, and will pour out!** Repeat this for B through F. You should end up with six FTM tubes, each inoculated with a separate culture.
4. When you have finished with your inoculations, you should place them in your bench cabinet to incubate until next time.

Exercise 5B: Inoculation of an Unsolidified Agar Medium Tube:

Materials: Test Cultures A, B, C, D, E, and F
6 TGYA tubes melted in the 60 C water bath on side bench (**Do not** get these all at once.)

1. If you have just finished exercise 5A, re-form your inoculating loop.
2. Go to the hot water bath set up on the side bench and retrieve **one** (I repeat: **one and only one**) of the tubes of TGYA medium. You will notice that it is still fluid, and this is a good thing. You know you are doing this part improperly if it solidifies before you are finished.
3. When you are back at your bench, as rapidly as you can without making mistakes, **aseptically** inoculate the tube with two loops of culture A (Yes, you have to go through the flaming routine for each one.).

4. When you have finished inoculating the medium, you need to quickly disperse the bacteria through it by rolling the tube between your hands for a few seconds. Do not shake or vortex the tube! When finished, label the tube with an A, your name, and the date.
5. Repeat steps 2 through 4 until you have six solidifying TGYA tubes, each with a different culture in it.
6. When finished, place the tubes you have inoculated in your bench cabinet for incubation until next time.

Lab Session 5

Background

I. Microbes and Oxygen

(See Brock 161 – 163)

Humans require oxygen to carry out the metabolic reactions on which we depend for life. Because oxygen is so important to us, we tend to think that it must be important for all organism, but this is not so. While there are no large, multicellular organisms that do not require oxygen, different microbial species display a wide variety of relationships with oxygen. There is really a continuum of oxygen requirements amongst microbial organisms, but we usually break it up into a number of discrete categories. Those organisms that require oxygen to live are called **obligate aerobes**, or simply aerobes. Much like you or I, they cannot carry out life processes without oxygen, though they often go into hibernation without oxygen, rather than rapidly dying. On the other end of the spectrum is a very large category of microorganisms that are killed by oxygen. These are called **obligate anaerobes**. In between, there are a number of intermediate categories. These are summarized in the table. In each case, the specific relationship to oxygen is largely governed by the metabolism of the organism. We will get more into this aspect of microbial oxygen requirements later in the quarter when we study microbial metabolism in general.

Category	Relationship With Oxygen
Obligate aerobes	Require oxygen for life
Microaerophiles	Require oxygen, but at a concentration lower than the 21% in the atmosphere
Facultative anaerobes	Grow better with oxygen, but can also grow without it
Indifferents	Grow equally well regardless of whether or not oxygen is present
Obligate anaerobes	Killed by oxygen

Table 5.1: **Oxygen Requirement Categories of Microorganisms**

There are two relatively easy ways in which to determine the oxygen requirements of a microbe. The first involves inoculating a tube of semi-solid media that contains a reducing agent, usually **thioglycolate**, that reacts with oxygen, removing it from the medium. The top of the medium is in contact with the atmosphere, allowing for rapid diffusion of oxygen into it. Below this oxygen is quickly scrubbed from the medium by the reducing agent. The result of this is an oxygen gradient that ranges from the atmospheric level of 21% at the very top, to zero, or **anaeroby** at the very bottom. If this medium is inoculated with an organism, it will grow in that part of the tube where the oxygen concentration is at a level it requires or can tolerate. The tubes depicted in figure 5.1 show the levels at which organisms of differing oxygen requirement will grow. Tube A shows the growth level of a strict aerobe, tube B that of an obligate anaerobe, and tube C that of a microaerophile. Tube D shows growth throughout the tube, which would be expected of facultative anaerobes and indifferents.

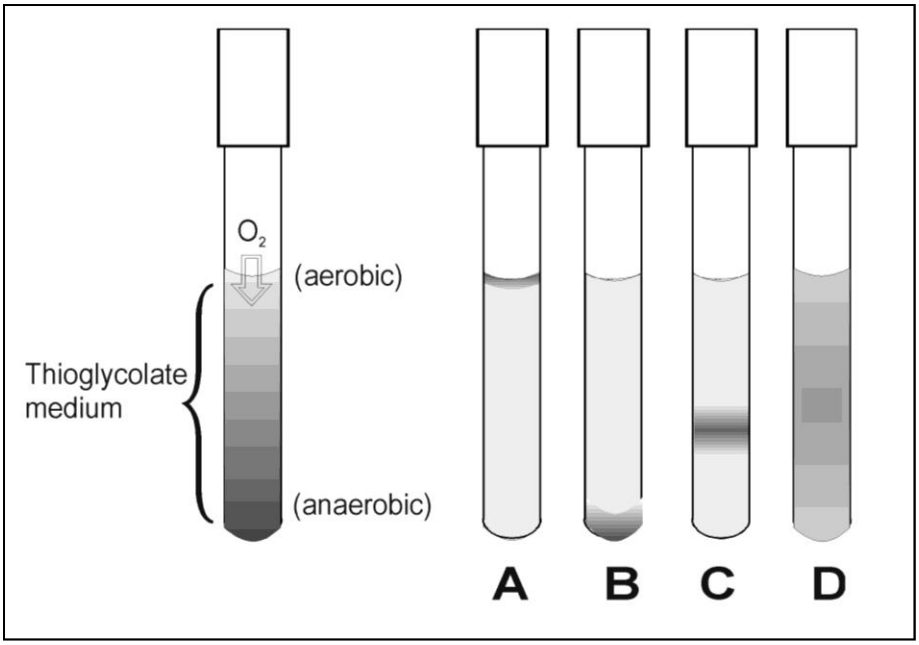


Figure 5.1: **The Thioglycolate Tube Method of Determining Microbial Oxygen Requirements**

The second method involves simply inoculating a tube of regular solid medium before it has cooled, thus allowing cells to be distributed throughout the

medium. When the medium solidifies, the solidity of the medium impairs oxygen diffusion through the tube, resulting in a natural oxygen gradient. The cells that are in the part of the medium with an oxygen concentration amiable to their growth then grow, and you read the tube just like in the thioglycolate medium.

As you have likely guessed, you inoculated these media during the last session. The FTM tubes, of course, were thioglycolate tubes, and the TGYA tubes represented media for the second type of oxygen requirement test. Today you are going to read these media according to figure 5.1. You are then going to make predictions on the basis of these oxygen requirement determinations as to which of the organisms tested will grow under atmospheric conditions, and which ones will grow under anaerobic conditions for part II of our investigation into anaerobes.

Anaerobic conditions are not exceptionally difficult to generate, making the growth and study of anaerobes much easier that might be supposed. Most of the equipment in use for generating anaerobic culturing conditions are modifications of those invented during the golden age of microbiology in the nineteenth century. Today, we will use one such piece of equipment, the GasPak jar, for the testing of the capacity of our organisms to grow under anaerobic conditions. The jar is an airtight plastic container into which plates inoculated with an organism are placed. A packet of chemicals, to which water is added, is then placed in the jar, and the jar is sealed. The chemicals in the packet react with water to generate carbon dioxide and molecular hydrogen. Palladium beads contained in a small chamber under the lid then act as a catalyst for the reaction of the hydrogen with the oxygen in the jar to form water. This depletes the oxygen in the jar, leaving an atmosphere of primarily nitrogen and carbon dioxide. Under these conditions, anaerobes, indifferents, and facultatives will do quite well, while aerobic organisms will be excluded. In today's exercises, you will streak the test organisms onto two sets of standard NA plates, as well as onto two sets of Brewer's agar plates, a medium specifically designed for the growth of many anaerobes. One set of each medium will be incubated in a GasPak Jar, and the other set in your bench under atmospheric conditions. Next session you will determine how well the two test media inoculated last time performed in allowing your to predict the reaction of the test organisms to the two conditions.

II. The Winogradsky Column

(See Brock 617 – 618)

The third exercise you will be doing today involves the construction of an experimental apparatus that we will be watching for the rest of the quarter as it develops. This is the Winogradsky column. Named for its inventor Sergei Winogradsky, a pioneer microbial ecology and biogeochemistry, the column provides an easy means of studying microbial ecosystems. It involves placing a volume of mud and water in large tube along with a few supplemental ingredients to better provide for microbial nutrition. Gradients of hydrogen sulfide and oxygen then are produced by the growth and activity of the organisms originally present in the mud. The result is a series of colored zones corresponding to regions along the gradients where certain groups of organisms are capable of growing, and represents a self-perpetuating, contained ecosystem. Today will only involve construction of the columns using mud from different locations. Observations will then be taken weekly as the quarter progresses, and you will periodically be told more about the workings of the system that develops, though you are by all means encouraged to read up on the column if you so choose. It is hoped that it will help you to learn a bit more about the activity of anaerobes in the environment, as well as about the close metabolic interdependence that characterizes microbial ecology.

III. Bacteriophage

(See Brock pp 238 – 239, 240 – 254)

So, naturalists observe, a flea
Has smaller fleas that on him prey;
And these have smaller still to bite 'em;
And so proceed *ad infinitum.* – Jonathan Swift

What is true of fleas is true also of microbes. Viruses are much, much smaller than your typical bacterium, and they constitute one of the major influences on bacteria and other microbes in the environment. Those viruses that prey upon bacteria are typically referred to as bacteriophage, and they are much like those viruses that can cause us an annoying sniffle as with the common cold, or a more serious case of bleeding out in the midst of a horrifying death as with Ebola. In the case of microorganisms, death can also be a result of infection. Today you will prepare a type of plate used to grow and study bacteriophage. The obstacle to propagation of viruses is that they are incapable of independent reproduction, requiring as they do a host cell to provide them with raw materials and cellular machinery. The solution is to dispense a volume of a viral suspension and a volume of a cell suspension into a warm solid medium while it is still liquid, and the pouring it on a suitable plate to form what is called an overlay over the medium already in the plate. When the cell and viral suspensions are mixed, there is an opportunity for the viruses to infect the cells (This requires that you have suitable cells for viral infection. Most bacteriophage can infect only a very, very limited number

of different types, species, or even strains of bacteria.). After the overlay sets, the infected cells are localized in the medium. Inside the infected cells, phage DNA rapidly cc-opts cellular machinery, using it to reproduce. Eventually, after a great many bacteriophage copies have been produced, the infect cells lyse, releasing the viral particles into the medium where they can infect more cells. After incubation, phage growth is evident as clearings in the overly that by that time is cloudy with bacterial growth. These clearings center on the original infected cell, and can be thought of as viral colonies, the meaning of which to microbiological study should be obvious to you from our work with bacterial colonies. The life cycles of bacteriophage will be further discussed tomorrow when we will observe the effect of phage infection on bacterial growth.

Procedures

Part 1: Follow Up from Session 4:

Examination of New Streak Plates:

Retrieve the new streak plates that you made last time. Show them to a TA. The TA will evaluate them, and give you any advice you need for them. Have him/her sign the appropriate place in the report section. Provide a commentary for your plates, focusing especially on anything that you did incorrectly and how to avoid doing so next time. You do not have to draw the plates.

Colony Counts for Viable Cell Count:

Retrieve the three plates you spread last time for the determination of population density of a broth culture of E. coli. Count the number of colonies on each plate and enter these numbers in the table in the report section near that into which your entered the absorbance data last time. There will be a class data sheet going around during class today. Be sure to enter your viable count and absorbance data into the appropriate places on it when it comes to you. Be sure to get a copy of this before you leave today, as you will do the lab report on the basis of the entire class's data, and not just your own. You will use these data to determine not only the concentration of the original broth culture from which the E. coli suspensions were made, but also the correlation between the viable count and turbidimetric measures.

Part 2: Today's Exercises:

Exercise 1: Anaerobes:

Work with the Same Partner as Last Time

1a: Examination of Tests of Organismal Oxygen Requirements:

Materials: FTM and TGYA tubes from last time

1. Examine the TGYA and FTM tubes you and your partner inoculated last time. Provide thorough descriptions of what the incubated tubes look like in the space provided in the report section.
2. Using the information given to you concerning these media during the lab lecture and in the background section for this session, evaluate the oxygen requirements of the six test organisms you worked with last time based on the appearance of the media. Fill in the appropriate spaces in the report section. Do any of the TGYA tubes disagree with their corresponding FTM tubes?
3. Based on the oxygen requirements you have now concluded the six test organisms to possess, write in the appropriate space in the report section your predictions for which ones will grow under aerobic conditions and which ones will grow under anaerobic conditions.

1b: Cultivation of Anaerobes

Materials: 4 NA Plates
4 Brewer's Agar Plates
Test Cultures A, B, C, D, E, and F

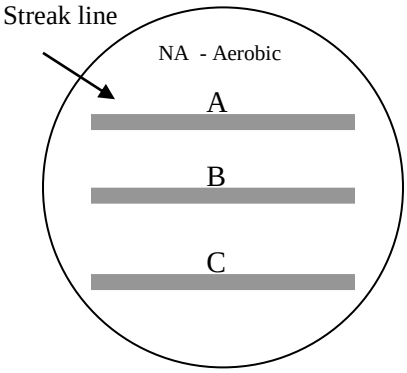


Figure 5.2: **Example of How to Streak Cultures onto Plates for Ex. 2**

1. Label two of the NA plates and two of the Brewer's agar plates as "Aerobic", and the remainder as "Anaerobic", being sure to also label each with your name and the date.
2. Take the two NA plate labeled as aerobic, and write on one of them in a column down the center A, B, and C (See the figure); on the other write D, E, and F in the same manner. Do this for all four sets of plates.
3. Aseptically streak the test cultures in a single line over the letter corresponding to it. You should end up with a streak of each test organism on each type of plate for each oxygen condition.
4. Take the plates you streaked for the anaerobic condition in the pile to go in one of the GasPak jars, and the plates for the aerobic condition can go in your bench cabinet.
5. Be sure to either participate in or watch the preparation of the GasPak jars at the end of the period.

Exercise 2: Preparation of Phage Plates for Next Session:
Work in Groups of Two.

Materials: 4 NA plates
4 tubes of soft top agar (Melted in water bath)
1 mL aliquot of BP (Bacteriophage) stock
Tube of E. coli B culture
6 tubes of sterile saline (9 mL each)
9 1 mL sterile pipette and blue pipump

1. Label your five tubes of saline as 1 through 4. Perform a dilution series according to the figure, using a fresh pipette for each transfer, and being sure to vortex each dilution tube before transferring from it.
2. Go to the water bath set up at the side bench and select your five tubes of soft top agar. Without removing them from the water bath, label them "Control", -4, -5, and -6. Also label your five plates with the same numbers (In addition to your name and the date.).
3. Using aseptic transfer technique, dispense 0.5 mL of E. coli B culture to each of the soft agar tubes using two 1 mL pipettes (Draw 1 mL of culture into one pipette and then use this 1 mL to make transfers to two of the soft top agar tubes. Make sure that you do flame the tubes as you uncap and cap them.).
4. To the control tube of agar, add no BP saline dilution. To the agar tube labeled at -4, add 1 mL from dilution tube 2, and so on as in the diagram. Use a fresh pipette for each transfer.
5. Once this is done, one by one take the tubes of soft top agar out of the water bath, quickly vortex , and pour over them over the surface of the plates with the corresponding number. Make sure to evenly distribute the soft top agar over the plates by using a gentle swirling motion. Set the plates aside for ten minutes to solidify.
6. When the plates are dry, turn them upside down and place them in your bench cabinet to incubate.
7. All used tubes should go in the back test tube racks for disposal.

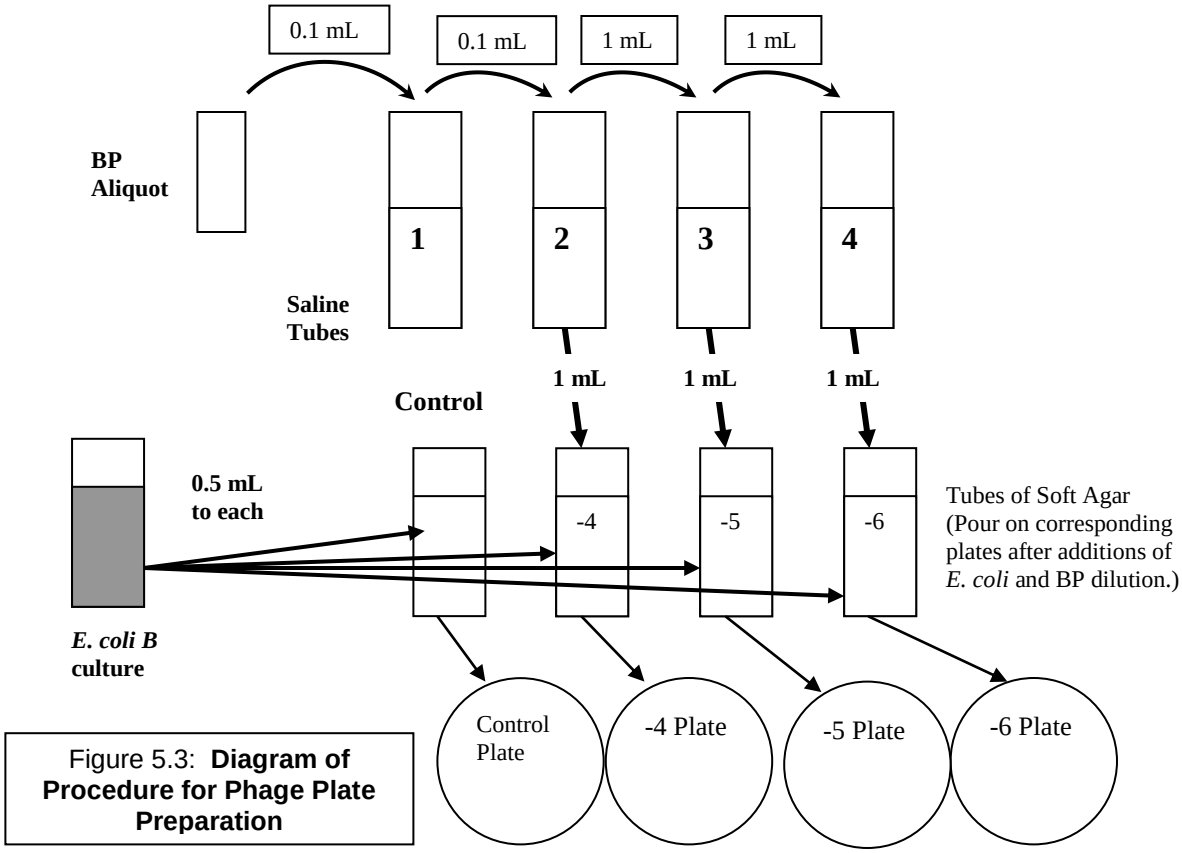


Figure 5.3: **Diagram of Procedure for Phage Plate Preparation**

Exercise 3: Construction of a Winogradsky Column:

There will be six groups for this exercise, with two groups for each of the three benches.

Materials for each group:

- Plastic column with rubber stopper
- Mud from assigned location
- Shredded paper
- Eggshells
- Calcium carbonate
- Baking soda
- Plastic wrap
- Rubber band
- Paint stirrer
- Beaker for mixing
- Pond water

1. Get in a group and help in the gathering of the above materials. The plastic column should be labeled with a group designation, the date, as well as the source of the mud you are using.
2. Place the rubber stopper in the column, and dump the shredded paper into it.
3. In the beaker, add about half of the mud, the calcium carbonate, eggshells, and some of the pond water. Stir this mixture, adding the water until the mixture has the consistency of paste (Thick oatmeal.).
4. Pour this into the column over the shredded paper. Use the paint stirrer to clean out the beaker and even out the mixture in the column.
5. Add the remainder of the mud to the beaker. Add the baking soda and water until you get the mixture to the same consistency as before.
6. Pour this mixture into the column on top of the previous one. Stir gently to get rid of air bubbles in the mud. The column should be 2/3 full at this point. Pour pond water onto the mud until the column is to within 2 cm of the top.
7. Cover the top of the column with plastic wrap and use the rubber band to secure it.
8. Put the column with those of the other groups in the hood at the back of the lab.

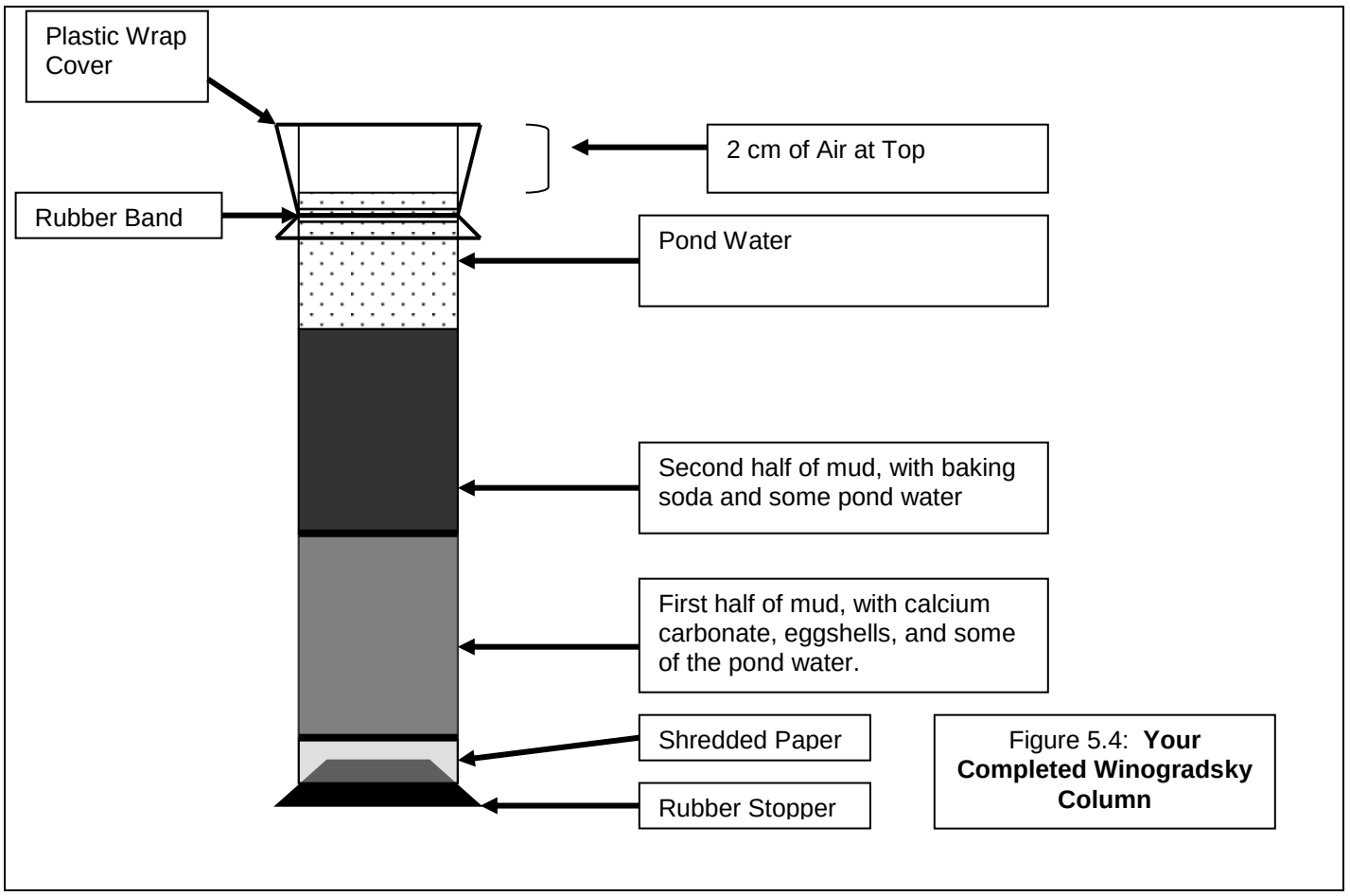


Figure 5.4: **Your Completed Winogradsky Column**

Lab Session 6

Please Note: Today's exercise and the follow up from the previous lab session should be done in the order indicated below. Yes, this is backwards, but there is a good reason for it.

Background

Kinetics of growth (bacteria and bacteriophage)

(See Brock, pp 142 – 145, 238 – 239, 240 – 254)

If individuals of any species are put into a situation where they can reproduce without any limitation for resources, their numbers will increase exponentially over time. This is an intrinsic property of living things, and reflects the fact that the rate of producing new individuals at any point in time is proportional to the number of individuals present at that time. [In mathematical terms, $\mathbf{dN = kNdt}$; integration yields $\mathbf{N_t = N_o e^{kt}}$]

With pure cultures of bacteria it is fairly easy to produce this situation: sterile medium is simply inoculated with a small number of cells and incubated. During exponential growth, the cell number is increasing by the same factor in every interval of a certain length. The culture may double its density every 35 minutes, for example. If so, this ratio (2.0) will be seen over every 35-min interval. To make this relationship more obvious, we plot the logarithm of cell number (or cell density) as a function of time. Exponential growth then appears as a straight-line increase (Region II on the graph below).

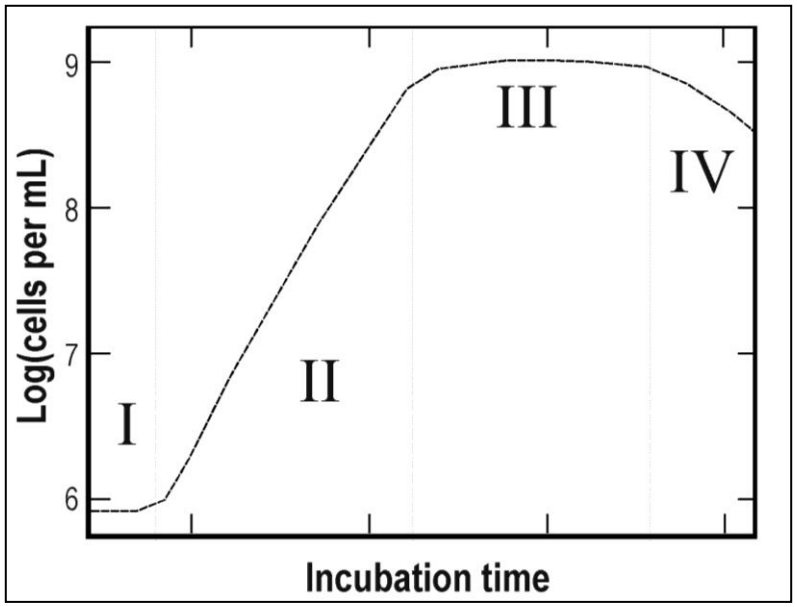


Figure 6.1: **Typical Microbial Growth Curve**

Before and after this period, however, other phenomena determine the shape of the growth curve. For example, when first inoculated, the cells may not grow for a period of time, resulting in a **lag phase** (Region I). After **exponential phase** (Region II), the culture has consumed most of its nutrients and accumulated metabolic by-products. The cells alter their metabolism and stop growing, resulting in **stationary phase** (Region III). If no new medium is supplied, cells eventually begin to die at a steady rate, seen as the **death phase** (Region IV).

The two middle phases of bacterial growth can be observed with turbidity measurements, which are much easier and faster than doing plate counts. Because turbidity measures the density of intact cells in suspension, it is also a sensitive way to detect **lysis** of cells. One set of teams will demonstrate this by infecting their culture with a lytic **bacteriophage**. At a high multiplicity of infection, nearly all bacterial cells in the culture will become infected shortly after the phage particles are added. The bacteriophage **lytic cycle** then begins in each infected cell and runs according to a well-defined "program" of events, culminating in the lysis of the host cell and release of many new bacteriophage particles. Since lysed cells do not scatter light, the end of the phage lytic cycle will be marked by a dramatic decrease in the turbidity of the infected culture.

Procedures

Part 1: Today's Exercise:

Exercise 1: Study of a Bacterial Growth Curve and the Effect of Phage Infection

We will work in groups for the growth curve exercise; groups on the middle bench will infect their cultures with phage. After inoculating their culture, each group will take up to four viable counts (dilution and spreading) and about twice as many turbidity measurements (absorbance readings). It is important to begin this exercise at the beginning of the lab period to allow enough time for growth to be measured. It will also be helpful to divide the work among individuals in each group, especially for the setting up process and the first few time points.

Materials for each group: 1 screwcap tube with 2 mL of *E. coli* culture
12 NA plates
8 tubes of 9.9 mL sterile water
8 tubes of 9 mL sterile water
1 indicator plate (NA plate with *E. coli* soft top overlay)
1 tube of phage suspension (For the Infected group only)
Sterile 1 mL pipettes and blue pipumps

1. Divide your plates into four groups of three, corresponding to three dilutions for each of four time points. Label each group with the following time points: 0, 40 minutes, 80 minutes, and 120 minutes, and within each group, label the three plates with the following dilutions: 10-5, 10-6, and 10-7. Divide the 8 tubes of 9.9 mL and 8 tubes of 9 mL of sterile water into four series of four, one series for each time point. The first two tubes in each series should be 9.9 mL of water, (label as -2 and -4) and the last two should be 9 mL (label as -5 and –6).
2. Get your group's culture from the instructor. As quickly as possible, aseptically transfer 0.1 mL of the *E. coli* culture in the screw-cap tube to the 9.9 mL tube labeled as "-2" in the first group of tubes (0 time point).
3. INFECTED GROUP: aseptically add 0.1 mL of phage suspension to the *E. coli* culture in the screwcap tube.
4. Re-cap the tube securely, wipe off the sides, and take an absorbance reading your group's assigned spectrophotometer (The spectrophotometers will already have been zeroed beforehand, so do not worry about that.). Enter the reading in the appropriate table in the report section.
5. As soon as this is done, place the screwcap tube in the 37 C incubator. (It may be necessary to wait a minute or two until several groups are ready).
6. RECORD the TIME when incubation began. This defines your starting point.
7. Vortex the tube of water to which your transferred the 0.1 mL of culture "-2". Aseptically transfer 0.1 mL from this first dilution tube to the second 9.9 mL tube "-4" using a new sterile pipette, and then vortex. Aseptically transfer 1 mL from tube 2 to the tube marked "-5" using a new pipette. Complete the dilution series as indicated in the figure.
8. Aseptically spread the four plates for this time point as indicated in the figure.

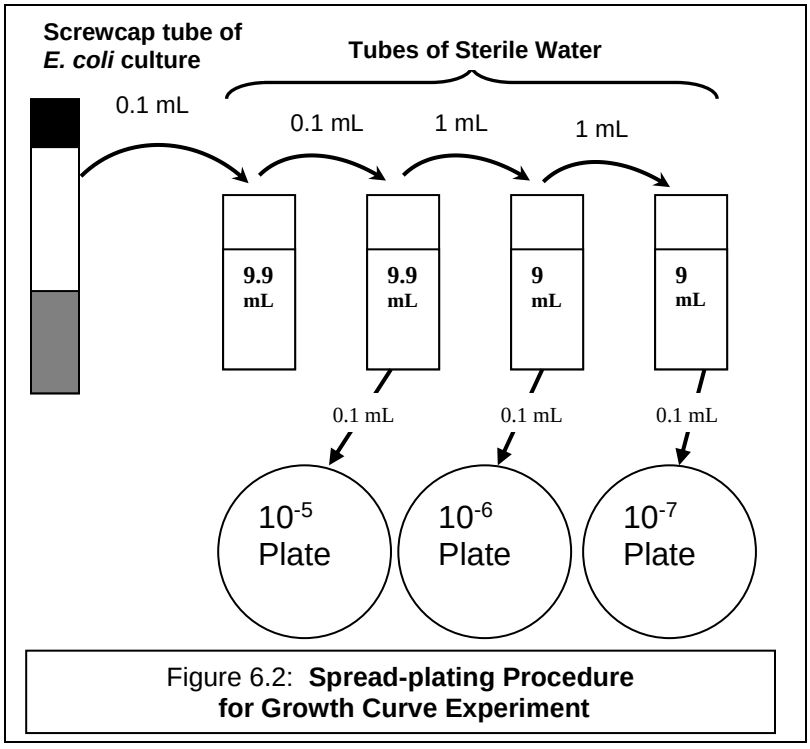


Figure 6.2: **Spread-plating Procedure for Growth Curve Experiment**

9. An absorbance reading of the culture should be taken every 20 minutes (as in Step 4) until the 160 minute mark. Each time the readings should be entered into the appropriate table in the report section of the manual. It is more important to record the exact time of each sample than to take the measurements exactly on time. However, whenever you take a measurement, be sure that you return the tube to the incubator as soon as possible.
10. Every 40 minutes (As you might have already guessed.), repeat the dilution and spread plating procedure USING THE NEXT SET OF TUBES until the 120-minute mark.
11. After the 160 minute mark has been reached, aseptically streak a loop of the culture in the screwcap tube onto the indicator plate (Be careful not to gouge the agar of the indicator plate. It is softer than regular agar, so you will need to use a lighter touch than usual.).
12. All plates spread or streaked should be placed in the bench cabinet of one of the group members for incubation unti next time. All used dilution and screwcap tube should be placed in the racks in the back of the lab for disposal.

Part 2: Session 5 Follow Up:

Do not work on the follow up portion until after you and your group have started exercise 1 of today's work!!!

Anaerobic Organisms

Those selected to help the TA's open the GasPak jars should follow the TA into lab next door. Please observe what happens when the jars are opened – especially the smell. Once the plates have been brought into the Micro lab and put at the front of the middle bench, retrieve the plates you and your partner streaked last time. Observe them and the plates that you incubated in your bench cabinet. Note your observations in the report section. Did the predictions you made for each test organism based on the results of the FTM and TGYA tubes pan out? Be sure to note from the board what each test organism was.

Phage Plates:

Remove the phage plates you prepared last time from your bench cabinet. Record your observations in the report section. Count the plaques on each plate, and use the dilution factors of the phage suspensions plated to determine the phage concentration that was present in the original phage suspension. Enter the data requested in the report section. This is significant because the phage stock you used to prepare these plates is the same that is being used today in the study of phage-induced lysis.

Lab Session 7

Background

Like every other living thing, microorganisms are intimately linked to their surroundings, constantly both impacting and being impacted by their environment. As you have no doubt learned previously from ecology, the environment of an organism may be divided into abiotic and biotic factors. You have already dealt with one abiotic factor in the exercises involing oxygen requirements, and then one biotic one in those dealing with bacteriophage. This session will represent the first part of an investigation into the other factors, both abiotic and biotic that impact microbial growth.

I. Microorganisms and Abiotic Environmental Factors

(See Brock pp 151 – 165)

Abiotic factors are those that arise from the chemical and physical properties of the habitat of an organism. Existing as they do as single cells, microbes are perhaps more impacted by these than are large organisms such as us. In general, the most important abiotic factors impinging on microbes are temperature, pH, and salt concentration. Somewhat less important, but by no means insignificant is the effect upon them by solar radiation, most significant being ultraviolet or UV radiation. This session, the class will be split into three groups, one each to investigate temperature, pH, and salt concentration, with everyone preparing for testing the effect of UV radiation.

A. Temperature

(See Brock pp 151 – 157)

Of all the physical aspects of the environment that impact an organism, the greatest is temperature. To understand the impact of temperature on bacteria and other microbes, one must understand two things. First, as they are generally single cells devoid of any way of regulating their internal temperatures, microbes tend to assume that of their environments. Second, every life process that takes place in a microorganism is carried out by the activity of enzymes. It would follow that the activity of the enzymes of the cell determines the activities of the cell. The way that these to factors relate is in the fact that enzymes are proteins that only possess catalytic activity if they are folded a proper way. Temperature impacts enzymatic activity by impacting the folding of the enzymes. As you can see in figure 7.1, there is, for any given enzyme, a certain temperature, the optimum, at which it is most active. As the temperature drops, the activity declines, mostly because of the kinetic aspects of the chemical reactions involved, but also because the folds of the enzymes begin to tighten. Eventually, and you will notice that the curve is rather gentle, activity stops as the cold inactivation minimum is reached. On the other hand, as the temperature increases, the enzyme begins to unfold, eventually reaching a point where its shape is lost and its catalytic activity ceases. Usually, this point, the heat inactivation maximum is reached before the enzyme becomes completely and typically irreversibly unfolded, or denatured. A curve describing the activity of a microbe over a given range of temperatures will closely track those of its enzymes due, so figure 7.1 can double in that capacity.

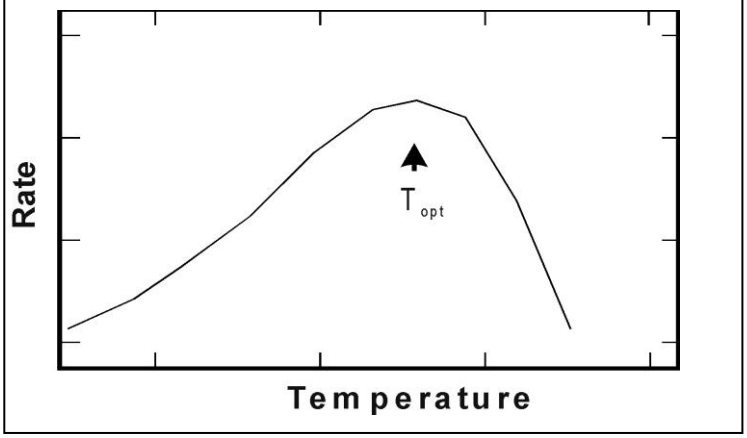


Figure 7.1: **Rate of Enzymatic Activity vs. Temperature**

While this curve typifies that seen with most enzymes and organisms, the temperatures of the different points upon it will vary from organism to organism, depending on its normal habitat. This is because evolution and natural selection will result in microorganisms having enzymes that have activity optima at the average temperature of their habitat. The range of activity of the enzymes above and below this optimum then determines the range of temperature over which a particular organism will grow. Though this should and in fact does result in a continuum of temperature ranges over which different organisms will

grow, there are a number of categories into which microbes are put based on their preferred growth temperatures. These are described in table 7.1. Please note that these are really three categories: psychrophiles, mesophiles, and thermophiles, with the extreme psychrophiles and thermophiles being subgroups.

Today, if you are group A, you will be inoculating media with a number of different organisms that will be incubated at various temperatures to demonstrate some of the variation in temperature preference.

Category	Growth Optima
Extreme psychrophiles	-20° C – 0° C
Psychrophiles	< 10° C
Mesophiles	10° C – 50° C
Thermophiles	>50° C
Extreme thermophiles	70° C – 113+° C

Table 7.1: **Categories of Organisms Based on their Preferred Growth Temperature**

B. pH

(See Brock 158 – 159)

Among the other abiotic factors, the one that is the most important is the concentration of hydrogen ions in the medium, generally referred to as its pH. Unlike with temperature, the selective permeability of microbial cell membranes means that they can regulate their internal pH, typically keeping it roughly neutral. However, like with temperature, there tends to a certain optimum pH for microbial growth, with a certain range over which growth is possible. There is much variation in the size of this range, with some organisms being capable of growth over very large pH values, while others require conditions that do not change much from their optimum. What primarily determines the size of these ranges is the variety of means by which the organism can maintain internal neutrality, though alteration of the proton concentration and charge differential across the cell membrane can impact transport proteins, metabolism, and the operation of flagella. Because small, localized shifts in pH are common, most microbes have simple membrane transport systems such a sodium/proton and potassium/proton antiporters that permit protons to be pumped out of or into the cell depending on the external conditions. Many microbes that must survive wide variations in pH must also have the capacity to produce specialized proteins that permit them to better adjust proton concentrations. If, however, the pH gets too far beyond the zone in which the organism can cope, the alteration of interior pH eventually leads to enzyme and cell membrane disruption and cell death. pH can also have an impact on the organism by affecting the oxidation states of nutrients and of electron donors (This will be covered in more detail during the metabolism section of the lab.).

As was true with temperature, organisms along a continuum of pH optima are divided into a number of different categories. These are summarized in table 7.2. Consider where organisms in each group might be found in the world. You will today be working with a number of organisms representing these groups, and will use them to inoculate a number of media with varying pH to see the effect.

C. Salt Concentration

(See Brock pp 159 – 161)

The effect of salt concentration on microbial growth is primarily due to its effect on the movement of water into and out of the cell. If the salt concentration inside the cell is higher than it is outside the cell, meaning that it is put in a hypotonic solution relative to its interior, then water will diffuse into the cell, causing it to swell. If the imbalance is great enough, the increase in water content can cause the cell to burst. The role of the cell wall possessed by most microbes is to prevent this from occurring. In the opposite situation, if the salt concentration outside the cell is higher than inside, or hypertonic, then water will diffuse out of the cell, causing the cell to shrink away from the cell wall in a process called plasmolysis. In isotonic situations, where the salt concentration is equal both outside and inside, there is, of course, no net flow of water inside or outside of the cell. In general, selective uptake of certain substances such as amino acids and potassium ions that will not interfere with cellular processes permit cells to maintain a slightly higher salt concentration internally so as to prevent dehydration. The ability of an organism to take up such solutes and its efficiency in doing so largely determines the range of salt concentrations it can survive, though at very high salt concentrations, the high levels of interior ion used to compensate can disrupt enzymes and cellular structures, providing an upper limit for most organisms. There do exist, however, organisms, primarily Archaea, that are halophiles, meaning that they are not only capable of growing with salt concentrations almost at the saturation point, but, in many cases, adaptations made to their enzymes have made them dependent upon these high concentrations.

As with the other abiotic factors covered, different organisms can grow under a variety of salt concentration ranges, and they are divided into a number of categories based upon this. These are summarized in table 7.3. Today, if you are in the salt concentration group, you will be inoculating a number of media with differing salt concentrations with different organisms, so as to determine the range of salt concentrations over which they will grow. As before, consider where these organisms might be found in the world.

D. Ultraviolet Light

(See Brock pp 272 – 273)

Ultraviolet light is radiation occupying the range of the electromagnetic spectrum between 40 and 400 nm in wavelength, or just past the lower limits of the visible spectrum. Few organisms are adapted to survive prolonged exposure to this radiation without substantial damage. Foremost amongst these are photosynthetic organisms such as cyanobacteria and plants that are exposed to large doses while collecting photosynthetically active light. The detrimental effect exerted by UV is largely due to its range including those wavelengths, centered on 260 nm, that are preferentially absorbed by DNA. When UV is absorbed by DNA, it causes neighboring thymine residues (The "T" in "ACTG".) bind together to form dimmers. These represent mistakes in the DNA code of the organism, and they can interfere in DNA replication, preventing reproduction and protein synthesis. Even if repaired, the result is often a mutation that alters the protein products of the affected genes. In microbes this leads to either mutants, or to cell death.

UV is used in some situations for sterilization purposes, though there are two major problems. First, UV has little penetrating power, being stopped by glass and even plastic. This allows it to only be useful in treating surfaces and air. Further, it has the same DNA-damaging effects on humans as it does on microbes, leading to burns and skin cancers (The tans that so many spend so much time working on are the result of radiation burns.), thus limiting its usefulness.

In general, most non-photosynthetic microbes prefer dark places and escape UV damage by avoiding UV altogether. Deinococcus radiodurans, for instance, is a wide found organism that is commonly found in clouds, where it receives heavy exposures. It has adapted by developing a large array of mechanisms of DNA repair. However, there are few exceptions beyond this. Endospores, however, do afford some protection not available to vegetative cells. In this session we will be irradiating three organisms, a Gram negative, a Gram positive, and Bacillus spores for varying amounts of time to examine the lethal effects of UV radiation on different bacteria.

II. Biotic Factors: Antibiotics

(See Brock pp 712 – 717, 719 – 723, 704 – 705)

Antibiotics are complex organic substances produced by living organism that inhibit or kill other organisms. While we tend to think of them only in a human context, they are actually commonly found in the world. A great many microbes produce them, though to what end is not always clear. Indeed many seem to simply be metabolic byproducts.

Due to this widespread and often copious production, they constitute one of the most significant biotic factors affecting the growth of all microorganisms, and not just that of human pathogens.

Alexander Flemming, a Scottish microbiologist discovered, by accident, the first antibiotic, Penicillin, in 1928. Since then, the use of antibiotics has revolutionized medicine and led to an explosion in the growth of the human population. What was not foreseen at the time was the rapid development of antibiotic-resistant bacterial populations. Resistance has two very simple bases. First, antibiotics work by a variety of mechanisms, some of which are mentioned in table 7.2. These mechanisms tend to be quite specific in their activity. As they work at the molecular level, this specificity means that, if the molecular target changes, then the antibiotic's effect may be decreased or even destroyed. This leads to increasing resistance in the microbes impacted by the antibiotic. The second is that the bacteria can evolve enzymes that degrade specific antibiotics, and the genes for these enzymes can spread through populations by plasmids (These are small, circular pieces of DNA that can be transferred from cell to cell through conjugation, of which we will learn later, and transformation.).

Antibiotic	Mode of Action
Ampicillin	Inhibits cell wall synthesis
Chloramphenicol	Binds to 50S ribosomal subunit
Erythromycin	Binds to 50S ribosomal subunit
Neomycin	Binds to 30S ribosomal subunit
Penicillin	Inhibits cell wall synthesis
Polymixin B	Inhibits membrane transport
Rifampin	Inhibits transcription by RNA polymerase
Streptomycin	Binds to 30S ribosomal subunit
Tetracyclin	Binds to 30S ribosomal subunit
Triple Sulfa	Structural analog of PABA
Ciprofloxacin (Cipro)	Blocks DNA gyrase activity, inhibiting DNA synthesis

Table 7.2: **Major Antibiotics and Their Modes of Action**

Resistance develops naturally as a consequence of natural selection. Whenever antibiotics are used on a bacterial population, such as that in an infected person, they impact those bacterial cells most sensitive to them, killing them off, and leading the population to shift to a makeup proportionally greater in less sensitive cells. Typically, the shift to this less sensitive population is not much of a problem, as they are few enough in number for the patient's immune system to finish off the infection. Problems arise in three situations, however. First, if the dosage of the antibiotic is too low to be effective, the population can slowly build up resistance to the point where they are resistant.

Second, if the course of antibiotic treatment is not seen through to conclusion, the premature ending of application can lead to a rebound in the bacterial population, this time with most of it being less sensitive or resistant (If another antibiotic is prescribed to treat the infection a second time, and the course of treatment is still not seen through to the end, the result is bacteria resistant to both antibiotics. This is the source of multiple drug resistant bacteria.). Third, the indiscriminant use of antibiotics, with them being prescribed for viral infections against which they have absolutely no impact, or their use in the cattle industry as a food supplement to increase growth, leads pathogenic bacteria being exposed to low levels of antibiotics in the environment and in those harboring small numbers of them normally leads also to the development of resistance.

It is thus very much necessary to have a means with which to determine which antibiotics will work against what bacteria so that the proper one may be prescribed to treat an infection. This is even more true when one considers that some antibiotics are narrow spectrum ones, meaning that they will only work against a few or even one species or strain of bacteria, while others are broad spectrum ones, meaning that they will be effective against a wide range of bacteria. Thus, even if resistance were not a factor, it would be necessary to establish the effectiveness of an antibiotic on different bacteria. It does the patient no good to prescribe him or her an antibiotic that will not work upon his or her infection. The standard way of doing this is to use what is referred to as the Kirby-Bauer method. This involves spreading a lawn-forming number of cells of the bacterium of interest on a plate and then placing disks impregnated with the antibiotics one wishes to test on the plate. The antibiotic will then diffuse out through the medium surrounding the disk, with the concentration decreasing as the distance from the disk increases. Bacterial growth will be impaired at a certain point around the disk where the concentration of the antibiotic is high enough to affect it. The result is circular areas around the disk within which the bacterium will not grow (See figure 7.1), called the zone of inhibition, and proportional in diameter to the bacterium's level of sensitivity to the antibiotic. This allows one to measure the diameter of the zone, and using a standardized chart, determine if the bacterium is sensitive, of intermediate sensitivity, or resistant to the antibiotic. Standardization for each antibiotic is necessary because different antibiotics will diffuse at different rates, affecting the diameter of the zone corresponding to the different levels of sensitivity.

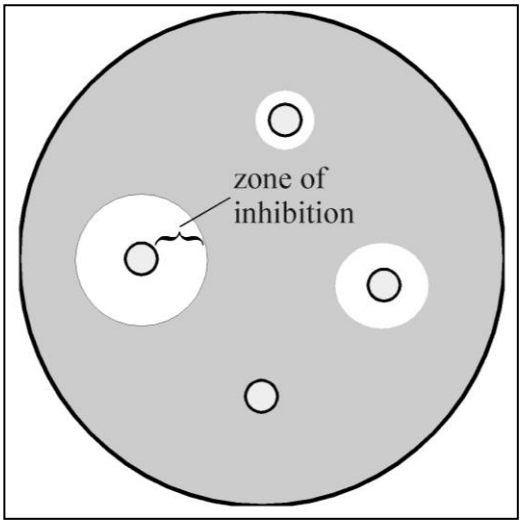


Figure 7.1: **Example of a Kirby-Bauer Test of Antibiotic Sensitivity**

Today you will be preparing plates for a Kirby-Bauer method test of six different antibiotics on four different bacteria.

Procedures

Part 1: Session 6 Follow Up: Growth Curve and Phage Infection:

Each group should count colonies on the dilution plates they spread last time. This should include examining the indicator plates streaked at the end of the period last session, and recording whether or not there is evidence of bacteriophage in the culture that was streaked out. Each group should then record their data, both from the colony counts and the spetrophotmetric readings from last time, on the class data sheet that will be passed around during the period. Copies of this data sheet will be made after they are filled out so each student will have data for all groups. Lab reports are to be completed using your group's data for the conditions you tested, and one other group's data for the condition you did not test.

Winogradsky Column:

Go to the hood in the back, and without disturbing it, examine the column your group constructed. Record your observations in the appropriate place in the report section (The Winogradsky column report is toward the end.). Be sure to note if any color bands are obvious (This is unlikely, but one never knows.).

Part 2: Today's Exercises:

Exercise 1: Abiotic Environmental Factors and Prokaryotic Growth

Section I: Temperature, pH, Salt Concentration, and Microbial Growth

For today's part of this exercise, you will work in the same groups as for the growth curve exercise. Each group will do only one of the three parts. The group for bench 1 will do exercise "A", that for bench 2 will do exercise "B", and that for bench 3 will do exercise "C" (Everyone, however, is responsible for knowing about all three, their principles, how they are done, and what the conditions for each were.). Work on the parts of this exercise in between the time points for the growth curve exercise.

A: The Effects of Temperature on Microbial Growth

Cultures: *E. coli*
Serratia marcescens
B. stearthermophilus
Sulfolobus acidocaldarius

Materials: 18 NB tubes (Including 3 screwcap tubes)
6 XT tubes (Including 1 screwcap tube)

1. Divide the NB tubes into three groups, one group each for *E. coli*, *S. marcescens*, and *B. stearthermophilus*. Label each tube in the different groups with its corresponding organism, making sure that one screw cap tube is in each group. Label the six XT tubes with *S. acidocaldarius*. Each of the six tubes in each group should be labeled with a different temperature: 5 C, 25 C, 37 C, 42 C, 55 C, and 75 C. The screwcap tubes should all be labeled for the 75 C condition.
2. Thoroughly vortex each of the culture tubes you have collected.
3. Using aseptic technique, transfer a loop of each culture into all six of the tubes for it.
4. When finished, take the tubes to the front of the middle bench, and place them each in the rack labeled with the appropriate temperature condition.
5. The culture tubes should then be place in the disposal racks in the back of the room.

B: The Effects of pH on Microbial Growth

Cultures: *E. coli*
S. aureus
Sulfolobus acidocaldarius
Bacillus alcalophilus

Materials: 3 pH 1 NB tubes, 1 pH 1 XT screwcap tube
3 pH 3 NB tubes, 1 pH 3 XT screwcap tube
3 pH 5 NB tubes, 1 pH 5 XT screwcap tube
3 pH 7 NB tubes, 1 pH 7 XT screwcap tube
3 pH 9 NB tubes, 1 pH 9 XT screwcap tube
3 pH 11 NB tubes, 1 pH 11 XT screwcap tube

1. As you gather the tubes listed, you should label each one according to what medium it is, and what pH it is (Otherwise, unless you can tell pH 3 from pH 9 by sight, you will be in trouble.). Then divide the NB tubes into three groups with each group of tubes including one of every pH condition. Label all the tubes in each of the particular groups with *E. coli*, *S. aureus*, or *B. alcalophilus* so that each of these three organisms has five tubes with five different pH conditions. The XT tubes should all be labeled with *Sulfolobus acidocaldarius*.
2. Thoroughly vortex each of the tubes of culture you have collected.
3. Using aseptic technique, transfer a loop of each culture into all six of the tubes for it, so that each organism has been used to inoculate a separate tube for each pH condition.
4. When finished, the NB tubes can be incubated in your bench cabinet, or else in those of your group members. The tubes inoculated with *S. acidocaldarius*, however, should be taken to the front of the middle bench, and placed in the rack labeled as for them.
5. The culture tubes should then be place in the disposal racks in the back of the room.

C: The Effects of Salt Concentration on Microbial Growth

Cultures: *E. coli*
S. aureus
Vibrio fischeri
Halobacterium salinarum

Materials: @ 0.5% NaCl: 4 NB tubes
@ 1% NaCl : 4 NB tubes
@ 2.5% NaCl: 3 NB tubes, 1 VB tube
@ 5% NaCl : 4 NB tubes
@ 7.5% NaCl: 4 NB tubes
@ 10% NaCl : 4 NB tubes
@ 25% NaCl : 3 NB tubes, 1 HB tube

1. As you gather the tubes listed, you should label each one according to what medium it is, and what its salt concentration it is to prevent confusion. Once this is done, group the media into seven groups with four media tubes in each, all with the same salt concentration. Please note that you should have 1 VB tube in the 2.5% NaCl group, and 1 HB tube in the 25% NaCl group. Label each tube in a salt condition group so that there is a tube specifically for each organism under study. Please be sure to label the VB tube in the 2.5% NaCl group with *Vibrio*, and the HB tube in the 25% NaCl group with *Halobacterium*.
2. Thoroughly vortex each of the tubes of culture you have collected.
3. Using aseptic technique, transfer a loop of each culture into all seven of the tubes labeled for it, so that each organism has been used to inoculate a separate tube for each salt concentration condition.
4. When finished, place the tubes in the bench cabinet of a group member to incubate until next session.
5. The tubes of culture that you used for your inoculations should then be placed in the racks in the back of the room for disposal.

Section II: Lethal Effects of UV on Bacterial Growth

Work alone on this part!!!

Materials: Culture assigned to you by your microscope #
NA plate (1)
Sterile cotton swab

1.
2. Label your NA plate with your name, the date, your assigned organism (See table 7.3), and your time point.
3. Using aseptic technique (Note: It is unnecessary to flame your swab.), dip your swab into your assigned culture. As you withdraw the swab, press and rotate it against the side of the tube to expel excess fluid.
4. Swab the plate to cover it with culture. The best way to accomplish this is to swab the plate from side to side, top to bottom, turn the plate a third of the way around, swab it again as before, turn it another third, and swab it again.
5. Take your plate over to the box set up on the side bench next to the UV box. The TAs will do the irradiating (The university is queasy about undergraduates irradiating anything – go figure.).

Organism	**Exposure Time**								
	10 sec	**20 sec**	**40 sec**	**80 sec**	**2.5 min**	**5 min**	**10 min**	**20 min w/out lid**	**20 min w/ lid**
Staph. aureus	1, 28	4, 31	7, 34	10, 37	13, 40	16, 43	19, 46	22, 49	25, 52
Serratia. marcescens	2, 29	5, 32	8, 35	11, 38	14, 41	17, 44	20, 47	23, 50	26, 53
***Bacillus megaterium* spores**	3, 30	6, 33	9, 36	12, 39	15, 42	18, 45	21, 48	24, 51	27, 54

Table 7.3: **Assignment of Organisms and Time Conditions for UV Exercise on Basis of Microscope Number**

Exercise 2: The Kirby-Bauer Method of Determining Microbial Sensitivity to Antibiotics

Work alone on this exercise!

Materials: 1 NA plate
Assigned culture
1 sterile cotton swab
Antibiotics dispenser
(At station)

Organism	*E. coli*	*S. aureus*	*Pseudomonas aeruginosa*	*Proteus vulgaris*
Microscope Number	4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52	3, 7, 11, 15, 19, 23, 27, 31, 35, 39, 43, 47, 51	2, 6, 10, 14, 18, 22, 26, 30, 34, 38, 42, 46, 50, 54	1, 5, 9, 13, 17, 21, 25, 29, 33, 37, 41, 45, 49, 53

Table 7.4: **Assignment of Cultures on the Basis of Microscope Number**

1. Note which organism is assigned to you on the basis of your microscope number per the table.
2. Aseptically dip the sterile cotton swab into the broth of your assigned culture. As you withdraw it from the tube, press the tip against the side of the tube to expel excess fluid.
3. Use the swab to cover the surface you your plate with culture. It is absolutely necessary to get the culture distributed as evenly as possible over the plate, so you should swab the entire plate from top to bottom using a rapid, tight side-to-side motion. Once this has been done, rotate the plate by a third, and then swab this way again. Turn the plate a third of the way around again, and swab it again. Dispose of the swab in a designated beaker of Lysol.
4. Briefly allow the plate to dry.
5. Go to one of the dispenser stations. Once you have the dispenser, remove the top from your plate, and place the dispenser over it (The circular bottom of the dispenser should just fit over the plate.). Press down on the plunger of the dispenser firmly yet gently (Break it and you've bought it.) until you hear and feel a click. Remove the top dispenser and place it aside for use by the next student.
6. At the same station there should be a beaker of ethanol, a lit Bunsen burner, and a pair of forceps (Tweezers). Dip the tips of the forceps in the ethanol, and carefully flame them. Use the now sterile forceps to gently tap the disks impregnated with antibiotics into place. You really just want to make sure the disks will stay in place when the plate is inverted. **You do not, you really do not, want to forcibly push the disks into the agar so that they break the surface!**
7. Invert the plate and place it in your bench cabinet to incubate until next session.

Lab Session 8

Background

I. Minimum Inhibitory Concentration of Antibiotics

(See Brock pp 704 – 705)

While the Kirby-Bauer method allows one to determine what level of sensitivity a bacterial pathogen shows toward an antibiotic, this information is worthless if one does not determine the concentration of the antibiotic at which it inhibits the antibiotic. This is crucial information to have. There is, of course, the potential threat of encouraging the development of resistant strains of the bacterium and not helping the patient if one gives too low a dosage to have an effect. There is also the threat of giving too high a dosage. Most antibiotics are toxic to humans at some level, and one of the reasons why a good antibiotic is so useful is because it will kill infecting bacteria before killing the patient. It is thus important to find a concentration of an antibiotic that is high enough to kill the pathogen, but not the patient. Kirby-Bauer plates do not allow this because one cannot quantify the concentration of antibiotics at various distances from the disk, and can thus not know the concentration at the point where the bacteria stop growing. The solution is to do a minimum inhibitory concentration, or MIC determination for the antibiotic with the bacterium of interest. In the standard form of this, one makes a series of broth tubes containing varying concentrations of the antibiotic, typically with two-fold increases in the concentration from one tube to another. To each of these tubes is added a standardized quantity of bacterial cells. The tubes are incubated and then all checked at the same time, with them being read according to whether or not the bacterium grew. The tube with the lowest concentration in which the bacterium did not grow is the MIC for that antibiotic for that organism. With this information, a doctor can determine the dosage necessary, given his or her physical characteristics, to combat the infection without killing the patient.

Today you will determine the MIC of the six antibiotics you tested with the Kirby-Bauer method with a few different bacteria.

II. General Characteristics of Bacteria I

You have likely by now guessed that most of the differences between bacteria are not readily apparent by simply looking at them. Indeed, the most distinct characteristics of bacteria and other microorganisms are to be found at a more fundamental level than that of their shape or appearance. You have learned something of this unseen diversity from exercises you have done previously, such as those covering oxygen requirements. In general, we divide the characteristics of bacteria into three categories: morphological characteristics, of which you have learned much already, and then cultural characteristics and metabolic or physiological characteristics. The next few labs will deal with looking at these characteristics.

A. Cultural Characteristics

Cultural characteristics are those macroscopic characteristics of bacterial growth. They are only apparent when there are large numbers of cells present. The most important of these, as you might have guessed, regards the appearance of bacterial colonies, their color, texture, shape, even their borders and whether or not they protrude upwards from the plate (Their elevation). Bacteria also tend to have characteristic patterns of growth when grown in other medium forms such as on slants or broths. While these characteristics do not often tell one much about the individual cells, they are of occasion of value.

B. Metabolic and Physiological Characteristics

(Brock pp 110 – 131, Chapter 17, See pp 811 – 814 for testing)

Metabolism can be understood to be the sum of all the chemical reactions that take place in a cell. It is here that the greatest diversity of bacteria may be seen outside of their DNA. Most of the characteristics that we will be studying today and in the next few sessions are metabolic in nature. As mentioned earlier in the context of the study of the effect of abiotic environmental factors on bacterial growth, all metabolic reactions that take place in a cell are carried out using enzymes. Organisms are limited in what they can do metabolically, what they can live off of, and thus where they are capable of living by the complement of enzymes encoded by their DNA. Due to this, bacteria will differ

greatly in their metabolic capabilities. This is not only interesting from the standpoint of understanding the means by which bacteria and other microorganisms live, but also because it provides us a way of building up a bank of characteristics about an organism that can be used to identify it from all others. This will come in handy later when we go about the identification of a bacterial unknown.

We have already studied one of the most significant metabolic capacities of bacteria when we looked at the abilities of great many of them to live without oxygen. At the time, the question of how they do this was glossed over. The answer is that there are two means by which certain bacteria manage to live quite well without oxygen. To understand this, we have to briefly discuss the biochemistry of how organisms generate energy.

In the biological world, there are generally two basic challenges: how to get materials for growth and reproduction, and how to generate energy. Both involve special interactions with the world outside the organism. For materials, there are photosynthetic organisms like plants and cyanobacteria that use power from the sun's light to build materials from scratch using carbon dioxide. Because they are able to do this, they are called autotrophs. Many other organisms, humans included, are heterotrophs, meaning that they must take in premade materials, with the ultimate source always being autotrophs. As for energy, this is generated from the breakdown of organic compounds; usually sugars, and again we run into the autotroph/heterotroph divide. Autotrophs will usually generate all the organic material they need for energy generation, while heterotrophs will again have to use premade materials. The big divide, however, is how organisms then extract energy from these organic compounds.

Biological energy is stored in the form of compounds with very energy-rich molecular bonds. Foremost amongst these is ATP. Energy production in organisms is thus concerned with how to constantly replenish the supply of ATP and other such storage molecules, and this is done through the careful breakdown of the organic compounds taken into the organism as food. In a typical biochemical pathway, a food substance will usually be converted to a sugar such as glucose and then broken down using glycolysis or a similar pathway. Glycolysis couples the regeneration of ATP to the breakdown of glucose and ends up producing two regenerated ATP molecules, and two molecules of pyruvic acid. This is called substrate-level phosphorylation. Electrons were also removed from the glucose as it was broken down, meaning it was oxidized, by an electron acceptor, NAD. To allow glycolysis to continue, the electrons accepted by the NAD (Called NADH once it has taken on the electrons) must be removed.

Oxidation and reduction can be confusing. The easiest way to remember them is with a simple mnemonic:
LEO goes GER, where:
LEO = Loss of Electrons is Oxidation
GRR = Gain of Electrons is Reduction
(It is silly, I know, but it works.)

Box 8.1: **Oxidation vs. Reduction**

There are two ways used by organisms to deal with regenerate the NAD used during glycolysis. The first is called fermentation, and it can only take place in the absence of oxygen. The second is called respiration, which may take place in the presence or absence of oxygen (For reasons we will soon get to.).

In fermentation, the electrons are dealt with by the reduction of an organic molecule, usually the pyruvate just generated from glucose, by NADH. Fermentation is one of the major ways in which microorganisms make a living without oxygen, and it is notable for its diversity. Organisms differ widely in the range of substances they break down and ultimately use in fermentation, for instance. Further, the range of byproducts of fermentation is great. In the reduction of organics by NADH, end products may include ethanol (grain alcohol), acetic acid (vinegar), carbon dioxide, butyric acid (A substance that gives rancid butter its characteristic smell.), hydrogen, and a great many other substances and compounds, many of which are virtually unpronounceable. Fermentation has been of enormous value in human history because of this diversity of end products, as well as a couple of other major features. First, two ATP are not much when it comes to the energy needs of an organism. Because of this, fermentative organisms have to break down and ferment a very large amount of food material to remain active, meaning that end products are produced in very, very large amounts (We have all had direct experience with this. Many of the bacteria in our intestinal track are capable of fermenting a large number of sugars, amino acids, and other substances we are incapable of breaking down with our own enzymes. When we eat foods with such substances that we have trouble with, such as the odd amino acids in beans and lactose in milk, they end up being fermented by our gut flora, thus producing gases and thereby, well, social discomfort, shall we say.). Second, the end products of fermentation tend to be toxic to most microorganisms in large amounts. Provided that there is not a mechanism to remove them, they build up until they kill the microbial population producing them. Due to this, fermentation has been a relatively easy way of making materials of value for the preservation of foodstuffs as well as disinfection (The ancients rarely drank straight water, preferring instead to drink diluted wine. The alcohol content of the wine would then kill the pathogens in the water. They did not know anything about this, mind you, other than it worked.).

Respiration involves a totally different and much more efficient way of dealing with the problem of regenerating oxidizing power. In it, the pyruvate left over at the end of glycolysis is sent through the tricarboxylic acid, or TCA cycle (This is also called the citric acid cycle, because citric acid is a major component of it that is regenerated

at the end of each turn of the cycle. Some organisms are able to live off of citric acid as the only food, or carbon source, with the citric acid being immediately shunted into the cycle for energy production. This is not true of all, however.). Over the course of this, the pyruvate is broken down into carbon dioxide. Carbon dioxide is the most oxidized form of carbon, meaning that while the pyruvate is broken down, it is also having its electrons stripped off, again by NADH, as well as similar electron carriers. Where are these electrons going? That question is the key to understanding respiration. The answer is that the electrons are ultimately being fed into the electron transport system (ETS). In the ETS, the electrons are passed off a number of times through a chain of different electron carriers, the cytochrome system. Their passage through the ETS results in the pumping of protons out of the cell, producing a chemical and electrical gradient that is exploited by the cell through the use of ATPase, a large enzyme that allows protons back into the cell, and harvest the energy this generates to regenerate ATP.

So what happens to the electrons, then? Ultimately, the electrons reach the end of the electron transport chain, where they are gotten rid of by the reduction of a terminal electron acceptor. In oxidative, or aerobic respiration, this terminal electron acceptor is oxygen, which is reduced to water. Aerobic respiration is common in the world, and used by all animals for the simple reason that it produces the most energy. Without getting into reduction potential and all that, it may simply be said that, if oxygen is the terminal electron acceptor, more protons can be pumped out of the cell and thus more ATP regenerated than is possible with any other terminal electron acceptor. In fact, at its most efficient, aerobic respiration may produce as many as 38 ATP for each molecule of glucose, compared to the mere 2 for fermentation (Did I not say that fermentation required a lot of fuel? See why?).

Terminal Electron Acceptor	**Reduced Product**
O2	H2O
NO_3^- (Nitrate)	NO_2^- (Nitrite), N_2O (Nitrous oxide), N_2 (Nitrogen gas)
SO_4^{2-} (Sulfate)	H_2S (Hydrogen Sulfide)
CO_2	CH_4 (Methane)
Fe^{3+} (Ferrous iron)	Fe^{2+} (Ferric iron)
S^0 (Elemental sulfur)	H_2S

Table 8.1: **Some Common Terminal Electron Acceptors and Their Products of their Reduction**

Aerobic respiration is not the only kind of respiration, however, and this is something that many students have a hard time learning. In fact, a wide variety of inorganic compounds may be used as the terminal electron acceptor, and it is this fact that allows certain microorganisms to utilize respiration without oxygen. The most common terminal electron acceptor after oxygen is nitrate (NO_3^-), which is reduced to nitrate (NO_2^-). When I said in the background to session 1 that there were bacteria that could breath uranium, this is what I meant: that there are those species that can use uranium as a terminal electron acceptor. Other terminal electron acceptors used by bacteria and other microbes are listed in table 8.1. Any time an organism can use a terminal electron acceptor other than oxygen, it can live quite well without oxygen (Indeed, if the organism can not use oxygen, then it will not be able to be active in the presence of oxygen.). To make things a bit more confusing, the same bacterium may be capable of using many different terminal electron acceptors, preferring, of course, to use the ones that will yield the most energy first, and even of using fermentation if necessary. It is all a matter of having the proper enzymes.

Today you will be inoculating media with which to test aspects of bacterial metabolism. The first one will involve the inoculation of another thioglycolate tube to remind you of how oxygen requirements are determined. You will also inoculate tubes of media designed to allow for the testing of the capacity an organism to ferment a number of different sugars. These are called Phenol Red Sugar tubes. They contain a nutrient broth in which only one particular sugar of interest is available for the organism to use. It also contains the pH indicator phenol red. The medium starts off slightly basic, and thus red. If the organism produces acids from the fermentation of the sugar, this will lower the PH of the medium, causing the indicator to turn the medium yellow. In each tube is also small, inverted, glass tube called a Durham tube, which is designed to trap gases that may be produced from the fermentation of the sugar. You will also use a special medium, the MR-VP medium, to test for the capacity of an organism to ferment glucose to a number of different end products. A tube of Simmon's Citrate slant will also be inoculated to test the capacity of the organism to utilize citric acid as its only carbon source. A nitrate broth will also be inoculated that is designed to allow one to test for the capacity of an organism to use nitrate as its terminal electron acceptor. Two other tests to be done with the media you will inoculate today concern specific enzymes involved in metabolism, and they will be explained next time.

III. Environmental Sampling

Today you will also have another chance to see the variety of microorganisms that are around you. You will be assigned either a general location or a substance to sample. If a location, you will be using a special medium designed for the direct sampling of surfaces. If a substance, you will be plating a sample of the substance on a plate of a special rich medium formulated for the growth of many different types of microbe. What you are to do with what you turn up will be discussed later.

Procedures

Part 1: Session 7 Follow Up:

Abiotic Factors I:

Temperature and Microbial Growth

If you were in the temperature group: Retrieve the tubes inoculated last time from the racks at the front of the middle bench, and examine them. For each organism and temperature condition, rate the growth in the tube 0 through 5, where 0 means that no growth is evident (There will sterile tubes available to which you can compare your tubes.), 5 means that there is thick, turbid growth evident (There will be such tubes available to which you can compare your tubes.), and 1,2, 3, and 4 are intermediate. The members of the group should please consult with one another on these ratings so that the numbers correspond to each other, and do not display individual biases. When finished, the group's data should be entered into a group data sheet that will be copied so that every student may have a copy.

If you were not in the temperature group: Make sure that you get a copy of the temperature group data sheet. You will be responsible for obtaining these data and using them to complete your report.

pH and Microbial Growth:

If you were in the pH group: Retrieve the tubes inoculated last time from the bench or benches in which they were incubated (Make sure to get the Sulfolobus tubes at the front!). For each organism and pH condition, rate the growth in the tube as 0 – 5, where 0 means that no growth is evident (There will be sterile tubes available to which you can compare your tubes.), 5 means that there is thick, turbid growth evident (There will be such tubes available to which you can compare your tubes.), and 1, 2, 3, and 4 are intermediate. The members of the group should please consult with one another on these ratings so that the numbers correspond to each other, and do not display individual biases. When finished, the group's data should be entered into a group data sheet that will be copied so that every student may have a copy.

If you were <u>not</u> in the pH group: Make sure that you get a copy of the pH group data sheet. You will be responsible for obtaining these data and using them to complete your report.

Salt Concentration and Microbial Growth:

If you were in the salt concentration group: Retrieve the tubes inoculated last time from the bench or benches in which they were incubated. For each organism and salt concentration condition, rate the growth in the tube as 0 – 5, where 0 means that no growth is evident (There will be sterile tubes available to which you can compare your tubes.), 5 means that there is thick, turbid growth evident (There will be such tubes available to which you can compare your tubes.), and 1, 2, 3, and 4 are intermediate. The members of the group should please consult with one another on these ratings so that the numbers correspond to each other, and do not display individual biases. When finished, the group's data should be entered into a group data sheet that will be copied so that every student may have a copy.

If you were <u>not</u> in the salt concentration group: Make sure that you get a copy of the salt concentration group data sheet. You will be responsible for obtaining these data and using them to complete your report.

Abiotic Factors II: Lethality of UV:

Retrieve the plate you prepared last time and examine it. You should record observations of your particular UV plate in the appropriate place in the report section (Please be sure to record your assigned organism and exposure time.). As you examine your plate, rate it on a scale of 0 through 5 according to the following:

0 = No growth
1 = no more than 10 colonies
2 = more than 10 colonies, but fewer than 50

3 = more than 50 colonies, but fewer than 150

4 = more than 150 colonies, up to the point where there are too many colonies to count, but not so many than individual colonies can be discerned

5 = confluent lawn of growth

There will be a class data sheet going around for this exercise. When it comes to you, you should record the rating you gave your plate in the appropriate place for your organism and exposure time. Copies of this data sheet will be made before the end of class. Be sure to get a copy of this before you leave. The class data should then be recorded in the appropriate tables in the report section. The lab reports should be done using the data for the entire class.

Kirby-Bauer Test of Antibiotic Sensitivity:

Take the plate you prepared last time from your bench cabinet, and obtain from the front of one of the benches a ruler. Measure the diameters of the zones of inhibition surrounding the antibiotic disks for your plate (i.e. The clearings that show inhibited bacterial growth.) in millimeters (Not centimeters or inches.) from one side to another. If no zone of inhibition is evident, then you should just measure the diameter of the disk. Record the diameters in the appropriate place in the report section. A class data sheet will be going around. Neatly record your data in the appropriate places, being sure to leave adequate space for others to write their data. Be sure to get a copy of the class data before the end of class today. The report is to be done using the class data, and not just yours. Use the antibiotic sensitivity table (8.1) provided to evaluate the Resistance, Sensitivity, or Intermediate reaction of the tested organism to the tested antibiotics using the averages of the class data as detailed in the report section.

Antibiotic		**Disk Code**	**Disk Potency**	**Resistant Diameter (mm)**	**Intermediate Diameter (mm)**	**Sensitive Diameter (mm)**
Ampicillin	**For Staph.**	AM	10 μg	<28	28	>28
	For Everything Else			<12	12 – 13	>13
Cloramphenicol		C	30 μg	<13	13 – 17	>17
Erythromycin		E	15 μg	<14	14 – 17	>17
Neomycin		N	30 μg	<13	13 – 16	>16
Penicillin G	**For Staph**	P	10 units	<28	28	>28
	For Everything Else			<12	12 –21	>21
Polymixin B		PB	300 units	<9	9 – 11	>11
Rifampin		R	5 μg	<24	24	>24
Streptomycin		S	10 μg	<12	12 –14	>14
Tetracyclin		T or TE	30 μg	<15	15 –18	>18
Triple Sulfa		SSS	300 μg	<13	13 –16	>16

Table 8.2: **RIS Determination of Test Organisms to Test Antibiotics Using the Recorded Zones of Inhibition**

Part 2: Today's Exercises:

Exercise 1: Determination of the Minimum Inhibitory Concentration of Antibiotics

Work in groups of two on this exercise (Depending on the number of people, some may have to work alone.).

The groups at each of the three benches will work with a single organism: **First bench:** ***E. coli***
Second bench: ***S. aureus***
Third bench: ***B. subtilis***

The groups at each bench will be numbered 1 – 6. Each group will be assigned one antibiotic with which to work:

Groups 1: Ampicillin
Groups 2: Triple Sulfa
Groups 3: Erythromycin
Groups 4: Polymyxin B
Groups 5: Streptomycin
Groups 6: Rifampin

Materials: Set of MHB tubes with concentrations of your assigned antibiotic:
(0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 ug/mL)

Be very careful to label these tubes as you get them so that you do not confuse yourself later!

Test tube rack
Broth culture of assigned organism
Sterile 1 mL pipettes and blue pipump
9.9 mL tube of sterile saline (1)
9 mL tube of sterile saline (1)

1. Obtain the materials listed above and set them out on your bench.
2. Aseptically transfer 0.1 mL of the broth culture of your bench's assigned organism to the 9.9 mL tube of sterile saline, and vortex. Aseptically transfer 1 mL from the 9.9 mL saline tube to the 9 mL saline tube, and vortex. This is your 10-3 dilution tube.
3. Aseptically transfer 0.1 mL of the 10-3 dilution tube each of the MHB antibiotic tubes.
4. When you are finished with the transfers, vortex the tubes and place them in your bench cabinet to incubate.

Exercise 2: Characteristics of Microorganisms: Cultural Characteristics and Metabolic Properties I:

Materials: Assigned Culture
1 NB tube
1 NA plate
2 NA slants
2 MR-VP tubes
1 Thioglycolate tube
Test tube rack
1 Simmon's Citrate slant
1 Nitrate broth tube
4 Phenol Red Sugar tubes (Dextrose, lactose, mannitol, and sucrose; label them as you get them – they all look the same!)

1. Collect the culture you have been assigned by your microscope number (See table 8.2). Please note that everyone should have their own tube of culture today.), and the media listed above. Be sure to label the media tubes as you collect them to avoid confusion. Also label each with your name, the date, and your organism.
2. Inoculate all the media you have collected with your assigned culture

Organism	Microscope Number
Pseudomonas aeruginosa	1, 5, 9, 13, 17, 21, 25, 29, 33, 37, 41, 45, 50
Staphylococcus. aureus	2, 6,10, 14, 18, 22, 26, 30, 34, 38, 42, 46, 51
Enterobacter aerogenes	3, 7,11, 15, 19, 23, 27, 31, 35, 39, 43, 47, 52
E. coli	4, 8,12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 53

Table 8.2: **Assignment of Cultures on the Basis of Microscope Number**

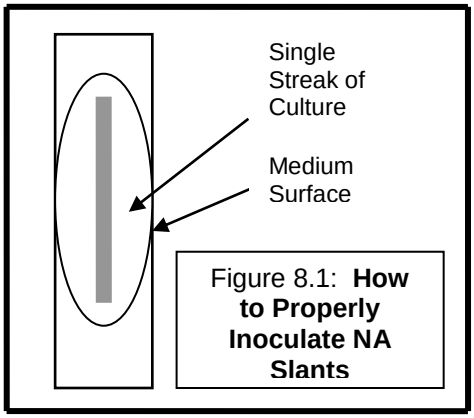


Figure 8.1: **How to Properly Inoculate NA Slants**

as follows:

a. Aseptically streak the NA plate for isolated colonies using whichever isolation technique you prefer.
b. Aseptically transfer a loop of culture to each of the following: the NA broth, the 2 MR-VP tubes, the Nitrate broth tube, the 4 Phenol Red Sugar tubes
c. Aseptically inoculate each of the 2 NA slants with a loop of culture by making a single streak up the surface of the slant of medium, being sure not to gouge the medium (See figure).
d. Use your inoculating needle to aseptically streak a sample of your culture on the surface of the Simmons Citrate slant, after which you will stab the needle straight down the middle of the agar to the bottom of the tube, distributing the culture through the agar. Withdraw the needle along the same path by which it entered.
e. Use your inoculating needle to aseptically stab-inoculate the Thioglycolate tube. Remember that you want to stab straight down, and then bring the needle straight out along the same path.

3. When you are finished with the inoculations, place all the media in your bench for incubation until next time. The tube of culture from which you made your inoculations should be placed in the back test tube racks for disposal.

Exercise 3: Environmental Sampling or Hunting the Wild Microbe

Each person should work on this individually. The location or substance you are assigned to sample is listed in the table below based on your microscope number. If you would prefer to sample a location or sample other than the one you have been assigned, please see one of the TAs.

Materials: 2 RODAC plates or 2 TSA plates

If you have been assigned a location to sample:

1. Collect your two RODAC plates. These plates have specially shaped, concave surface that allows the medium to be easily pressed against a surface for sampling.
2. Go to the general location to which you have been assigned on the basis of your microscope number. Please note that these are general locations only.
3. Find two surfaces in the general location you have been assigned that interest you for whatever reason (Have you ever wondered how well they clean the tables in the dining pavilion? How sanitary are those toilet seats? Et cetera.), and sample them. To do this, you simply remove the top from the RODAC plate, and then firmly, yet gently press the surface of the medium to the surface to be sampled (You do not want to press so hard that the medium cracks), and then replace the top to the plate. Sample the second surface in the same way using the second RODAC plate.
4. Once you have sampled the two surfaces you have chosen, return to the lab (If you have had to leave it.). Tape the tops of the plates to the bottoms, and then label the two plates with your name, the date, the location, and the surface sampled.
5. Place the plates in the box at the front of the middle bench to be incubated.

If you have been assigned a substance to sample:

Location or Substance to Sample	**Microscope Number**
Bathrooms	1, 12, 23, 34, 45
Dining Pavilion	2, 13, 24, 35, 46
Outside (Try to avoid objects humans touch regularly.)	3, 14, 25, 36, 47
Personal Items (No body parts)	4, 15, 26, 37, 48
Rieveschl (Lab excluded)	5, 16, 27, 38, 49
Spoiled Milk	6, 17, 28, 39, 50
Spoiled Pork	7, 18, 29, 40, 51
Spoiled Ground Beef	8, 19, 30, 41, 52
Spoiled Chicken	9, 20, 31, 42, 53
Feline Fecal Matter	10, 21, 32, 43, 54
Canine Fecal Matter	11, 22, 33, 44, 55

Table 8.3: **Assignment of Location or Substance to be Sampled by Microscope Number**

Note: Please be careful while sampling your substances. All of them have been previously found to harbor pathogens.

1. Collect your two TSA plates. Label the backs with your name, the date, and the substance you will be sampling.
2. Go to the proper station for the sampling. Here you will find tubes with a specificed dilution of broth derived from the substance, sterile pipettes, a beaker of Lysol for used pipettes, a Bunsen burner, a hockey stick, and a beaker of ethanol.
3. Use a sterile pipette to aseptically transfer 1 mL of the dilution to each of the two plates you have. When finished, discard the pipette in the used pipette beaker.
4. Aseptically spread each plate using the hockey stick and ethanol provided.
5. Take the plates back to your work station and let them sit until they dry.
6. When dry, take the plates to the front of the middle bench and place them inverted in the box to be incubated.

Lab Session 9

Background

I. Metabolic and Physiological Characteristics II

(See Brock pp 111 – 113, 601 – 603, 604 – 605, 811 – 814)

Last time you learned about the differences in how microorganisms go about generating the energy they need for their cellular processes, and how these differences can impact their oxygen requirements. You also inoculated a number of media aimed at allowing you to test a small part of the metabolic and physiological capabilities of a few bacteria with which we have worked this quarter. This focus on metabolism continues into today's session.

Last time it was mentioned that the enzymes an organism produces determine its metabolic capabilities, but few individual enzymes were actually mentioned. This was intentional, as it is better to get an overview of metabolism before looking at the individual enzymes involved. Today, however, individual enzymes, and how to test for them, are the topic of interest.

It must be first said that all enzymes are produced inside the cell, but that they may be divided into two groups based on where they catalyze the reactions for which they are specific. The first group are those called endoenzymes. These are enzymes that are to be found only inside the cell, and are only active in the catalysis of reactions that occur there. These include the enzymes that are active in the metabolic pathways of glycolysis, fermentation, and the TCA cycle that we mentioned last time. The second group are those enzymes called exoenzymes, and these are enzymes that are transported out of the cell to catalyze reactions there. Many exoenzymes are involved in the degradation or modification of substances in the environment of the cells. Some of these destroy toxins that would harm the cell, such as antibiotics and pesticides and such. Others are involved in breaking down large, compex molecules that can not be taken into the cell or utilized in their normal forms. However, once broken down by exoenzymes, their degradation products can be easily taken in for use in metabolism, both as raw materials for cell growth and reproduction and as food sources.

Frequently, the large, complex molecules broken down are polymers, long, repeating chains of smaller, often identical molecules called monomers. The most important polymers are proteins, made up of a large number of amino acid monomers, and polysaccharides, made up of large numbers of simple sugar molecules. The reactions that degrade these polymers usually require the presence of water, and function by adding the water to a molecular bond, in the process breaking it. This type of reaction is called hydrolysis, and the enzymes that carry them out are called hydrolases. It is important to note that endoenzymes can also be hydrolases.

Today will be concerned with tests for a number of endo- and exoenzymes. Among the endoenzymes are catalase, oxidase, tryptophanase, phenylalanase, urease, and cysteine desulfuase. Among the exoenzymes are gelatinase, amylase, caseinase, and lipase. We will look at each of these in turn.

Oxygen is a substance toxic to all linving things. This may sound odd considering what was discussed last time concerning its role in aerobic respiration, and the fact that we die without it. Despite this, it is very much true. Oxygen is a very powerful electron acceptor (The reason why it is the best terminal electron acceptor possible for respiration.), and, when it is partially reduced, it can form oxygen free radicals, superoxides, and peroxides. Hydrogen peroxide is probably the most familiar to you, being conmonly used as a disinfectant and component of mouthwashes and toothpastes. The reason why it is used in these roles, and the reason why all the reduced oxygen compounds mentioned are so dangerous is because they can very readily strip away electrons from important molecular components of cells, rapidly killing them. If an organism is to survive under aerobic conditions, it has to have a complement of enzymes that will degrade these reduced oxygen compounds to prevent and repair the damage they do. Among the most important types of enzymes that do this are is catalase, which breaks down hydrogen peroxide into oxygen and water. Catalase is both an endo- and exo-enzyme, being active both inside and outside the cell. If you have ever used hydrogen peroxide on a wound, you have not doubt noticed the bubbling that results from this. The bubbles are oxygen produced as catalases in your blood break down the peroxide. This bubbling upon application of hydrogen peroxide makes it a very easy enzyme to test for. Last time you inoculated two slants with your test organism, and today you will be using one of them to test for catalase activity by dripping hydrogen peroxide on the growth.

Last time it was mentioned that a final cytochrome in the electron transport system is responsible for the ridding of the cell of the electrons collected from the TCA cycle by using them to reduce oxygen. This is cytochrome C, and enzyme you have likely heard of because it was one of the earliest molecules to be used as a molecular clock for the examination of the relations between species. It is also called oxidase. Oxidase is also a very important enzyme because its presence or absence is used to differentiate between a large number of bacteria. You will be testing for its production by your test organism from last session using the growth of it on the NA plate you streaked. A similar enzyme, nitratase, is responsible for the placing of electrons on nitrate when it is used as a terminal electron acceptor in

anaerbobic respiration. One of the things you can discover from the nitrate reduction test you inoculated last time is whether or not the organism you are studying produces nitratase.

An important group of endoenzymes have the role of degrading amino acids so that they may be used for energy production. Two that you will be inoculating media to test for the presence of today are tryptophanase, phenylalanase, and cysteine desulfurase. Tryptophanase breaks down tryptophan (This is the amino acid present in large amounts in turkey, and that is a precursor to the neurotransmitter serotonin. Serotonin is involved in sleep regulation, and may explain why turkey makes one feel sleepy.) into indole and pyruvic acid. The pyruvic acid may, of course be used immediately in the TCA cycle or fermentation, while indole may be further broken down to ultimately produce pyruvic acid, or may be modified for the synthesis of tyrosine and phenylalanine. The degradation of indole can take time, however, and the test for tryptophanase works by detecting indole in the test medium after incubation. Phenylalanase, then, breaks down phenylalanine (A precursor to the neurotransmitters epinephrine, or adrenaline, and norepinephrine.) into phenylpyruvic acid and ammonia. The ammonia may then be used to synthesize other amino acids, and the phenylpyruvic acid can either be broken down into pyruvic acid or may be used in biosynthesis as well. The test medium you are inoculating today has phenylalanine present in large amounts, and the degradation of it by phenylalanase will be detected next time by testing for the presence of phenylpyruvic acid. Finally there is cysteine desulfurase. Cysteine is one of the few sulfur-containing amino acids, and when it is broken down to pyrivic acid and ammonia in the course of metabolism, this sulfur has to go somewhere. Indeed, in its degradation, the first step is carried out by cysteine desulfurase, which cleaves the molecule into a-amino-acrylic acid and hydrogen sulfide. Hydrogen sulfide is a toxic gas that gives the characteristic odor to rotten eggs, swamps, and flatulence (It is also of great importance in the Winogradsky column ecosystem. Hint, hint, wink, wink, nudge, nudge.). The medium to test for this cleavage that you will be inoculating today contains iron salts that react with hydrogen sulfide to produce a very dark precipitate of iron sulfide.

We finally come to a few major hydrolytic exoenzymes that degrade polymers into monomers for transport into the cell for metabolic usage. The first is gelatinase, an enzyme that belongs to a class of proteases that break down protein. As you might have surmised, gelatinase specifically degrades gelatin. The medium you will be inoculating to test for its production by your organism contains gelatin as the only solidifying agent. If your organism produces gelatinase, then you should find the the medium to have been liquified during incubation (See figure 9.1). Amylase is a hydrolase that breaks down the polysaccharide starch, made up of a great number of glucose molecules as monomers. Amylase production is tested by streaking the test organism out on a plate of a medium containing large amounts of starch. Amylase production may be tested by looking to see if the starch in the medium surrounding the growth of the organism has been broken down, as you will learn next time. Casein is a protein found in milk that is responsible for its white color. If a bacterium produces caseinase, which degrades casein into a variety of amino acids and smaller polypeptides that are clear, and is grown on a medium containing casein, then the result will be the clearing of the medium around where the bacterial growth has occurred. Finally, there is lipase. Lipase hydrolyzes fat molecules, or triglycerides, into fatty acids and glycerol for transport into the cell and use in both energy production and the synthesis of structures such as the phospholipids of the cell membrane. The production of lipase is tested for by growing the organism of interest on a medium such as spirit blue agar that contains vegetable oils. If lipase is produced then one either observes a clearing of the oils on the surface of the medium around the growth, or a dark precipitate formed by the lowered pH from the fatty acids around the growth.

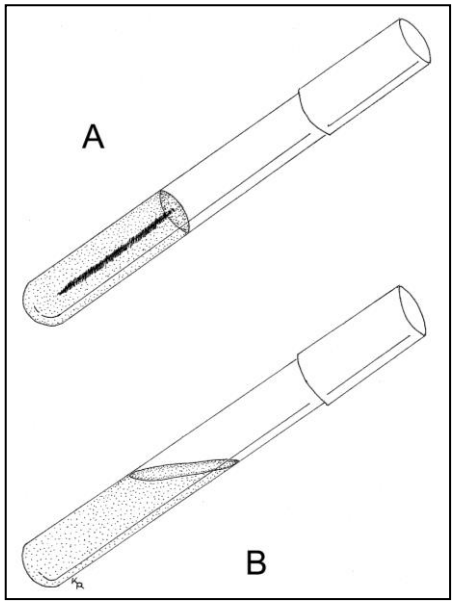


Figure 9.1: **Liquefaction of a Tube of Gelatin Medium by Gelatinase**

Finally, you will also be testing for the production of urease. Urea a nitrogen-containing compound frequently used by animals as storage compound for potentially toxic nitrogenous waste. Certain types of bacteria are able to degrade urea using urease. This enzymatic degradation results in the formation of carbon dioxided and ammonia. The medium used to test for this enzyme contains urea, as well as phenol red. The medium is specially buffered so that it is close to neutral, and has a yellowish-peach color to it. If the enzyme is produced and urea is hydrolyzed, this will release ammonia into the medium, raising the pH and causing the indicator to turn the medium a bright pinkish-red.

II. Environmental Sampling

Today you are also going to be looking at the results of your samplings from last session. While you are examining your plates, especially if you sampled a location, think of the different kinds of organisms that were likely present in such a place, and how, prior to this class you likely never gave them much mind. To add to your thoughts on this, consider also that a medium will only allow to grow those organisms for which it provides proper nutrition. No medium can be so complete as to permit the growth of all the microbes present even in a small space. Indeed, studies where the number of colonies that arise on rich media have been compared to the number of cells observed under the microscope have shown that perhaps as little as 1% of the microbial diversity may be shown on a plate of a given medium. In short, however disgustingly crawling with life the thing you sampled might appear to be, it is likely a good deal worse. Today you will be focused on examing the diversity of microorganisms you were able to catch with your sampling method, and you will do this both by looking at the colonies that have resulted, and by doing Gram stains of four of them. Four of colonies that are shown to be bacterial will be streaked out for next time…when your real study of one of them will begin.

Procedures

Part 1: Session 8 Follow Up:

MIC Testing of Antibiotics:

Get out the antibiotic tubes that you inoculated last time with the organism assigned to your bench. You should observe the tubes and rate them as + or – growth. There will be a class data sheet going around. When it gets to you or your partner, make certain that your data goes on it. Copies will be made of the data sheet before the end of class today. Make sure that you get a copy before leaving class, as the lab report will be based on the class data, and not just your own.

Characteristics of Microorganisms:

Evaluate the media you inoculated last time according to the criteria detailed below:

Record all data collected today in the appropriate places in the report section!!!

The guide to biochemical tests is to be found on the pages following the instructions for this session. Whereever it is referred to below, you will need it to read the test involved.

- ***Steak plate:*** Evaluate the morphology of the resulting colonies according to their color, size, form, elevation, opacity, and margins (See handouts). When you are finished with this, keep the plate so that you can use the colonies on it to test if the organism produces oxidase according to the instructions in the guide to biochemical tests.
- ***First NA slant:*** Evaluate the growth according to its color, opacity, and form (See handouts).
- ***Second NA slant:*** Use the growth on the slant to test for catalase production by the organism as detailed in the guide to reading biochemical tests.
- ***NB tube:*** Evaluate the amount of the growth of you organism, as well as its surface, subsurface, and sediment characteristics (See handouts). **This evaluation should be done before you vortex.**
- ***Thioglycolate tube:*** Evaluate the growth in the tube and use it to determine the oxygen requirements it posesses.
- ***Phenol Red Sugar Tubes****:* Evaluate according to instructions in the guide to biochemical tests.
- ***MR-VP tube:*** Put one aside, and do not use it this session. For the other, evaluate according to the instructions for the methyl red test in the guide to biochemical tests.
- ***Nitrate broth tube:*** Evaluate production of nitrase according to the instructions in the guide to biochemical tests.
- ***Simmon's Citrate Slant***: Evaluate according to instructions in the guide to biochemical tests.

Environmental Sampling:

Retrieve your plates from the box at the front of the middle bench. Examine them, and record your observations in the proper place in the report section. Be sure that your observations include a good description of the different types of colonies you observe, including shapes, sizes, textures, and colors. When you are done, save your plates for use in exercise 1 later on.

Winogradsky Column

Go to the hood in the back, and without disturbing it, examine the column your group constructed. Record your observations in the appropriate place in the report section. Be sure to note if any color bands are obvious, and if there have been any changes since your last observation.

Part 2: Today's Exercises:

Exercise 1: Selection of Environmental Isolates for Further Study

Materials: 4 NA plates
4 clean microscope slides

Please be extra careful when working with any environmental isolates. There is the possibility that some of them may be serious pathogens.

1. Examine your plates carefully, and identify four colonies that are not mold-like in appearance (Colonies of this description are most likely of fungi, and we are not interested in them.) that you find interesting.
2. You need to make smears that you will then Gram stain for each of these colonies chosen. When you make your smears, however, you need to make sure that you use only part of each colony. Why? Well…
3. With the remaining portion of each colony, you need to streak for isolated colonies on the NA plates (So you will end up with one smear and one streak plate for each colony chosen.). Use either the quadrant streak or the T-streak; whichever one you feel more comfortable with. You might find it helpful to turn back to session 3, where you first learned how to do streak plates.
4. When finished, examine the stained smears under oil immersion, draw a representative field of view for each, and provide detailed commentary on what you see in the appropriate place in the report section. If you find that the organisms composing a colony present as Gram-positive cocci far larger than any you have seen before, ask a TA to look at it, as it is likely that what you have found is a yeast.
5. When you are finished, dispose of the slides in one of the Lysol bins, and place the plates you have streaked in the box at the front of the middle bench for incubation.

Exercise 2: Characteristics of Microorganisms: Metabolic Properties II:

Materials: 1 Starch Agar plate
1 Skim Milk Agar plate
1 Sprit Blue Agar plate
1 NA plate (If you have been assigned *S. aureus*.)
1 Gelatin tube
1 Phenylalanine slant
1 SIM tube
1 Tryptone broth tube
1 Urea broth tube

Organism	Microscope Number
Enterobacter aerogenes	1, 5, 9, 13, 17, 21, 25, 29, 33, 37, 41, 45, 50
E. coli	2, 6,10, 14, 18, 22, 26, 30, 34, 38, 42, 46, 51
Pseudomonas aeruginosa	3, 7,11, 15, 19, 23, 27, 31, 35, 39, 43, 47, 52
Staphylococcus. aureus	4, 8,12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 53

Table 9.1: **Assignment of Cultures on the Basis of Microscope Number**

1. Collect the culture you have been assigned by your microscope number (See table 9.1. Please note that everyone should have their own tube of culture today.), and the media listed above. Be sure to label the media tubes and plates as you collect them to avoid confusion. Also label each with your name, the date, and your organism.
2. Inoculate all the media you have collected with your assigned culture as follows:

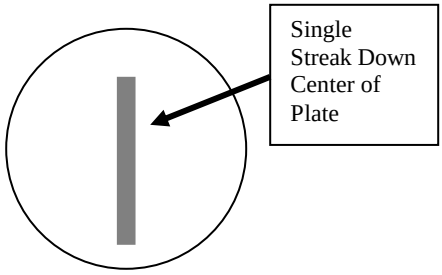


Figure 9.1: **Proper Way to Streak Starch Agar, Skim Milk Agar, and Spirit Blue Agar plates for Exercise 2**

   a. Aseptically streak a loop of culture onto each of the Starch Agar, Skim Milk Agar, and Spirit Blue Agar plates. You should not streak for isolation!!! Instead, you should make a single streak up the center of the plate (See figure 9.1).
   b. Aseptically transfer a loop of culture each to the Tryptone broth tube and the Urea broth tube.
   c. Aseptically streak a loop of culture up the surface of the medium for the Phenylalanine slant.
   d. Use your inoculating needle to aseptically stab inoculate both the SIM tube and the gelatin tube with a sample of your culture. Stab straight down, and withdraw the needle along the same path.
   e. If you have been assigned *S. aureus*, you should streak a loop of culture for isolation on the NA plate.

3. When you are finished with the inoculations, place all the media in your bench for incubation until next time. The tube of culture from which you made your inoculations should be placed in the back test tube racks for disposal.

Preparation for Session 10:

Next session we will be going over how to use two systems to rapidly use a number of biochemical tests to identify a certain class of unknowns. There will be six groups for this, with two groups for each bench (All on the left side of one bench will be a group, and all on the right side will be the second group.). One of the two systems we will be working with takes only 4 hours to develop, so a couple of people from each group will need to volunteer to come in three to four hours before class starts to set up this system so it can be read during the class. Discuss amongst each other who will be coming in, and let the TAs know who this will be. Be sure that the chose group members do come in. The entire group will be penalized if they do not show up. The chosen group members should familiarize themselves with exercise 3 A of session 10's work before they come in.

Handy-Dandy Guide to Reading Biochemical Tests

(See Brock, table 24.3 on pp 812 – 813)

Sugar Fermentation Tests: The Phenol Red Sugar Tubes:

What is tested: Whether or not an organism ferments a sugar, and whether or not it produces gases, acids, or both from such fermentation. The sugar in the medium is also the only available carbon source, so if the tube does not display any turbidity, it is likely because the bacterium cannot utilize the sugar. You should be sure to note whether or not the broth is turbid in your commentary.

Indications:	*Tube has turned yellow* →	The organism has produced acid from the fermentation of the sugar in the medium
	Bubble in the Durham tube →	The organism has produced gas from the fermentation of the sugar in the medium
	Tube is still red or orangish →	The organism has not produced acid from the fermentation of the sugar in the medium
	No bubble in the Durham tube →	The organism has not produced gas from the fermentation of the sugar

Differential Fermentation of Glucose: MR –VP Tubes:

Remember that the MR – VP tubes are used for both the Methyl Red and Voges – Proskauer tests. This is why they are always inoculated in pairs. Remember to only use one tube for each of the tests, and not to use the same tube for both. Please also note that the tube on which you perform the Voges – Proskauer test must be allowed to incubate for a longer period of time than that on which you perform the Methyl Red test.

Part I: The Methyl Red Test:

This test should be done one session after the tube was inoculated.

What is tested: Whether or not an organism is a mixed acid fermenter of glucose.

What to do: Add a whole dropper of methyl red and vortex the tube thoroughly

Indications:

*What a **positive** looks like* → The tube turns red

What this tells you: The organism has produced mixed acids from the fermentation of the glucose in the medium

*What a **negative** looks like* → The tube turns brownish

What this tells you: The organism has not produced mixed acids from the fermentation of glucose, but it may produce 2,3-butanediol, ethanol, or some other such non-acid products from fermentation instead.

Part II: The Voges – Proskauer Test:

This test should be done two sessions after the tube was inoculated.

What is tested: Whether or not an organism ferments glucose to produce 2,3-butanediol, ethanol.

What to do: Transfer 1 mL of broth from the tube to an empty, new tube, which you can get in the front. to the broth in this new tube, you should add **a whole cartridge of VPA solution** and **5 drops of VPB solution**, and then **vortex every 20 seconds for 5 minutes**.

Indications:

*What a **positive** looks like* → The broth turns red

What this tells you: The organism has fermented the glucose in the medium to produce 2,3-butanedioland ethanol instead of producing the mixed acids that the methyl red test is designed to detect.

*What a **negative** looks like* → The broth turns brownish

What this tells you: The organism has not fermented the glucose in the medium to form 2,3-butanediol and ethanol. This may mean that it fermented the glucose to form mixed acids, in which case your methyl red test should have shown positive, or it might mean that it ferments glucose, but does not produce anything these tests are designed to detect, or it may not ferment glucose at all.

Chemical Make-up of the Voges – Proskauer (Barritt's) Reagents:

VPA: 6 g α – naphthol per 100 mL 95% ethanol

VPB: 16 g KOH per 100 mL dH_2O.

Citrate Utilization Test: The Simmon's Citrate Slant:

What is tested: Whether or not an organism is capable of utilizing citrate as its sole carbon source (Citrate is the only carbon source available in the medium.).

Indications: *What a **positive** looks like*: The tube has turned a very intense cornflower or Prussian blue color.
What this tells you: The organism has used the citrate in the medium as its sole carbon source.

*What a **negative** looks like*: The tube has not changed in color, remaining a lovely yellowish-green.
What this tells you: The organism has not used the citrate in the medium as its sole carbon source.

Catalase Production Test:

This test is performed using a plain NA slant with growth on it. If you are using the same slant that you have for examination of the cultural characteristics of your organism, be sure to evaluate it growth and growth pattern prior to performing this test, as it will destroy the usefulness of the slant for that purpose.

What is tested: Whether or not an organism produces catalase, which breaks down peroxides.
What to do: Get a dropper bottle of hydrogen peroxide from the front and drip a full dropper on the slant.

Indications: *What a **positive** looks like* → The hydrogen peroxide fizzes when it hits the growth.
What this tells you: The organism has produced catalase, which is breaking down the hydrogen peroxide into water and oxygen gas (This is what is in the bubbles).

*What a **negative** looks like* → Nothing happens.
What this tells you: The organism has not produced catalase, so the hydrogen peroxide is not broken down into water and oxygen gas.

Note: When you are finished with the hydrogen peroxide, take it back to the front, but make absolutely certain that the cap is loose. If the cap is on tightly, the bottle may explode (Yes, as in *BOOM!* followed by glass shrapnel flying through the air. Please do not be responsible for anyone in here get microbiology wounds more serious than a few stains!!!).

Nitrate Reduction Test: The Nitrate Broth Tube:

What is tested: Whether or not an organism can utilize nitrate as an electron acceptor in anaerobic respiration, or if it uses nitrate for any other purpose.
What to do: Add **3 drops of Nitrate Test Solution A** and **3 drops of Nitrate Test Solution B** to your nitrate broth tube from the session before.

Indications: *What a **positive** looks like*: The tube turns a dark red.
What this tells you: The organism has reduced the nitrate in the tube to nitrite in anaerobic respiration.

*What a **negative** looks like*: The tube does not change color
What this tells you: At this point it is hard to say anything definitive. You know the organism does not reduce nitrate to nitrite, but there might be something more going on…

Chemical Make-up of the Nitrate Test Solutions:

Nitrate Test Solution A:
8 g sulfanilic acid per liter of 5 N acetic acid

Nitrate Test Solution B:
5 g dimethyl-α-naphthylamine per liter of 5 N acetic acid.
Note: ***Nitrate Test Solution B is a possible carcinogen. Do not get any of it on your hands!!!!!***

If your test turns up negative, you have to go a step further to confirm the negative.

Why confirm a negative result? A positive test turns red because there is nitrite now in the broth due to the reduction of nitrate. However, just because the test shows there to be no nitrite in the tube, this does not mean that the organism did not use the nitrate in some way. The confirmation of the negative result is thus a way to make certain that there is nitrate still in the broth, indicating the organism has not used it at all.

What to do: Add **a pinch of zinc dust** to the tube and vortex it.

Indications: *The tube turns red* → This is a ***true negative***. The zinc changes nitrate to nitrite, which then reacts with the test solutions A and B that you added earlier to form the red color.
What this tells you: The organism has not done anything with the nitrate.

The tube does not change color → This is a ***false negative***, meaning that there was neither nitrate nor nitrite in the tube.
What this tells you: The organism uses nitrate in some way, but we really do not know what it does with it aside from not converting it to nitrite (For instance, it might produce nitrogen gas instead, or perhaps it uses the nitrate in the production of amino acids.).

Oxidase Production Test:

This test will be done using what is referred to as the filter paper method. A station at the front of the middle bench will be set up for this test.

The Chemical Make-up of the Oxidase Test Reagent:
1 g dimethyl-ρ-phenylenediamine hydrochloride per 100 mL of dH2O

What is tested: Whether or not an organism produces Cytochrome C oxidase, the cytochrome that usually reduces oxygen gas to water.
What to do: Go to the station in the front and get a filter paper disk. Drip a few drops of oxidase reagent on the disk and take it back to your bench. Aseptically transfer a loop of culture from the streak plate you made last session to the part of the disk with the reagent on it.

Indications: *What a **positive** looks like:* The filter paper and culture turns dark red to purple/black in less than 30 s.
What this tells you: The organism has produced cytochrome C oxidase.

*What a **negative** looks like*: The filter paper and culture do not turn dark red or purple/black in less than 30 seconds. It will likely turn colors some time after this period no matter what, but it if does take this long, then it should be counted as a negative anyway.

What this tells you: The organism has not produced cytochrome C oxidase.

Starch Hydrolysis Test: Starch Agar Plate:

What is tested: Whether or not an organism is capable of hydrolyzing starch into its constituent glucose monomers for use in cellular metabolism.

What to do: Use the dropper in your staining box to flood the plate with Gram's Iodine (Yes, the same as what you use in doing Gram staining.).
What this does: Gram's Iodine reacts with starch to form a complex with a dark, brownish-purple color, allowing you to see where there is starch still in the medium, as well as where there no longer is any.

Indications: *What a **positive** looks like*: The plate turns a brownish-purple color, but with a prominent clear area, almost like a halo, surrounding the streak where the bacteria have grown.
What this tells you: The bacteria growing in the streak have produced the exoenzyme **amylase**, which they have secreted into the medium around them. This enzyme has hydrolyzed the starch in the medium closest to the streak into its constituent glucose monomers.

*What a **negative** looks like*: The entire plate turns a brownish-purple color, with no clearing at all (The area under the streak may appear to be clear, but this is likely because the bacterial growth in the streak is so thick that the iodine can not penetrate it to react with any starch underneath.).
What this means: None of the starch in the medium has been broken down, meaning that the bacteria growing in the streak have not secreted any of the exoenzyme **amylase**, and have not hydrolyzed any of the starch.

Casein Hydrolysis Test: The Skim Milk Agar Plate:

What is Tested: Whether or not an organism is capable of producing the enzyme caseinase, an exoenzyme that breaks down the protein casein, the primary protein in milk that is responsible for the white color associated with it (Hence the skim milk agar plates, which are made with milk, are white.).

What to do: Simply look at the plate.

Indications: *What a positive looks like*: There is an obvious clearing of the medium surrounding the bacterial streak.
What this tells you: The bacteria growing on the plate have secreted casienase into the medium, which has hydrolyzed the casein responsible for the white color, thus resulting in the clearing. Thus, the organism is caseinase positive.

What a negative looks like: There is no clearing at all apparent around the growth on the plate.
What this tells you: The bacteria growing on the plate have not secreted any casienase into the medium, so there has been no loss of the white color of the medium.

Lipid Hydrolysis Test: The Spirit Blue Agar Plate:

What is tested: Whether or not an organism produces the enzyme lipase, an exoenzyme that breaks down lipids into their components.

Indications: *What a positive looks like*: 1. There is a dark blue precipitate along or around the streak of growth
2. There is an obvious clearing of the oils along the surface of the agar around the growth.
What this tells you: The organism has produced lipase, which has hydrolyzed the lipids in the medium surrounding the bacterial growth.

What a negative looks like: There is no dark blue precipitate or clearing of the oils on the surface of the medium surrounding the bacterial growth.
What this tells you: The organism has not produced any lipase and has not hydrolyzed the lipids in the medium surrounding the bacterial growth.

This test can be tricky to read, so if you are in any way uncertain, please ask a TA.

Tryptophan Hydrolysis Test: The Tryptone Broth Tube:

What is tested: Whether or not an organism produces the enzyme tryptophanase, which hydrolyzes the amino acid tryptophan into indole, pyruvic acid, and ammonia.

What to do: Add a full dropper of Kovac's reagent to the tube and vortex.

The Chemical Make-up of Kovac's Reagent:
10 g of p-dimethyl-aminobenzldehyde
150 mL of 1-pentanol
50 mL HCl

Indications: *What a positive looks like*: A very red liquid layer forms that overlays the surface of the broth.
What this tells you: The organism has produced tryptophanase, and hydrolyzed the tryptophan in the medium.

What a negative looks like: A liquid layer overlaying the broth does develop, but it is yellowish or tan.
What this tells you: The organism has not produced tryptophanase, and has not hydrolyzed the tryptophan in the medium.

Urea Hydrolysis: The Urea Broth Tube:

What is tested: Whether or not an organism is capable of producing the enzyme urease that breaks down urea into ammonia and carbon dioxide.

Indications:

*What a **positive** looks like*: The tube has turned hot pink.
What this tells you: The organism has produced urease, which has broken down the urea in the medium, releasing ammonia that has raised the pH, causing the indicator to turn the medium the hot pink color.

*What a **negative** looks like*: The tube has either not changed color, or has become a peachish color.
What this tells you: The organism has not produced urease, and no ammonia has been released to raise the pH of the medium.

Hydrogen Sulfide Production Test: The SIM Medium Tube

What is tested: Whether or not an organism can produce the enzyme cysteine desulfuase that breaks down the amino acid cysteine to produce pyruvic acid and hydrogen sulfide.

Indications:

*What a **positive** looks like:* A very distinct and obvious black precipitate has developed in the tube.
What this tells you: The organism has produced cysteine desulfurase, which has broken down the cysteine in the medium, and produced hydrogen sulfide that has reacted with iron salts in the medium to produce the black precipitate.

*What a **negative** looks like*: The tube has not really changed in appearance, and no black precipitate is evident.
What this tells you: The organism has not produced cysteine desulfurase, and has not produced any hydrogen sulfide from the break down of cysteine.

Phenylalanase Production Test: The Phenylalanine Slant:

What is tested: Whether or not an organism is capable of producing the enzyme phenylalanase that is capable of breaking down the amino acid phenylalanine into phenylpyruvate and ammonia.

What to do: Add a dropper full of 10% ferric chloride to the slant and then use your loop to mix it with the growth on the slant (i.e. Stab the surface repeatedly.).

Indications:

*What a **positive** looks like*: The resulting mixture at the top of the slant turns green in less than 5 minutes.

What this tells you: The organism has produced phenylalanase, which has broken down the phenylalanine in the medium.

*What a **negative** looks like*: No color change is evident.
What this tells you: The organism has not produced pheynalanase, and has not broken down the phenylalanine in the medium.

Coagulase Production Test:

This test is specific for Staphylococcus aureus. If you have not been assigned this organism, you do not need to do this test.

What is tested: Whether or not an organism is capable of producing the enzyme coagulase that catalyzes the coagulation of blood plasma. This test is used to clearly differentiate S. aureus from other, similar staphylococcal species.

What to do: Go to the station designated for this test. There you should small plastic tubes containing 0.5 mL of rabbit plasma. Take one back to your station and use your loop to aseptically transfer 2 to 4 colonies from your streak plate to the tube. Put the tube in a foam rack floating in the 37 C water bath on the side bench. The tubes will be in the water bath for 24 hours, and then put in the refrigerator. You will then read them during the next lab session.

Indications:

*What a **positive** looks like*: The plasma in the tube has formed a clot (i.e. coagulated.).
What this tells you: The organism has produced coagulase, and is likely to be Staph. aureus.

*What a **negative** looks like*: The plasma in the tube is still very liquid, with no evidence of a clot being present.
What this tells you: The organism has not produced coagulase.

Lab Session 10

Background

I. Identification of Bacteria

(See Brock pp 811 – 814)

"It is easy to identify bacteria, but it is very hard to identify them correctly."
-- Dr. Thomas Tornabene

As we have discussed previously, most of the diversity of bacteria is to be found at the level of the enzymes they produce and metabolic reactions they are capable of carrying out. As we have been learning about facets of the metabolism of our test organisms, you have not only learned about, for instance, what sugars they can ferment, or what enzymes they produce, you have been also identifying characteristics by which they might be identified. Think of the game of Guess Who. In the game, each player picks a face out of the crowd of faces, and then uses yes or no questions about the identifying features of the faces to determine the face chosen by the other player. The same principle applies to bacteria and other microorganisms, only the questions are along the lines of, "Can you produce acid, but not gas from the fermentation of lactose?" instead of, "Are you wearing a hat?" Provided that you gather enough test results and get enough identifying characteristics, you can usually come very close to identifying the organism you have in hand using these simple biochemical tests, just as you can potentially identify a particular human being if you have gathered enough facial features possessed by this person.

The use of such basic morphological and biochemical test were at one time the only way that one could go about identifying an organism, and you might wonder why we are even bothering to go over it in this great day of DNA sequencing and PCR (The argument could be made that, as the results of the metabolic tests depend on the presence or absence of enzymes, and as enzymes are coded by genes in and organism's DNA, then, by finding out what metabolic characteristics that organism has, one roughly <u>is</u> sequencing its DNA to identify it.). The truth of the matter is that, on a large scale, morphological and biochemical testing for identification is a good deal easier and cheaper than the DNA-based methods. For this reason, it is still used by health professionals in major hospitals, as well as by public health, sanitation, and criminal investigation scientists. Thus, it is still of relevance even in this shining land of the future, though we will cover DNA sequences next time. You will be doing a quasi-demonstration of this today, as you go about how CDC scientists identify *B. anthracis*, the organism that causes anthrax.

II. The Way of Tradition: Bacterial Identification Using Bergey's Manual

(See Brock pp 348 – 349)

Prior to the advent of DNA sequencing and molecular techniques like PCR, the only way one had of identifying a bacterium was to do a battery of metabolic tests like we have been doing, combine them with basic information about Gram stain reaction, cell morphology, motility, and such, and then use the results to make the identification. As has been mentioned earlier, this method is still used when DNA-based methods are impractical. How? The answer to that question is *Bergey's Manual of Systematic Bacteriology*, with which you should already be somewhat familiar from using it previously to look up information about some of the bacteria we have studied. The *Manual* was born at the direction of the organization that is now called the American Society for Microbiology, with the first edition being published in 1923. It is named after Dr. David H. Bergey, the original head of its editorial board, and the creator of the Bergey's Manual Trust, which now oversees the publication of the *Manual*. It is a compilation of known characteristics of all known bacteria and archaea (At least what was known for all that was known at the time of the last edition.). It is organized according to the shared characteristics of the organisms it covers so as to make it "easy" to identify an organism once you have a sufficient number of characteristics in hand.

You will be using the *Manual* to identify one of the bacteria that you select from your environmental plates today, and which you will be putting through a number of test in the next few sessions. While the manual is divided into four volumes, you will likely only need to use volumes I and II, which cover Gram negative and Gram positive bacteria, respectively. While intimidating to be sure, the manual is really not all that bad to use. Once you have the Gram stain of your organism, you know in what volume to look. You will then find section headings that classify the organisms in those sections according to a few common features that should be easy enough to obtain from the tests we do (i.e. Cell morphology, oxygen requirement, oxidase production, endospore production, motility, et cetera.). Once you have the section, you turn to it, and you should soon find a chart listing characteristics of the groups of organisms covered in that section. You then use this chart to narrow down the possibilities to usually a single genus. You turn to the chapter on that genus, and you should soon find a chart giving the characteristics of its consituent species, and then you do the same thing until you have identified your unknown.

Easy in theory is much different from easy in practice, however, and you are likely to run into a few problems. The biggest one is likely to be misreading your tests. It is not often that you will run into a false positive, but it is much more likely that you will run into a false negative (The reasons why should be clear with a little thought about how the biochemical tests work.). It is also possible to manage to catch a bacterium of a species covered in the *Manual*, but that does not fit the profile given for it exactly. Then there is the big bugaboo that we have been trying to emphasize for a while now: if you are not sufficiently careful with your aseptic technique, you will get contamination, and if you culture is contaminated, then none of the tests apply. Try to keep these things in mind as you work with your unknown and go about the tests and procedures necessary to identify it.

III. Miniaturized Multitests: Rapid ID of Enteric Bacteria

(See Brock p 814)

While the use of biochemical tests and such in conjunction with Bergey's Manual to identify an unknown bacterium may still be quite useful, there are times when it is simply too slow, inconsistent, and requiring of too much preparation and materials. Just imagine, for instance, how many tubes poor Julie and Cathy have had to make for these tests? When dealing with certain situations, especially those in a major hospital, or in a sanitation department, traditional biochemical tests simply are not good enough. The problem is that in these situations, DNA sequencing is not wholly appropriate or cost effective either. These problems were solved with the advent of miniaturized multitests. These are compact apparatuses that contain multiple media and reagents for the testing of many of the standard biochemical characteristics of bacteria. They thus dispense with the glassware and other such bulky equipment that can be so problematic, but they have a further advantage in that they are much, much faster, not to mention more reliable than traditional tests. One kind, the Micro-ID system takes only four hours to develop, while the other we will work with, the Enterotube II system, requires only 24 hours. Further, after the necessary incubation time, one simply reads the tests according to a chart, determines an identification code based on the results, and looks up this code in an interpretation guide to make the final identification. Miniature multitests also tend to have the benefit of not requiring as large an inoculum as traditional tests, and as the time it takes to grow enough bacteria on a medium to provide sufficient inocula can be a significant factor in the time it takes to make an identification, the value of this feature should be clear.

Organism	Disease Caused
Enterobacter aerogenes	Urinary tract infections
Salmonella typhi	Typhoid fever
Shigella dysenteriae	Bacterial dysentery
E. coli O157:H7	Food poisoning; bloody diarrhea
Klebsiella pneumonia	Bacterial pneumonia; septicemia
Yersinia pestis	Plague (AKA Black Death)
Proteus mirabilis	Urinary tract infections
Serratia marcescens	Septicemia; pulmonary disease

Table 10.1: **Major Pathogens of Family Enterobacteriacaea and the Diseases they Cause**

Despite these advantages, miniature multitests do not currently exist that would permit one to identify just any bacterium that one runs across. These multitests take a good deal of money to develop in the first place, and then the demand for them has to be high enough to make their fabrication on a large scale worthwhile. Due to this, most miniaturized multitests, the ones we will look at included, are for a limited number of highly medically significant bacteria. It is not cost effective for them to be developed and produced for any other group. One is stuck using the traditional method with all its problems if one is trying to identify something along the lines of a lithotrophic thermophile from the bottom of the ocean. In the case of the systems we will be examining, the group of medically important bacteria they are designed for is called the *Enterobacteriacaea*.

The family *Enterobacteriacaea* is a large group of Gram negative bacteria that share the characteristics of all being rods that are also oxidase negative. Members of the family include a large number of organisms commonly found in the digestive tracts of animals, and for that reason, the family is often also called the Enterics. While most of the *Enterobacteriacaea* are harmless, and often quite needed and beneficial, there are many species within that cause serious medical problems. Among the more significant pathogens are those listed in table 10.1. Given that it includes such a rogues gallery of pathogens, it should be clear why they were seen to justify the development and production of miniaturized multitests that would specifically identify them. It should also be noted that many of these organisms are very rapid in causing disease (The pneumonic form of plague, caused by *Yersinia pestis*, is recorded to have killed in as little as two hours in some cases.), so the speed of identification afforded by miniaturized multitests can be crucial in determining the proper course of treatment for a patient infected by one of them.

Procedures

Part 1: Follow Up to Session 9

Characteristics of Microorganisms: Metabolic Properties I:

Get out the second MR – VP tube you inoculated during lab session 8. You will need to do a Voges-Proskauer test on it according to the instructions given in the guide to biochemical tests. Record your observations and the results of the test in the proper place in the report section.

Characteristics of Microorganisms: Metabolic Properties II:

Get out the media you inoculated last time. Evaluate each according to the instructions in the guide to biochemical tests. If you were assigned *S. aureus* as your test organism last time, you should use the streak plate you prepared to do a coagulase test. The instructions for this are to be found in the guide to biochemical tests also. Record all data collected from these test media in the appropriate part of the report section.

Environmental Isolates:

Examine the four streak plates you made last time for the environmental isolates you chose. Select a colony from each, and use them to make smears. Gram stain these smears to verify that none of them are yeasts. Keep exercise 1 for today in mind as you examine the smears under oil immersion. You do not have to draw what you see under oil immersion, but be sure to provide a good description of what you see in the appropriate part of the report section.

Part II: Today's Exercises:

Exercise 1: Criteria for the Identification of *Bacillus anthracis*:

Work in groups of two on this exercise. You will have 30 minutes tops on which to work on this exercise. At the end of this time, the TAs will call time, and there will be a brief discussion of the findings and their relation to future work. You will also be instructed as to whether or not you should seek medical attention or a notary public for the certification of your will.

Materials: Culture of possible B. anthracis
Clean microscope slides
Bottle of malachite green

1. While one of you is collecting materials, the other should set up a steam bath using a tin can, tripod, wire mesh, and Bunsen burner as in lab session 3 when we learned about acid fast and endospore staining.
2. While the bath is getting heated up, prepare four smears of the bacterial culture.
3. When the bath and the smears are ready, one of you should perform a spore stain on one of the smears as gone over in lab three. Follow the instructions from lab session 3 as before to do this.
4. The one of you not performing the spore stain should do a Gram stain on one of the other smears.
5. When the stainings are complete, observe the smears under oil immersion and record the results observed in the report section.
6. At some point during the above, either you or your partner should go to either the back or the side bench. You should find a microscope with a capsule stain of the organism already in focus under oil immersion. Record whether or not the organism possesses a capsule. You should also find a TTC motility medium culture of the organism that has been incubated prior to class. Record your interpretation of the growth as indicating that the organism is motile or non-motile.
7. Wait until time is called.

Exercise 2: Selection of an Environmental Isolate for Further Study

Materials: 2 NA slants

From the four isolates you streaked last time, select one that you like best, that calls out to you, which is especially weird, or that just plain puts its hand up. You will be working with this one a good deal more, so be sure that it is one that you feel you can bond with. Before doing anything else, however, get the slide that you made of this isolate earlier and find a good field of view under oil immersion. When you have done this, get a TA to come and look. The TA will be the one who will have to okay this isolate. If he/she approves of it, make sure that he/she initials the appropriate place in the report section.

Once an isolate has been chosen and approved, choose a colony of it, preferably the one that you used to make the smear, and aseptically streak it onto the two NA slants you have collected for this exercise (Be sure that you use the same colony to inoculate both slants!!). Be sure that you do this precisely as you did the slants last session.

Exercise 3: Using Miniaturized Multitest Systems:

For this exercise you should work in groups made up of everyone on your side of the bench you work at. There should be six groups total, two for each bench.

A. Inoculation of the Micro-ID:

Materials: Micro-ID
3.5 mL Tube of sterile saline
Sterile 1 mL pipette and blue pipump
Plate of assigned unknown organism
Tube of MacFarland #2 turbidity standard

1. Gather the materials listed and take them to your bench.
2. Use your loop to aseptically add colonies, vortexing after each addition, of the unknown to the tube of saline until it is as or more turbid than the MacFarland #2 standard.
3. Open the Micro-ID and lay it flat on the bench. Label it will the name of your group, the date, and the letter of the unknown you are using.
4. Use the sterile pipette to transfer 0.2 mL of the saline colony suspension to each well of the Micro-ID.
5. Close the lid of the Micro-ID and set it upright. Tap it until all the disks in the bottoms of the wells are moistened with the saline suspension of colonies.
6. Set the Micro-ID in its rack, and place it in the 37 C incubator to incubate until after class has started. The tube of saline suspension should go in one of the racks in the back of the lab for disposal. Save the plate of the unknown for later use. To keep it safe, put it in your bench.
7. Leave the lab and go about your business.

B. Reading of the Micro-ID:

Materials: Pre-inoculated Micro-ID set
Micro-ID code sheet, and results chart
2% KOH dropper bottle

1. Place the Micro-ID flat on the desk and open the lid. Add 2 drops of 20% KOH to the VP (Voge-Proskauer) well ONLY. Close the lid, turn the Micro-ID upright, and gently tap it on the desk to bring the KOH down to the disk in the well.
2. Rotate and gently shake the Micro-ID so that the upper disks on the first five wells get wet. Make certain that you get all of them, or your results will suffer.
3. Read the test wells and use table 10.2 to determine if they are reacting as positive or negative. Read the upper disk for the first five test wells, but the lower disk for all the others. Do not read the VP well for at least 10 minutes. Fill out the chart in the report section as you go.

4. For each of the five sections of the chart that you use to convert the results of your Micro-ID to an identification code, add together the numeric values listed for each positive test (Score all the negative results as zero.). This will give you a five digit code.
5. Look up the five digit code you have generated in the ID book. If the code is there, record the ID, and any atypical reactions. If the code is not there, go back and make sure you have recorded the correct positive/negative for each test (You might have some ambiguous results. Look carefully at them.). If this gives you a new code number, look it up.
6. When you are finished and everyone in the group has had a chance to examine the Micro-ID, it should be disposed of in the biohazard bag in the back.

Test Abbreviation	Full Name	Active Ingredients in Test	Positive Reaction	Negative Reaction
VP	Voges-Proskauer	Glucose Sodium Pyruvate Arginine α-Naphthol Derivative	Pink to Red	Light yellow
N	Nitrate Reduction	Potassium Nitrate Sulfanic Acid α-naphthylamine Derivative	Red	Colorless to Light Pink
PD	Phenylalanine Deaminase	Phenylalanine Ferric Chloride	Green	Light Yellow
H2S	Hydrogen Sulfide	Sodium Thiosulfate Lead Acetate	Brown to Black	White
I	Indole	Tryptophane p-Dimethylaminobenzaldehyde	Pink to Red	Light Yellow to Orange
OD	Omithine Decarboxylase	Ornithine Brocresol Purple	Purple to Red-Purple	Amber to Yellow
LD	Lysine Decarboxylase	Lysine Bromthymol Purple	Purple to Red-Purple	Amber to Yellow
M	Malonate Utilization	Sodium Malonate Bromthymol Blue	Green to Blue	Yellow
U	Urease	Urea Cresol Red	Orange to Red-Purple	Yellow
E	Esculin Hydrolysis	Esculin Ferric Ammonium Citrate	Brown to Black	No color change or Beige
ONPG	β-Galactosidase	ONPG	Light Yellow to Yellow	Colorless
ARAB	Arabinose Fermentation	Arabinose Bromcresol Purple	Yellow to Amber	Red-Purple to Purple
ADON	Adonitol Fermentation	Adonitol Bromcresol Purple	Yellow to Amber	Red-Purple to Purple
INOS	Inositol Fermentation	Inositol Bromcresol Purple	Yellow to Amber	Red-Purple to Purple
SORB	Sorbitol Fermentation	Sorbitol Bromcresol Purple	Yellow to Amber	Red-Purple to Purple

Table 10.2: **Tests of the Micro-ID Miniaturized Multitest System with a Guide to the Reading of Them**

C. Inoculation of the Enterotube II System:

Materials: Enterotube II
Plate of assigned unknown (Same as for Micro-ID)

1. Remove the plastic tips from either end of the Enterotube. Under the blue cap should be the looped "handle" part of the wire running through the tube, and the inoculating tip of the wire should be under the white cap.
2. Touch the inoculating tip of the wire to a ***single*** colony of your assigned unknown (The tip is already sterile, so you **do not** have to flame it.).

3. Using the looped "handle", slowly draw the wire through the Enterotube, twisting as you do so – do not draw the wire all the way out of the end.
4. Push the wire back though the tube, again twisting it as you do so. You will notice a notch in the wire near the loop as you do this. Re-insert the wire only to this notch, and then bend the wire at the notch to break off the loop. Use the piece of wire broken off with the handle to poke holes in the notched sides of the last eight test compartments (ADON through CIT). Do not poke any holes in any of the other compartments, or you will not get viable results from them.
5. Replace the blue and white endcaps and you are done. Label the Entertotube with the name of your group and the date, and place it in the box for it at the front of the middle bench for incubation. The Enterotube system requires 24 hours to develop. It will be put in the refrigerator tomorrow so that you can read it during the next session.

Be sure that you take down the letter designation of your unknown culture. If you do not have this on your report, you will not be given credit for it!

Lab Session 11

Background

I. Modern Methods of Bacterial Identification

A. Agglutination and Serotyping

(See Brock pp 822 –827)

Antibodies are intrinsically 'multivalent', in the sense that they have more than one antigen-binding site per molecule. This property enables them to cross-link two molecules or particles that contain the antigenic determinant they recognize. Conversely, if the antigen is multivalent (i.e., contains more than one antigenic determinant per molecule or particle), it will be able to cross-link two antibodies, so that the combination of antigen and antibody will tend to form a large, insoluble network (See figure 11.1). In the human body, this lattice formation provides an important defense against toxins and pathogens, by preventing their spreading, and concentrating them for phagocytic destruction. In the laboratory, it is used in liquid solution or gels to provide a visual signal that an antigen-antibody reaction has occurred.

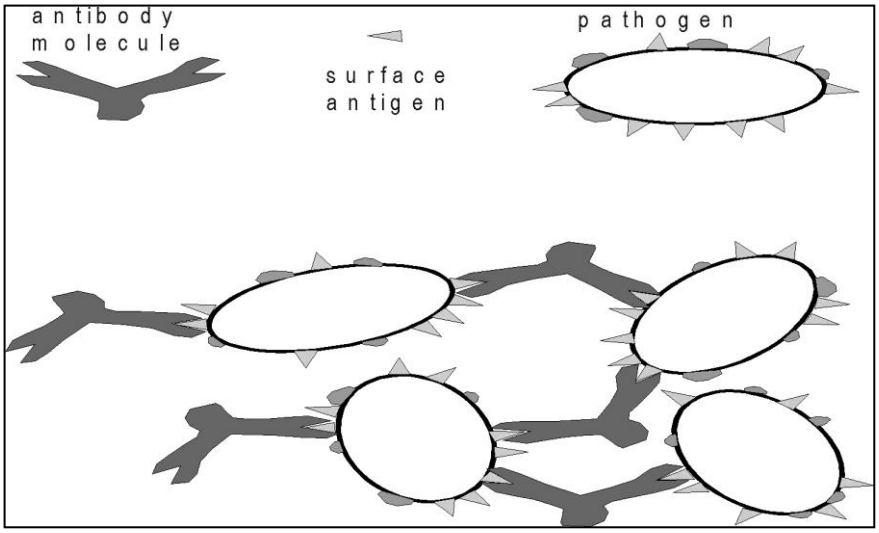


Figure 11.1: **Antibody Cross-linkage and Lattice Formation**

When the multivalent antigen occurs on a cell (or particle of similar size), the antibody cross-links the cells into large aggregates. This process, called agglutination, converts a uniformly turbid suspension into a clear solution with large clumps. Because it is sensitive, rapid and readily visible, agglutination is used for blood typing and in a variety of immunological test kits, including those for drugs and pregnancy. It is also well suited for the identification of bacterial pathogens, because antibodies raised against bacterial cells usually agglutinate only that species or sub-species.

B. DNA Sequencing

(See Brock pp 305 – 306, 332 – 336)

The recipe for an organism is contained in its DNA, and if one knows the sequences of nucleotides, As,Ts, Cs, and Gs, then one can hypothetically know all about it, right? Well, no, it does not quite work that way, but there is still a lot of value to DNA sequence just the same. One of the major outcomes from the invention of feasible methods of determining DNA sequencing in the 1970s was the ability to identify bacteria and other microorganism with far greater certainty that was possible before. This is because, even when two different species have the same genes, and there are a large number of genes shared by most organisms, the exact sequence of those genes are going to differ between the two. If a database is made of enough gene sequences from enough organisms, then, if one has an unknown, one should be able to determine the sequence of a given gene, feed that sequence into a database, and have the identity of the unknown come back to you. This has, in fact, been done, and is possible.

The problem with DNA sequencing, however, is that there is a lot of DNA in an organism; any organism. It is not feasible to sequence the entire genome of an organism just to identify it. The solution has been a focus on the specific finding, sequencing, and cataloging only a limited number of genes. The most carefully catalogued and most often used gene sequences are those of the 16S ribosomal RNA, or 16S rRNA gene. Remember that ribosomes are composed of two subunits, both of which are made up of both protein and RNA. The 16S rRNA is the RNA constitute of the small ribosomal subunit. All organisms have to have ribosomes to make proteins, so this means that all organisms have 16S rRNA genes (In eukaryotes, they are a bit larger, and referred to as 18S rRNA genes.), so you know for certain

when you are getting sequence from an unknown organism that it will have this gene; something that would not necessarily be true with another gene. Also unlike some other genes, there will generally be only one allelic version of the 16S in an organism. This allows scientists of identify an organism as something new by shear dint of discovering and sequencing a bit of its DNA containing its 16S sequence. Indeed, for many prokaryotes, the 16S is all that is known of the organism.

Identification is not the only thing that can be accomplished with the sequence of an organism's 16S rRNA gene. Since the 16S rRNA is involved in transcription, and is thus crucial to the organism, it does not change as quickly in sequence (Because if the sequence were changed too much, an organism's offspring might find their 16S gene to be nonfunctional, and they would be dead. And dead creatures are not known for reproducing much.) as most other genes. They still change however, and this is a key to their use. You see, if you compare the differences in the sequence of a gene from two different organisms, provided they are not tremendously closely related, then one can determine relatively how closely related they are. If you do this with enough organisms (Using powerful computer software.), you can construct an outline, called a phylogenetic tree, showing the relative relationships between the organisms based entirely on the differences in their gene sequences. The problem is that this tree gets fuzzy and hard to resolve when the gene for which you are comparing the sequences changes so often in its base pair composition that the differences get overwritten, and prevent you from seeing the actual degrees of difference between the organisms. This is why the evolutionary stability of the 16S is so useful. Because it changes so slowly, or is conserved so well, and because it is roughly 1500 base pairs in length, meaning it has a lot of information in it (This is actually the reason why it started being used to begin with. There are two other rRNA genes: the 5S and the 23S. The 5S gene, at only 120 base pairs, was small enough to sequence easily, but too short to be useful. The 23S had a lot of information, but, at about 2900 base pairs, it was too large to easily sequence at the time.) it can be used to determine the relationships between organisms that are very, very different from each other. The universal tree of life showing the evolutionary relationships between all living things that was shown in figure 1.1 was constructed from 16S sequences.

It should be apparent now that one can not only identify an organism on the basis of its 16S sequence, but one can use it to determine where the organism fits in on the universal tree of life. One can determine if it is a bacterium, archaeon, or eukaryote, and, if one looks at the characteristics of its close neighbors on the tree, one can make very good guesses as to its characteristics of morphology, metabolism, and ecological role. In those situation in which all you have is the 16S sequence of an organism, the value in this should be clear.

You are going to be given the 16S sequence of an organism and, using a database of 16S sequences, you are going identify the organism it came from.

Procedures

Part I: Follow Up to Session 10:

Enterotube II Sytem:

Each group needs to retrieve the Enterotube that was inoculated during the last session, and score the results according to the following:

1. Examine the coloration of all test compartments except for the Indol (IND) and the VP tests, and score them as positive or negative according to the table. Record the results in the table in the report section.
2. When you have finished reading the other tests, you will need to read the Indol (IND) test. Position the Enterotube so that the GLU-GAS test compartment is facing upwards, flame a loop to red hot, and immediately use this to melt a small hole in the plastic of the H2S/IND compartment. Use a dropper to add one or two drops of Kovac's reagent to the compartment through this hole (You need to read the other compartments before this because the Kovac's reagent may leak into the other compartments.). Watch for the development of a red color within ten minutes of the addition of the reagent.
3. Look in the report section for the sheet that you will use to convert the test results into an identification code that you will use to identify your unknown. For each positive test, circle the number below its abbreviation. Add up the numbers circled in each section. Enter the sum of the numbers circled for each section in the boxes below the sections. You now have a five-digit identification code for your unknown.
4. Find the interpretation guide for the Enterotube II and look up this code to identify your unknown. If the number does not match any in the guide, go back and reexamine any ambiguous tests to make sure that you did not read them incorrectly. Generate the new identification code if necessary and look it up. Enter the identified name of your unknown in the report section and answer the questions.

Be sure to note the letter of your unknown in the report section so that you can get credit for it.

Abbreviation	Test	Positive	Negative
GLU-GAS	Glucose Fermentation	Yellow	Red or Orange
	Gas Production	Separation of the wax overlay from the surface of the medium	No wax separation
LYS	Lysine Decarboxylase	Purple	Yellow
ORN	Ornithine Decarboxylase	Purple	Yellow
H2S/IND	H2S Production	Black precipitate has formed	No Black precipitate
	Indole Formation	Pink color upon addition of Kovac's reagent	No color change after addition of Kovac's reagent
ADON	Adonitol Fermentation	Yellow	Orange
LAC	Lactose Fermentation	Yellow	Orange
ARAB	Arabinose Fermentation	Yellow	Orange
SORB	Sorbitol Fermentation	Yellow	Orange
VP	Voges-Proskauer	Development of red color	No color change
DUL-PA	Dulcitol Fermentation	Yellow	Green
	Phenylalanine Deaminase	Black to smoky gray color	No change
UREA	Urease	Reddish-purple	Yellow
CIT	Citrate Utilization	Blue	Green

Table 11.1: **Tests of the Enterotube II Miniaturized Multitest System with a Guide to Reading Them**

Winogradsky Column

Go to the hood in the back, and without disturbing it, examine the column your group constructed. Record your observations in the appropriate place in the report section. Be sure to note if any color bands are obvious, and if there have been any changes since your last observation.

Part 2: Today's Exercises:

Exercise 1: Identification of an Unknown Bacterial Organism:

Step 1: Separation of your Working and Storage Stocks:

Retrieve the two slants you inoculated with your unknown last time. Choose one of them to be your working stock. This is the slant from which you will be doing a great many inoculations in the next few sessions. The remaining slant will then be your storage culture. Place it in the racks set aside for the storage cultures. They will be refrigerated to preserve them in case they are needed.

Step 2: Official Gram Stain of Your Unknown:

Materials: 1 microscope slide

Make a smear of your unknown and Gram stain it. Be careful while you are staining it, as this will be the "official" Gram stain of your unknown, and you will need the information you get from this later on. Record the Gram stain reaction in the appropriate part of the report section.

Step 3: Inoculation of Media for Cultural Characteristics and Metabolic Properties I:

Materials: Test tube rack
1 NB tube
1 NA plate
2 NA slants
2 MR-VP tubes
1 Thioglycolate tube
1 Nitrate broth tube
4 Phenol Red Sugar tubes (Dextrose, lactose, mannitol, and sucrose; label them as you get them – they all look alike!)
1 Simmon's Citrate slant
1 TTC motility medium tube
1 Endospore medium slant (If your organism is a Gram positive bacillus)

1. Collect the media listed above. Be sure to label the media tubes as you collect them to avoid confusion. Also label each with your name, the date, and "unknown".

2. Inoculate all the media you have collected with the working stock of your working culture as follows:
   Note: Remember that you are going to be using culture growing on a solid medium. This means two things: 1. The growth is very, very concentrated. You do not need to use much each time you inoculate the media today. 2. Be careful not to gouge the agar in the tube when you are getting samples for inoculations.

   a. Aseptically streak the NA plate for isolated colonies using whichever isolation technique you prefer.
   b. Aseptically transfer a sample of culture with your loop to each of the following: the NA broth, the 2 MR-VP tubes, the Nitrate broth tube, the 4 Phenol Red Sugar tubes
   c. Aseptically inoculate each of the 2 NA slants with a sample of culture using your loop by making a single streak up the surface of the slant of medium, being sure not to gouge the agar as you do so. If you need to inoculate an endospore medium slant, you should do so with this same procedure.
   d. Use your inoculating needle to aseptically stab inoculate the Gelatin tube, Thioglycolate tube, and TTC motility medium tube. Remember that you want to stab straight down, and then bring the needle straight out along the same path.
   e. Use your inoculating needle to first streak your unknown over the surface of the Simmon's Citrate slant, and then stab it down to the bottom of the tube.
3. When you are finished with the inoculations, place all the media in your bench for incubation until next time.

Exercise 2: Identification of Bacterial Unknowns Using Agglutination and Serotyping:

The goal of this exercise is to use a specific antiserum to determine whether an unknown bacterial culture is *Salmonella*.

Materials per group: Antiserum (70 uL)
Suspension of a positive control (*Salmonella enterica* serovar Typhimurium)
Suspension of an unknown
Suspension of a negative control (E. coli)
Pipettor

1. Using a wax pencil, draw three large but well-separated circles on a microscope slide; label them with the symbols "+", "u" or "-" outside each circle.
2. Carefully deposit 20 uL of the corresponding bacterial suspension in the center of each circle. Try to avoid spreading the drop.
3. USING A CLEAN TIP EACH TIME, pipette 20 uL of antiserum into the center of each cell suspension, and MIX the two solutions by pipetting up and down several times. Limited stirring or moving the tip around during this process may also help, but try to avoid spreading the drop.
4. After a few minutes, examine each drop against a dark background. The "+" test should show a clumping of the cells resulting in a grainy appearance, whereas the "-" test should be a uniformly turbid suspension. Describe the actual appearance of these tests on the laboratory report form.
5. Record the number and the agglutination result (pos or neg) for your unknown.

Exercise 3: Sequence-Based Identification of Microbial Unknowns: 16S rRNA Gene Sequence

Make certain that the TAs have your email address. Either tonight or tomorrow, you will be emailed the 16S rRNA gene sequence of an unknown microbial organism. You will need to copy this sequence from the email. Go to the Ribosomal Database website at: http://rdp.cme.msu.edu/html/
Once the page has loaded, if you look halfway down the page, you will notice a link that says: "Click here to enter the preview site." Click this link.
When you get to the new page, you will see two links in the middle of the page. Click the one that reads, "Sequence Match".
Paste the 16S gene sequence you were sent into the data box.
Click the submit data box.
If you scroll down the result page, you will see that the results display as a sort of tree, with a number of boxes stemming out from each other diagonally across the page, and ending in a list of organsims' binomial names. To the left of each name is a series of columns of numbers. The first column from the left should be blue, and next to it should be a column of red numbers. The red numbers represent the score of the hit, where 1.0 means that the 16S sequence for that organism was 100% like that of the sequence of your unknown. The organism that shows a 1.0 similarity score is your unknown. Record the name of this organism.
Answer the questions in the report section. Please note that this report is due at the start of the next session.

Lab Session 12

Background

I. DNA Damage and Repair

Please see prologue to exercise 2 for background.

Procedures

Part 1: Follow Up to Session 11:

Characteristics of your Unknown I:

Get out the media you inoculated for your unknowns last time. As you did in lab nine with a known organism, you are going to collect data from your media, recording your observations, and results for each in the appropriate places in the report section. Evaluate the media according to the criteria given below:

- ***Steak plate:*** Evaluate the morphology of the resulting colonies according to their color, size, form, elevation, opacity, and margins (See figure 1 of lab 9). When you are finished with this, keep the plate so that you can use

the colonies on it to test if the organism produces oxidase according to the instructions in the guide to biochemical tests.

- ***First NA slant:*** Evaluate the growth according to its color, opacity, and form (See figure 2 of lab 9).
- ***Second NA slant:*** Use the growth on the slant to test for catalase production by the organism as detailed in the guide to reading biochemical tests.
- ***NB tube:*** Evaluate the amount of the growth of you organism, as well as its surface, subsurface, and sediment characteristics (See figure 3 of lab 9).
- ***Thioglycolate tube:*** Evaluate the growth in the tube and use it to determine the oxygen requirements it posesses.
- ***Phenol Red Sugar Tubes****:* Evaluate according to instructions in the guide to biochemical tests.
- ***MR-VP tube:*** Put one aside, and do not use it this session. For the other, evaluate according to the instructions for the methyl red test in the guide to biochemical tests.
- ***Nitrate broth tube:*** Evaluate production of nitrase according to the instructions in the guide to biochemical tests.

TTC motility medium tube: Evaluate the growth of the organism in the tube, and determine whether or not it is motile.

Part 2: Today's Exercises:

Exercise 1: Identification of an Unknown Bacterial Organism:

Part 4: Inoculation of Media for Metabolic Characteristics II:

Materials:
- 1 Starch Agar plate
- 1 Skim Milk Agar plate
- 1 Sprit Blue Agar plate
- 1 NA plate (If your unknown is a Gram positive coccus.)
- 1 Gelatin tube
- 1 Phenylalanine slant
- 1 Simmons Citrate slant
- 1 Tryptone broth tube
- 1 Urea broth tube
- 1 SIM tube

1. Collect the media listed above. Be sure to label the media tubes and plates as you collect them to avoid confusion. Also label each with your name and the date.
2. Inoculate all the media you have collected with from the working stock of your unknown as follows:
   a. Aseptically streak a loop of culture onto each of the Starch Agar, Skim Milk Agar, and Spirit Blue Agar plates. You should not streak for isolation!!! Instead, you should make a single streak up the center of the plate (See figure).
   b. Aseptically transfer a loop of culture each to the Tryptone broth tube and the Urea broth tube.
   c. Aseptically streak a loop of culture up the surface of the medium for the Phenylalanine slant.
   d. Straighten out your loop to form a needle and use this to aseptically streak a sample of your culture on the surface of the Simmons Citrate slant, after which you will stab the needle straight down the middle of the agar to the bottom of the tube, distributing the culture through the agar. Withdraw the needle along the same path by which it entered.
   e. Use your inoculating needle to aseptically stab inoculate the SIM tube with a sample of your culture. Stab straight down, and withdraw the needle along the same path.
   f. If you have been assigned S. aureus, you should streak a loop of culture for isolation on the NA plate.

When you are finished with the inoculations, place all the media in your bench for incubation until next time.

Exercise 2: Bacterial Genetics I: DNA Damage and Repair:

Work in groups of two on this exercise.

(See Brock pp 273 –274, 439)

The goal of this exercise is to demonstrate some basic biological properties of DNA damage, using an auxotrophic (nutrient-requiring) strain of *E. coli* as the test subject. Specifically, we hope to measure i) the rate of killing by UV light, ii) the induction of mutations by UV light, and iii) partial reversal of UV effects by subsequent exposure to visible light ("photoreactivation").

To avoid some of the problems of handling suspensions, the *E. coli* cells will be plated first and then exposed to UV for different lengths of time. Killing will be measured on fully supplemented ("+Ura") medium, which allows all viable cells of the uracil auxotroph to grow. Mutagenesis will be measured on similar medium lacking uracil. On this "-Ura" medium, the orginal E. coli strain cannot grow, but reverse mutants (revertants) can grow and form colonies. Revertants are normally rare in pure cultures, but damaging the cell's DNA triggers their formation at much higher frequencies.

To show the full effect of UV radiation, plates must be protected from visible light after exposure. Alternatively, photoreactivation can be observed if an identical set of plates is illuminated with fluorescent lights after UV but before incubation.

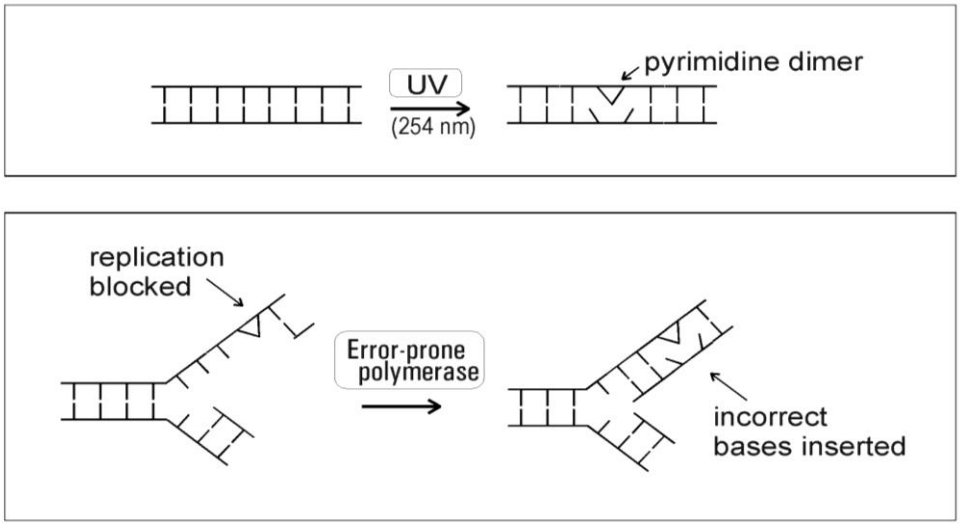


Figure 12.1: **Damage of DNA by UV Radiation and Its Subsequent Repair**

Important Terms and Concepts

short-wave UV (UV-C)	repair options	quantitative assays
thymine dimers	- nucleotide excision	- killing
blocked DNA replication	- photoreactivation	- photoreactivation
mutant	- error-prone bypass	- mutagenesis
revertant		
auxotroph		

Materials
E. coli strain YA289, 1 mL (undiluted and 10^{-1}) or (10^{-3} and 10^{-5})
– Ura plates (2)
+ Ura plates (2)
Pipettor

One student in each group should assay killing, while the other assays reversion. Both will test for photoreactivation.

Killing Assay:

1. Draw a single vertical line on the back of each of eight "+Ura" plates; label the right side of each plate "10^{-3}" and the left side "10^{-5}".
2. Carefully pipette 50 uL (0.05 mL) of the 10^{-5} E. coli suspension on the corresponding half of each plate. Spread the suspension so that it covers as much of that half as possible, without coming too close to the dividing line.
3. Repeat for the 10^{-3} suspension, and allow all the plates to absorb the liquid.
4. Group the plates into pairs. Label each pair with one of the UV doses specified by the instructor, then label one of the two plates "-PR" and the other one "+PR".

Reversion Assay:

1. Draw a single vertical line on the back of each of eight "-Ura" plates; label the right side of each plate "undil" and the left side "10^{-1}".
2. Carefully pipette 50 uL (0.05 mL) of the 10^{-1} E. coli suspension on the corresponding half of each plate. Spread the suspension so that it covers as much of that half as possible, without coming too close to the dividing line.

3. Repeat for the undiluted suspension, and allow all the plates to absorb the liquid.
4. Group the plates into pairs. Label each pair with one of the UV doses specified by the instructor, then label one of the two plates "-PR" and the other one "+PR".

Both Students:

5. Make sure that all plate surfaces are dry, and then take both sets of four plates to the UV lamp. An instructor will help with the UV irradiation (caution: avoid exposing your face to the UV). Note that the lids of the plates must be removed for irradiation, because plastic and glass do not transmit UV-C.
6. As each pair of plates is removed from the UV chamber, replace the lids and stack the "-PR" plates under a square of aluminum foil. When all four of these have been collected, wrap the stack in foil. Label it with your name and the type of assay (killing vs. reversion). An instructor will collect these stacks for incubation.
7. Put the four remaining +PR plates under fluorescent lights for 30 min, as shown by the instructor. Leave lids on during this treatment.
8. After photoreactivation, bundle the +PR plates with tape. Label with your name and the assay performed (killing or reversion), and take them to the front of the middle bench for incubation.

Lab Session 13

Background

I. Bacterial Genetics II: Conjugation

Please see prologue for exercise 2 for background on conjugation.

II. Koch's Postulates and the Etiology of Disease

(See Brock pp 12 – 14)

We speak quite easily today of certain microorganisms as causing diseases. It is hard to keep in mind that, until just the last century and a half or so, no one really could quite say what caused disease. Epidemics seemed to strike without warning, kill enormous numbers of people, and then disappear. There were certain areas that seemed particularly prone to such epidemics, and were thus to be avoided unless you lived there. People just did not know, and this was frightening. If you do not know what causes a disease, after all, it is hard to know what to do to avoid it, or even really how to treat it. This lack of understanding led to supernatural explanations of disease causation: you got sick because a fever demon had breathed on you, because you were under a spell, because you did something to anger a god, or you did nothing, but a god just did not like you for some reason. This led to all sorts of odd practices and rituals aimed at keeping evil spirits at bay or else trying to keep in the good graces of relevant gods. For instance, during the Black Death, groups of flagellants would parade through city streaks whipping themselves in a collective penance for the evils that had brought the plague that was wiping out whole cites and a quarter of Europe's population. Because cats were thought to be evil, they were killed to, something that likely made things worse, as the plague was carried about by rats and their fleas. A lot of suspected witches were also burned, both then and in later years during other epidemics.

Attempts at scientific explanations would occasionally pop up. Aristotle and other Greek philosophers and physicians, for instance, thought disease to be caused by little animals that could be passed from person to person (Explaining what had been noted for a long time: diseases are often contagious.), and recommended such precautions as boiling drinking water and keeping clean. Two other widespread ideas were that disease had its origin in foul airs called miasmas from swamps, caves, forests, and graveyard, or that it is due to an imbalance in the four humors (Blood, black bile, yellow bile, and phlegm) of the body. Strangely, it was these last two "scientific" theories that came to be accepted and survived through the dark ages. Both explanations were held by many doctors and men of science until the nineteenth century (The continuation of the humor theory of disease is why bleeding was a treatment for most everything during the time.).

The little organism theory began a revival when the advent of inoculation against such diseases as small pox in the eighteenth century showed that something could be transferred from person to person that would cause illness (Keep in mind that inoculation for smallpox involved a doctor transferring material from the pustules of a sick person to a healthy person to cause a mild form of the disease.). That this something was a physical thing, however, was not really demonstrated until the early nineteenth century, when it was shown by such individuals as Joseph Lister, Ignaz Semmelweis, and Oliver Wendell Holmes that chemical disinfectants could prevent the transfer of such infectious material. This was put to the test in the 1830s and 1840s, when it was discovered that, if surgeons were to wash their hands before operating, their patients would get sick less often.

The question still remained as to what the infectious stuff was. There were those who thought of it as just disease particles, but others had a more radical idea: that diseases were caused by microorganisms, and it was the transfer of them from a sick person to a healthy person that caused the healthy person to become ill. One of the early proponents of this idea was Louis Pasteur, the killer of spontaneous generation, who had done much work for the government of France on problems in the wine industry. It was noted that wineries would occasionally catch "diseases" that would make multiple batches of wine taste bad or have peculiar odors. Pasteur determined that such winery "diseases" were caused by microorganisms other than the yeasts that should have been there that would get into the fermentation vats, and throw off the fermentation process. He also discovered that a protozoan was causing a disease amongst French silkworms. Fungi had previously been shown to cause diseases in silkworms and potatoes, but no one thought to make the jump that Pasteur did when he suggested that maybe microbes could also cause disease in humans and other higher animals. This suggestion was what we now call the germ theory of disease.

Few accepted Pasteur's claim. There were many who refused to disbelieve the miasma and humor theories. Bacteria had been noted in the blood and other fluids of patients with certain illnesses, but it was unclear what they were doing there. Many thought that bacteria did not cause disease, but that diseases somehow attracted them. The solution to this controversy of the actual connection between microbes and disease was formulated by a small town German doctor by the name of Robert Koch. Robert Koch was a self-taught microbiologist who had developed an interest in anthrax, which was a problem for some of the local livestock farmers. He had noticed that those animals that were infected with the disease showed the presence of a large number of bacilli in their blood and body fluids, with their concentration getting even higher after the animals death. Koch theorized that these bacilli were the germs of the disease and set up an experiment to test this. He found a way to infect mice with the bacillus, and noticed that each time a mouse was infected, it developed anthrax. He then did a serial infection, where he transferred the infection through a series of twenty mice. In each case, he was able to observe the bacilli after, but not before the mice were infected. From the twentieth mouse, he removed a bit of the spleen, and used this to inoculate a beef broth culture. Again, he noticed the bacilli to be present. He then injected some of this culture into a group of healthy mice, not injecting any into a second group. The injected group developed anthrax, but the control group did not. Further, the injected group displayed the presence of the bacillus, but the control did not. He named the bacterium he had found *Bacillus anthracis* and published his findings in 1876 to much skepticism, but slowly won people over. In some cases, he did not win over people until a few years later, when he was able to establish a bacterial cause of tuberculosis.

Though he never explicitly stated them, the core or Koch's experiments was made up of a series of rules that have come to be known as Koch's postulates. These are basic rules for establishing whether or not a suspect microbe really causes a disease, and are still used today, though with some trouble on occasion. They may be stated as follows:

1. The suspected microorganism must be present in every individual with the disease, but must never been seen in healthy individuals.
2. The suspected microorganism must be isolated from the diseased and grown in a pure culture.
3. The same disease as before must result when one inoculates healthy individuals with the pure culture of the suspected microorganism.
4. One must then be able to re-isolate the suspected microorganism from the newly diseased individuals.

You might say the postulates are merely common sense ways of linking cause to effect, and that is true, but Koch was the first one to use them for disease, and it stands as perhaps his most important contribution to medicine. It

was not his last one, either. In addition to discovering the causative organisms of anthrax and tuberculosis, he also developed the tuberculin test that is still used today, discovered the organism that causes cholera and made this the basis of recommendations that form the basis of water sanitation. He also did extensive studies on malaria, plague, typhus, East Coast Cattle Fever, and a number of other diseases. His work was not limited to medicine, and his contributions to microbiology included advancements in methods of photographing microorganism, pure culture technique, microbial enumeration, staining, solid media, and it was from his subordinates that we got the use of the Petri plate and agar. For his lifetime of work, he was awarded the Nobel Prize for Physiology in 1905. Not bad for a man you have likely never heard of and to whom you might well owe your life, huh?

Today you will be starting a quasi-demonstration exercise designed to test Koch's postulates and help you to understand how they work.

Procedures

Part 1: Follow Up from Session 12:

DNA Damage and Repair:

Retrieve the plates you prepared last time. Count the colonies on each of half of each plate, and record the information in the table in the report section. Make sure that you also get a copy of your partner's data.

Characteristics of Your Unknown II:

Get out the media you inoculated last time. Evaluate each according to the instructions in the guide to biochemical tests. If your unknown is a Gram positive coccus, then you should use the streak plate you prepared to do a coagulase test as you learned earlier (If you have forgotten, the instructions are in the guide to biochemical tests.). Record all data collected from these test media in the appropriate part of the report section. When you are finished, go on to Exercise 1 of today's exercises. Be sure to also perform a Voges-Proskauer test on the remaining MR – VP tube.

Winogradsky Column

Go to the hood in the back, and without disturbing it, examine the column your group constructed. Record your observations in the appropriate place in the report section. Be sure to note if any color bands are obvious, and if there have been any changes since your last observation.

Part 2: Today's Exercises:

Exercise 1: Use of Bergey's Manual to Determine the Identity of Your Unknown

You now have determined a fairly large number of characteristics for your unknown. The next step is to start using them to determine the identity of your unknown. If you have not already done so, you need to start looking through *Bergey's Manual* to do this. A good idea, and one that you will need to act on anyway for the report on the unknowns, is to make out a flow chart that shows how your are narrowing down the possibilities for your unknown. Each step on the chart should represent the reduction in possible organism that your unknown could be.

Try making this flow chart as you look through *Bergey's Manual*. There will be a couple of copies circulating through the lab today; try to look through them during class. There are also copies in the Biology-Chemistry library that you can use outside of class.

As you are looking through *Bergey's*, if you find that a test or two that we did not do in class would be useful in the identification of you unknown, please let Julie know. She will be able to tell you if we have the materials needed for these tests, and if she can prepare them for your use. Please let her know of any media requests today so they can be

ready next session. Please remember that we do not have the resources to do any sequencing to determine your unknown's identity.

There are extra media from the earlier tests we performed. If any of the results you obtained for your unknown were ambiguous and you would like to try the test again, please go ahead and inoculate a new set of media for those tests. The extra media should be found along the side bench.

As always, if you have any problems, please let a TA know so he/she can help.

Exercise 2: Bacterial Conjugation:

(See Brock pp 291 – 297)

Transfer of DNA directly from one bacterial cell to another is termed "conjugation", because it requires the two cells to adhere and form a transient connection. Transfer proceeds in one direction, from the "donor" to the "recipient", and depends on a number of genes encoded on a plasmid. Furthermore, the plasmid is normally the only DNA that is transferred, so that conjugation can be considered the means by which plasmids disseminate themselves among bacterial populations.

Sometimes a self-transmissible plasmid becomes integrated into the donor chromosome. In this case, the plasmid will transfer large regions of the bacterial chromosome to the recipient cell during conjugation. After transfer, homologous recombination can replace various sections of the recipient chromosome with corresponding sections of the transferred donor DNA. If the donor and recipient differ with respect to the functional state of genes in these regions, then genetic recombinants resulting from this mating can be identified. This is normally done by plating the mixture of donor, recipient, and recombinant cells on a medium that allows only recombinants to grow and form colonies.

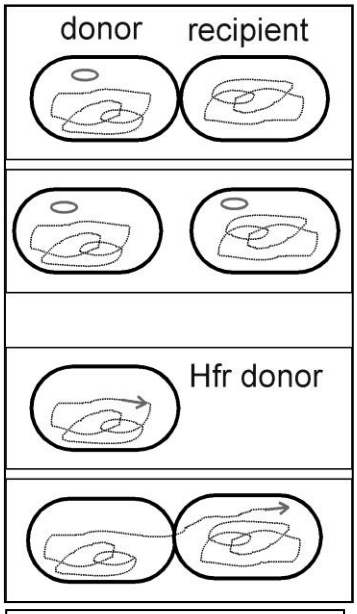


Figure 12.1: **Conjugation**

The goal of this exercise is to demonstrate the transfer and recombination of chromosomal genes for an *E. coli* Hfr (High frequency of recombinants) donor to a recipient strain. The recipient has genetic markers, in the form of mutations that inactivate each of three biosynthetic genes (*thr*, *leuB*, and *hisG*). As a result, the strain requires the amino acids threonine, leucine, and histidine in its growth medium. The donor has no mutations in these genes, so if those chromosomal regions are transferred into the recipient, some cells can substitute the functional copy by recombination and gain the ability to grow without the corresponding amino acid. The recipient also has a mutation in a gene (*rpsL*) that makes it resistant to the antibiotic streptomycin. Because the donor lacks this mutation, it cannot form colonies on any plate containing streptomycin.

Conjugation is initiated by mixing donor cells and recipient cells at a ratio of about 1:10, and incubating at 37º with minimal agitation. The Hfr cells begin transferring DNA almost immediately, at a rate of about 50 kb per minute. Transfer begins from a particular point on the chromosome and proceeds in a particular direction. For this reason, certain genes are transferred sooner and at a higher frequency than others, reflecting their order on the chromosome. In order to detect this directionality, we will sample the culture at two times (10 min and 60 min) and evaluate transfer of the *thr*, *leuB* and *hisG* genes independently of each other. This will be done by spreading the cell mixtures on plates containing streptomycin (+Str) but lacking either threonine (-Thr), leucine, (-Leu), or histidine (-His).

[Students work in pairs and take turns sampling and plating cells (A vs. B)]

Materials:
- Tube of donor cells (*E. coli* YA289)
- Tube of recipient cells (*E. coli* AB1157)
- Sterile Erlenmeyer flask with foil cap
- 2 sterile tubes with caps (18 mm)
- 4 "-Thr" plates
- 4 "-Leu" plates
- 4 "-His" plates

1. Organize and label the selective plates with the sample to be plated and student name; the 12 plates should correspond to the table on the report sheet. Also get one tube each of donor and recipient cells and label them.
2. Student A: Plate 0.1 mL of the donor cells on each of the three types of selective medium (see report sheet). Student B: Plate 0.1 mL of the recipient cells on each of the three types of selective medium.
3. Pipette 5 mL of the recipient culture into the sterile flask. Pipette 0.5 mL of the donor culture into the flask, and swirl thoroughly but briefly. After this point, avoid any vigorous shaking or swirling of this flask.
4. As soon as possible, put the flask into the 37º water bath provided and start timing the transfer.

Student A:

5. At 10 min, transfer about 0.5 mL to one of the sterile tubes. To avoid disturbing the mating mixture itself, leave the flask in the water bath, and withdraw the sample with the pipettor provided.
6. Vortex the sample tube for 30 sec to disrupt DNA transfer.
7. Immediately plate 0.1 mL of the sample on the first selective plate. Repeat for the remaining selective plates (see report form).
8. After the liquid has soaked in, stack the plates, tape the stack, and label with your name.

Student B:

At 60 min, repeat steps 6-8.

Exercise 3: Koch's Postulates, Part I:

Koch's Postulates will be tested through a series of demonstration exercises that will go on from today through session 16. The demonstrations will primarily involve you examining the specimens set up on the side bench through the next few sessions, as well as watching while Julie and other brave volunteers (Including you if you wish!) work on them.

We obviously can not use humans in demonstrating Koch's Postulates (Strictly speaking, we can, but humans are difficult to obtain and expensive to maintain as test subjects, and there is that whole thing about professional ethics, the law, and such. Of course, in the past human subjects, usually volunteers were used to study diseases using Koch's postulates. This is something to keep in mind as you study epidemiology.) , so we will be using cockroaches. Basically, the situation is this: there is an unknown disease called the Red Death that is savaging the roach population. We have on the side bench a colony of roaches that has not yet had an outbreak of Red Death. To prevent spread of the disease through the population, immigrants to the colony are quarantined for a period to ensure that they do carry the disease. Unfortunately, it seems that a number of those currently in quarantine are affected by the disease. This demonstration exercise centers on the use of Koch's Postulates to establish whether or not the Red Death is caused by a microorganism, and if so, what sort of microorganism it is. Julie will do most of the work, but if you would like to help out, please volunteer.

For today you should examine the roaches in the two roach containers, paying close attention to the differences between the sick and healthy roaches. Remember that the population in the container labeled as the colony is free of the disease, while the population in quarantine is affected. Record your observations in the appropriate place in the report section.

Today, Julie will remove a sample from the hemocoels, or body cavities, of a number of roaches, both healthy and diseased, and streak these samples out onto TSA plates. These will be incubated to observe what grows up on them, and if there is any obvious difference between the microflora of the healthy roaches and that of the diseased roaches. Be careful to observe her as she does this, noting how she does the transfers. Record your observations in the report section. If you would like to help her, please volunteer before this part of the demonstration is carried out.

Exercise 4: Sign Up for Group Projects:

As you should know by now, a good amount of the work remaining this quarter will be done in groups working independent of one another. You should by now be familiar with the three group projects available. Today there will be a sign up sheet going around for these projects. The sheet will have a brief description of the three projects, so if you do not remember them exactly, this is not a problem. Next to your name, you need to put down your first and second choice for a project. When you come in next session, you will be told of the project on which you will be working.

Lab Session 14

Background

None.

Procedures

Part 1: Follow Up to Session 13:

Identification of Unknowns:

If you re-inoculated any test media last time due to ambiguity in the results you originally obtained for them, read them as you did earlier according to the instructions in the guide to biochemical tests. Be sure to carefully record your observations and results in the report section.

Conjugation:

Collect the media you prepared last time. Count the colonies that have arisen and record the data in the table in the report section.

Part 2: Today's Exercises:

Exercise 1: Identification of Unknowns, cont.

If you requested any new test media from Julie, obtain them from her and inoculate them as appropriate from your working stock. Incubate them in your bench cabinet unless the instructions in Bergey's manual specify otherwise. Be sure that you carefully note in the report section what these tests are, what they are testing for, how you inoculate them, and how you will read them.

Exercise 2: Koch's Postulates; Part 2:

The plates Julie and others streaked last time will be set out for observation. Please record your observation in the appropriate places in the report section. Pay careful attention to any differences you observe between the plates streaked from the healthy roaches, and those streaked from the diseased roaches.

Julie examined the plates yesterday, and inoculated a broth culture from the odd, red colonies that showed up on some of the plates. This might be the organism that causes the Red Death. To find out, she will be injecting samples of this broth culture into a group of healthy roaches to see what happens. A control group will be injected with sterile broth. If you would like to volunteer to aid Julie, please do so before she starts. While the roaches are being injected, please be careful to record exactly what is being done in the report section.

The effect of the injections will be observed next time.

Exercise 3: Group Project Planning

Today you will each be given a packet corresponding to your assigned project group. Today, after the other exercises are complete, the three groups will be taken aside and told something more about the projects. After this time, each group should go over the materials and make plans for the next few lab sessions, during which we will be working primarily on these projects. You should also during this time come up with a plan for what you are going to do, and who is going to do what. You should prepare these plans in a brief written form. At the end of the period, the group will present the plans and ideas to the project instructor, and also turn in a copy of this preliminary plan. A more detailed plan is due at the start of class next session when work will begin

Lab Session 15

Background

Please see the project packets for any relevant background material.

Procedures

Part 1: Follow Up to Session 14:

Identification of Unknowns:

If you inoculated media for new tests last time, you will need to retrieve them and read them as indicated in Bergey's manual. Identify your unknown to the best of your ability using these results in conjunction with those already in hand.

Winogradsky Column

Go to the hood in the back, and without disturbing it, examine the column your group constructed. Record your observations in the appropriate place in the report section. Be sure to note if any color bands are obvious, and if there have been any changes since your last observation.

Part 2: Today's Exercises:

Exercise 1: Group Projects I:

Today you will be primarily working on the core exercises of your project. Material relating to them should be in your project packet.

Exercise 2: Koch's Postulates; Part III:

Last time, healthy roaches were injected with either sterile nutrient broth, or with a broth culture of the microbial organism that produced red colonies on the plates streaked from roaches with the Red Death. Today, you need to observe these roaches and note which of them appear to be diseased, and which appear to be healthy. Carefully note differences between those roaches injected with just sterile broth, and those injected with the broth culture of the suspected organism. Answer the questions in the report section.

Today, Julie will be streaking out new plates with samples taken from the hemocoels of the roaches tested last time to see what turns up. As usual, if you would like to help, please volunteer before she starts.

Lab Session 16

Background

None. See project packets for relevant background materials.

Procedures

Part 1: Session 15 Follow Up:

Group Projects

Gather relevant data from last session.

Part 2: Today's Exercises:

Exercise 1: Group Projects, continued:

See project packets.

Exercise 2: Koch's Postulates; Part IV:

Last time, Julie and others streaked out plates from samples taken from the roaches injected with either a broth culture of the organism potentially responsible for the Red Death, or with sterile broth. These plates are laid out on the side bench. Examine them and note the differences, if any, between those taken from the roaches injected with the sterile broth, and those injected with the culture. Record your observations in the report section, and complete the questions. A few broth cultures were inoculated from the mysterious red colonies were inoculated. Each person should make a Gram stain of one of these broths and record the results in the report section as well.

The primary question at issue is that of whether or not the Red Death is a disease caused by an organism, and if the red microbe is the causative organism.

Lab Session 17

Background

None. See group packets for relevant background materials.

Procedures

Part 1: Follow Up from Session 16:

Group Projects:

Gather relevant data from last session.

Winogradsky Column

Go to the hood in the back, and without disturbing it, examine the column your group constructed. Record your observations in the appropriate place in the report section. Be sure to note if any color bands are obvious, and if there have been any changes since your last observation.

Compare your column to those of others, as well as those set up by Julie and the TAs before the quarter started.

After everyone has gathered data regarding appearances and bands, follow Julie's instructions on disposing of the material in the tubes and subsequent cleaning.

Part 2: Today's Exercises:

Exercise 1: Group Projects, continued:

See project packets.

Lab Session 18

Background

None. See project packets for relevant background materials.

Procedures

Part 1: Follow Up from Session 17:

Group Projects:

Gather data from last session.

Part 2: Today's Exercises:

Exercise 1: Group Projects, the Thrilling Conclusion:

See group packets

APPENDIX: QUANTITATIVE DILUTIONS

Dilutions are expressed as a ratio, such as 1:2 or 1:10. This is the ratio of the **initial volume (V_i)** of the sample to the **final volume (V_f)** of the sample. The "sample" may be an antibiotic (as in an MIC determination) or it may be a suspension of bacterial cells (as in a viable count determination), but it is usually transferred by pipette. The **volume transferred in the pipette defines V_i**, while **this volume plus the volume in the receiving tube defines V_f**.

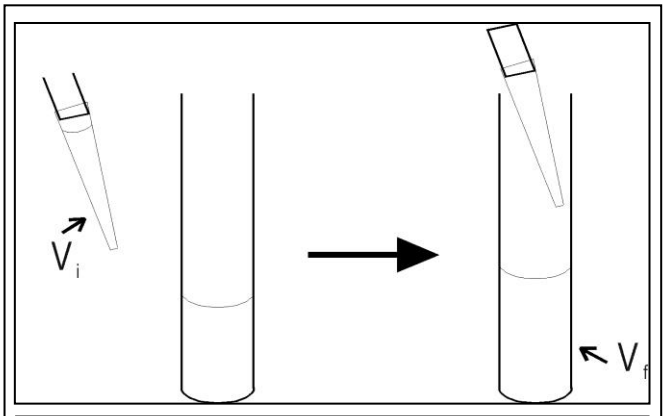


Figure A.1: **V_i vs. V_f**

So, a 1:2 dilution involves pipetting a volume of sample into an equal volume of diluent, (1:1+1) a 1:10 dilution involves pipetting a volume of sample into 9 volumes of diluent, (1:9+1) and a 1:100 dilution involves pipetting a volume of sample into 99 volumes of diluent (1:99+1).

all

and

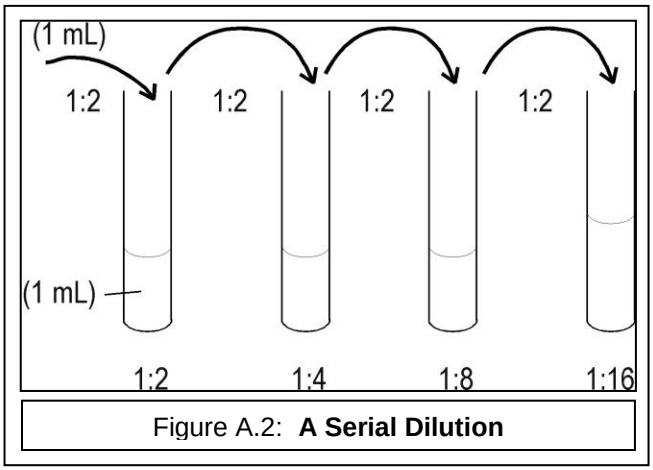


Figure A.2: **A Serial Dilution**

A number of dilutions can be strung together, and the overall dilution is the product of the steps in the series up to that point. Thus, in a serial 1:2 dilution with 4 tubes, the last tube represents a 1:16 dilution of the original sample, the third tube is a 1:8 dilution (See figure A.2).

Plate counts

Determining viable counts of bacteria or bacteriophage typically requires huge overall dilutions (factors of one million or greater). These are usually done in several steps of 1:100 and/or 1:10, and the overall dilution is usually expressed in scientific notation, such as 10^{-6}.

Three numbers are needed to calculate the viable titer (cell density) of a culture or phage suspension: the overall dilution that was plated, the volume that was plated, and the number of colonies or plaques that formed. Suppose, for example, that 0.1 mL of a 10^{-6} dilution was spread on a plate and yielded 250 colonies. The fact that 250 colonies formed from 0.1 mL means that the tube in question had a titer (or density or concentration) of 2,500 cells per mL (250/0.1). The fact that this tube was the 10^{-6} dilution means that its titer (or density or concentration) is, by definition, 10^{-6} the titer of the original sample. Therefore, the original sample contained 2.5×10^9 cells per mL ($2{,}500/10^{-6}$ or $2{,}500 \times 10^6$).