THE ART OF MICROBIOLOGY

A LABORATORY MANUAL

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NAME:

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Chapter 1
Introduction

AN AESTHETIC EXPLORATION OF MICROBIAL ECOLOGY

Much of the time when we talk about bacteria, we talk about how they behave in pure cultures, or in conspicuous single-organism infections. For instance, we might talk about what an *Escherichia coli* growth curve looks like, or what a patient's prognosis is during a *Clostridium tetani* infection. However, in the real world, bacteria live as members of communities comprised of other bacteria, viruses, archaea, eukaryotic microbes, plants, and animals. The behavior of organisms in complex communities is often very different than their behavior in simpler laboratory environments, or in "monocultures" like those experienced during active infections. This is because in communities, organisms respond to each other's presence and the effects that other species have on the environment.

MICROBIAL ECOLOGY

The study of microbes in their natural environments is called ***microbial ecology*** and is an important facet in understanding how microbes fit into our world. The activities of natural microbial communities control Earth's atmosphere and climate, the growth of crops and other plants, the productivity of the oceans, and can enhance or destroy the health of humans and other animals. **In this course, we will practice the basic skills of microbiology while working to make new discoveries in microbial ecology.** Working in teams of 2 or 3 classmates, you will isolate unknown bacteria from environmental samples and use a combination of molecular methods, biochemical tests, and *artistic visualization* to learn about the ecologies of your organisms.

Microbial ecology has a long history, and a number of influential early microbiologists such as Sergei Winogradsky and Martinus Beijerinck worked with complex environmental samples. However, in the century following the acceptance of the germ theory of disease (largely driven by the work of Robert Koch and Louis Pasteur), most microbiological research was done on pure cultures of organisms known to cause illness. This focus on pure culture was partly historical -- it just happened to be the way that famous scientists like Koch and Pasteur preferred to work -- but it was also technological. Prior to the 1990s there simply weren't many good ways to

understand what was going on in complex microbial communities. Winogradsky was able to detect certain *metabolisms* in mixed cultures -- for instance, by observing the production of methane or hydrogen gas -- but he was unable to determine which of the many microbes in his samples was responsible. Microbiologists knew that complex communities were responsible for many important environmental functions, and they suspected that the complex "natural flora" of the human body was important for human health, but they had very little success in understanding what these mixed groups were doing metabolically. Therefore, they focused on what they *could* study, which was pure cultures.

POLYMERASE CHAIN REACTION (PCR) AND THE MICROBIOME

This all changed in the 1990s, when the development of the ***polymerase chain reaction (PCR)***, DNA sequencing technology, and the unraveling of the structure of the ribosome yielded a new way to study microbes. Different species of microbes can be identified by the unique DNA "barcodes" contained in their ribosomal RNA gene sequences, and these barcodes can be detected in complex natural samples and used to determine "who's there" and in some cases also "what they are doing". The efficiency of DNA sequencing has improved exponentially since the adoption of **"next generation"** sequencing technologies in about 2006, and now scientists are able to routinely count millions of individual cells of all species in natural communities. The science of molecular microbial ecology remains in its infancy, but it has already revolutionized our understanding of the human ***microbiome*** and its role in both health and disease. It is certain that these new technologies will filter down into patient-level medical practice in the near future, and as a consequence tomorrow's medical professionals will have a need to understand ecological principles their predecessors were able to ignore.

PETRI DISH ART AND SCIENTIFIC INQUIRY

In this course, you will use a combination of pure culture and simple mixed-culture methods to learn something about the ecologies of bacteria that have been freshly isolated from natural samples. Based on their identification barcodes and a suite of experiments related to their metabolic and physiological abilities, you will construct a hypothesis about the ecological roles of your isolates, and then you will test this hypothesis using experiments of your own design. **Your reports, your data, and your isolates will be available for future research by other students and/or professional scientists.** Our goal here is to learn something both *new* and *interesting* about your samples, and your ultimate goal should be to

generate ***at least one dataset of professional, publishable quality***. There is a non-zero possibility that the work you generate in this course could lead to a professional publication, with you and your teammates as co-authors!

"The work you generate in this course could lead to professional publication."

In addition to standard pure-culture experiments, you will also be undertaking an unusual activity: **painting with bacteria**. Many bacteria produce exotic shapes and colors when growing in petri dishes, and you will use this fact to create living artworks based on your own personal designs. **These paintings are simple mixed-culture experiments** and you will use them to provide ecological depth to the observations you make in your pure-culture experiments. It's also like painting with invisible ink, so have fun, be creative, and worry about the science part later!

This course is going to be a lot different than other laboratory courses you've taken. There are no pre-planned demonstrations; everything you are going to do is new research. Nobody knows the answers; not you, not your TAs, not your professors. Some things will be easy, others will be frustrating and require you to troubleshoot and "think outside of the box" to figure out solutions. There's no way to get data from other people or "coast" through difficult problems -- ***you have to get results to get credit for this course.*** Sure, it sounds a little intimidating, but we're all here to help each other out, and at least it will be interesting!

One key to success (and low stress) in this course is to keep a firm eye on where each day's work falls in the "grand scheme" of the semester. The flow chart in **Figure 1.1** shows how the different chapters in this lab manual fit together. There are 5 worksheets that you will turn in: each of these will ask you to summarize and explain several experiments in a way that begins to tell a "story" about your microbes. Each worksheet will also give you some practice writing like a professional scientist. All of this will lead up to a final report that will be entirely based on your own work. You'll take the observations from the semester and synthesize them into a novel hypothesis about your microbes and how they interact with their environment and each other – and you'll actually do the experiments to test this hypothesis, possibly making discoveries entirely new to science.

The point of this course is to let you experience what it's like to do **"real science",** and to learn some microbiology in the process. Stay focused on the central goals -- developing real world skills and creating new knowledge about the natural world -- and try to enjoy yourself!

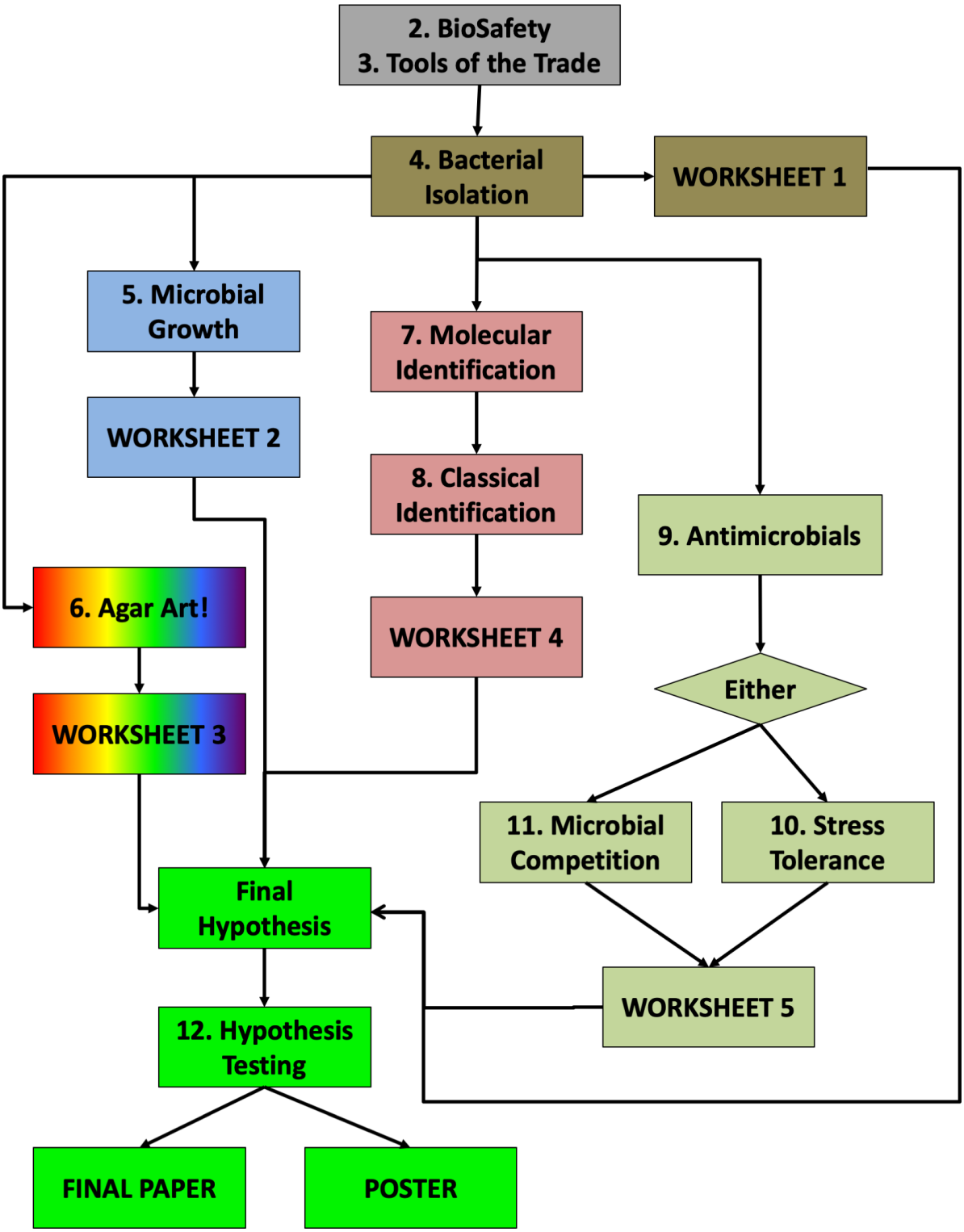

Figure 1.1. Outline for the reports and techniques you will be doing in the Biology of Microorganisms Laboratory class.

Chapter 2
BIOSAFETY

Guidelines for Biosafety in Teaching Laboratories

AMERICAN SOCIETY FOR MICROBIOLOGY

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Biosafety level 2 (BSL2) guidelines for teaching laboratories.

Preamble: Educators need to be aware of the risks inherent in using microorganisms in the laboratory and must use best practices to minimize the risk to themselves, students, and the community. The following guidelines are designed to encourage awareness of the risks, uniformity in best teaching practices, and safety of the students. These guidelines are not mandatory, but are designed to promote best practices in the teaching laboratory. Use of organisms that require BSL2 facilities is not recommended for typical K-12 settings unless these facilities are available. BSL2 is suitable for organisms that pose moderate individual risk and low community risk for infection. When good microbiological techniques are used, these organisms rarely cause serious disease, and effective treatment for laboratory-acquired infections is available. Best practices must be adopted to minimize the risk of laboratory-acquired infections and to train students in the proper handling of organisms that require BSL2 procedures. Students should always demonstrate proficiency in laboratory techniques using organisms that require BSL1 practices before being allowed to handle organisms that require BSL2 practices. The practices set forth in these guidelines fall into six major categories: personal protection, laboratory physical space, stock cultures, standard laboratory practices, training, and documents. For ease of use, the requirements and practices are brief. Explanatory notes, sample documents, and additional resources can be found in the appendix.

Personal Protection Requirements

- Wear safety goggles or safety glasses for normal laboratory procedures involving liquid cultures that do not generate a splash hazard (e.g., proper pipetting, spread plates, etc.). Use safety goggles and face shields or safety goggles and masks when performing procedures that may create a splash hazard. If work is performed in a biological safety cabinet, goggles and face shields/masks do not need to be worn.
- Wear closed-toe shoes that cover the top of the foot.
- Wear gloves when handling microorganisms or hazardous chemicals.
- Wear laboratory coats.

Laboratory Physical Space Requirements

- Require all laboratory space to include:
  - Nonporous floor, bench tops, chairs, and stools.
  - Sink for hand washing.
  - Eyewash station.
  - Lockable door to the room.
- Follow proper pest control practices.
- Keep the storage area for personal belongings separate from work area.
- Keep a working and validated autoclave in the building or arrange for licensed waste removal according to local, state, and federal regulations..
- Post biohazard signage
  - wherever cultures are used and stored.
  - on the door to the room.
  - on any containers used to transport cultures.
- *Recommended: Have a biological safety cabinet. The biological safety cabinet is required when large volumes of culture are used or when a procedure will create aerosols.*

Stock Culture Requirements

- Only use cultures from authorized, commercial, or reputable sources (e.g., an academic laboratory or state health department). Maintain documents about stock organisms, sources, and handling of stock cultures.
- Obtain fresh stock cultures of microorganisms annually (e.g., purchased, revived from frozen stock cultures, etc.) to be certain of the source culture, minimize spontaneous mutations, and reduce contamination.
- Keep stock cultures in a secure area.

Standard Laboratory Practices

- Wash hands after entering and before exiting the laboratory.
- Tie back long hair.
- Do not wear dangling jewelry.

- Disinfect bench before and after the laboratory session with a disinfectant known to kill the organisms handled.
- Use disinfectants according to manufacturer instructions.
- Do not bring food, gum, drinks (including water), or water bottles into the laboratory.
- Do not touch the face, apply cosmetics, adjust contact lenses, or bite nails.
- Do not handle personal items (cosmetics, cell phones, calculators, pens, pencils, etc.) while in the laboratory.
- Do not mouth pipette.
- Label all containers clearly.
- Keep door closed while the laboratory is in session. Laboratory director or instructor approves all personnel entering the laboratory.
- Minimize the use of sharps. Use needles and scalpels according to appropriate guidelines and precautions.
- Use proper transport vessels (test tube racks) for moving cultures in the laboratory and store vessels containing cultures in a leak-proof container when work with them is complete.
- Use leak-proof containers for storage and transport of infectious materials.
- Use microincinerators or disposable loops rather than Bunsen burners.
- Arrange for proper (safe) decontamination and disposal of contaminated material (e.g., in a properly maintained and validated autoclave) or arrange for licensed waste removal according to local, state, and federal regulations.
- Do not handle broken glass with fingers; use a dustpan and broom.
- Notify instructor of all spills or injuries.
- Document all injuries according to university or college policy.
- Keep note-taking and discussion practices separate from work with hazardous or infectious material.
- Use only institution-provided marking pens and writing instruments.
- Teach, practice, and enforce the proper wearing and use of gloves.
- Advise immune-compromised students (including those who are pregnant or may become pregnant) and students living with or caring for an immune-compromised individual to consult physicians to determine the appropriate level of participation in the laboratory.

Training Practices

- Be aware that student assistants may be employees of the institution and subject to OSHA, state, and/or institutional regulations.
- Conduct extensive initial training for instructors and student assistants to cover the safety hazards of each laboratory. The institution's biosafety officer or microbiologist in charge of the laboratories should conduct the training.
- Conduct training for instructors whenever a new procedural change is required.
- Conduct training for student assistants annually.
- Require students and instructors to handle microorganisms safely and responsibly.
- Require students to demonstrate competency at BSL1 before working in a BSL2 laboratory.
- Inform students of safety precautions relevant to each exercise before beginning the exercise.
- Emphasize to students the importance of reporting accidental spills and exposures.

Document Practices

- Require students to sign safety agreements explaining that they have been informed about safety precautions and the hazardous nature of the organisms they will handle throughout the course.
- Maintain student-signed safety agreements at the institution.
- Prepare, maintain, and post proper signage.
- Document all injuries and spills; follow university policy, if available.
- Make Material Safety Data Sheets (MSDS) available at all times; follow institutional documentation guidelines regarding number of copies, availability via print or electronic form, etc.
- Post emergency procedures and updated contact information in the laboratory.
- Maintain and make available (e.g., in a syllabus, in a laboratory manual, or online) to all students a list of all cultures (and their sources) used in the course.
- Keep a biosafety manual specific to the laboratory and/or course in the laboratory.
- Keep a copy of the current version of *Biosafety in Microbiological and Biomedical Laboratories* (*BMBL*) in the laboratory.

Chapter 3
TOOLS OF THE TRADE

Microbiologists use a number of specialized tools to do their work. Probably the first one you think about is the microscope -- but, perhaps surprisingly, it doesn't play a big role in day-to-day microbiology. We'll be using microscopes later on in this course, but in this exercise we will be learning about the tools we use to manipulate bacterial cultures. We will also learn perhaps the most important lesson of all -- how to perform **aseptic technique**, or how to work with bacteria without either contaminating your cultures or exposing yourself to the organisms.

First, lets look at the different tools.

a) ***Autoclave.*** *Most of the special techniques used by microbiologists involve trying to create pure cultures of microbes -- or at least cultures where the only microbes present are the ones we want to study. This is always a problem because there are microbes everywhere in the world, on every surface as well as floating around in the air. The first trick to working with pure cultures is to start with* ***sterile*** *conditions -- media (the stuff we grow bacteria in) and glassware where all the microbes have been killed, and that have been sealed up to prevent new bacteria from getting in. The primary way we sterilize things is with the autoclave, which is essentially a big pressure cooker. By using steam under pressure, the autoclave can kill every microbe -- including their resistant spores -- leaving media and glassware sterile.*

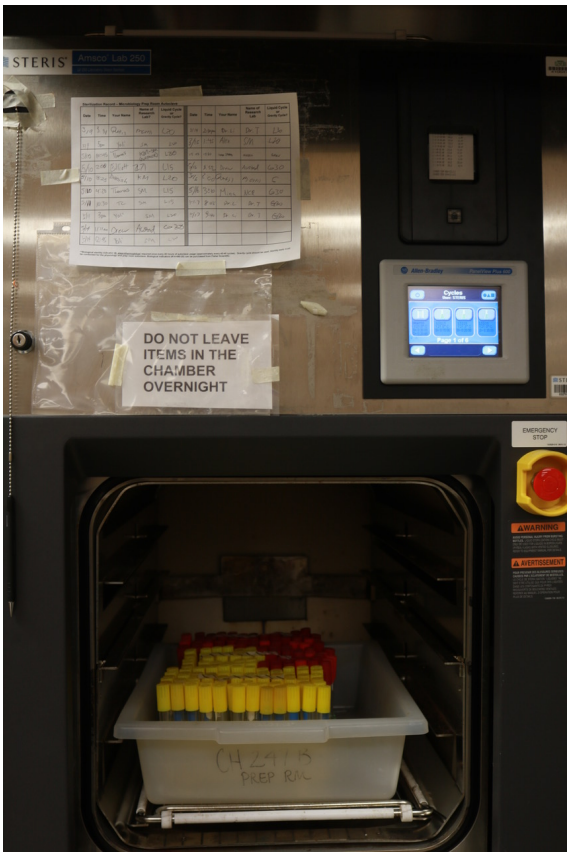


Figure 3.1 An Autoclave.

b) ***Broth culture media.*** *One of the two major types of bacterial growth medium is the* broth culture*. Broth media are basically water with various dissolved nutrients that bacteria can use. Broth media is usually autoclaved in 1L or larger bottles and then distributed to empty test tubes that were autoclaved separately.*

c) ***Agar plates.*** *The other major kind of medium is the agar plate (Fig 3.2). Plates and broth are pretty much the same, except that plates are solidified using* agar agar*, which is an extract from red algal seaweeds from the genus* Gelidium*. To make plates, you first mix up a batch of broth medium, and then add some amount of granulated agar (usually to 1.5% concentration). The agar doesn't dissolve until the medium goes through the autoclave, where the heat melts it. After autoclaving, the hot agar is poured into pre-sterilized plastic petri dishes, where it solidifies when it cools to about 40º C.*

d) ***Bunsen burner.*** *When working on an open lab bench, microbiologists use a Bunsen burner to stay clean (Fig. 3.2). It serves two purposes. First, you can use it to sterilize spreaders and loops (see below) by directly heating them. Second, it creates convective air currents that keep dust (and microbes) from settling on anything near the flame, giving you a "force field" around your work area.*

e) ***Inoculating loop.*** *This is a thin piece of wire filament on the end of a heat-shielded handle, twisted around into a small loop at the "business end" (Figure 3.2). The wire can be rapidly heated red-hot in the Bunsen burner flame, completely sterilizing it. It can then be used to collect and transfer bacteria from one culture to another. There are a number of similar tools, including* **inoculating needles** *(like a loop but without the loop) and sterilized wooden dowels or toothpicks.*

f) ***Cell spreader.*** *This bent glass rod -- often called a "hockey stick" because of its shape (Figure 3.2) -- is used to spread bacteria across the surface of an agar plate. It is first dipped in ethanol, which is then lit on fire in the Bunsen burner to kill off any microbes.*

g) ***Disinfectant.*** *Always keep a spray bottle of disinfectant around. You should wipe down your work bench before and after every work session, and you can also use the disinfectant to take care of small culture spills. Common disinfectants include ethanol and quaternary ammonium compounds.*

h) ***Micropipette.*** *This device (Figure 3.2) is used for moving small, very precise volumes of fluid around. Usually, they move "microliters" of fluid*

-- one microliter (μL) is 1/1,000,000 of a liter, or a 1/1000 of a milliliter, so 1000 μL = 1 mL. The micropipette uses disposable plastic tips that are autoclaved in sealed boxes, preventing cross-contamination between samples as well as contamination from the environment. You will use three types of micropipette in this class -- the P1000, P200, and P20. They differ in the range of volumes they can move.

i) ***Vortex mixer.*** *This device is used to rapidly and thoroughly mix the contents of a test tube. You just gently press the bottom of a test tub into it and it shakes it in tight circles, creating a spinning vortex that quickly mixes up the contents.*

OK, that's who the players are. Now let's see how they work.

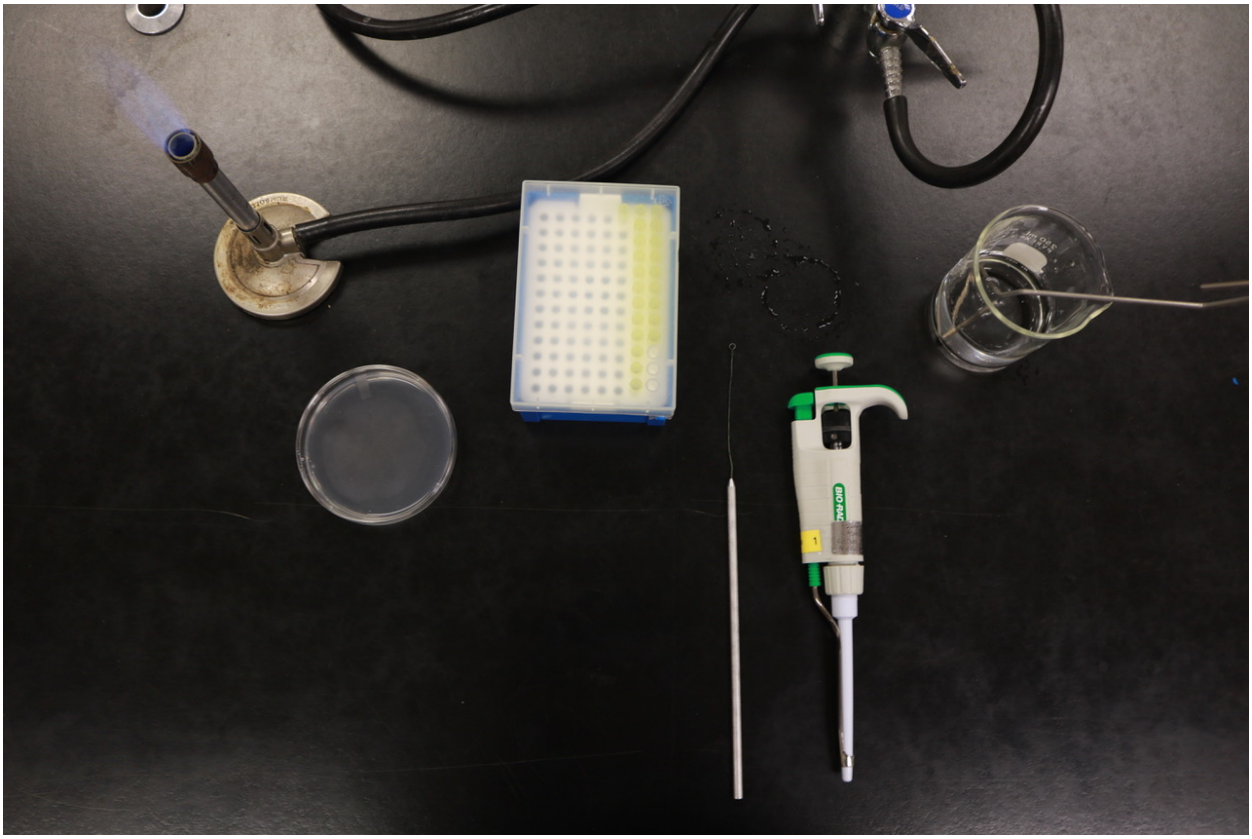


Figure 3.2. A typical workspace setup. From left to right: Bunsen burner, agar plate, box of micropipette tips, inoculating loop, P200 micropipette, and a beaker of ethanol containing a cell spreader.

Exercise A.

PIPETTING (WITH A SIDE OF STATISTICS)

The micropipette has four important parts. The sterile **disposable tip** is the only part that actually comes into contact with a sample. The **plunger** is how you make it pick stuff up into the tip and push it back out again -- push the plunger down to expel the contents of the tip, and release the plunger to pull stuff up into the tip. The **micrometer** shows you how many microliters the micropipette is set to pick up when you release the plunger; you can change the setting by turning the plunger. Finally, the **ejector** lets you get rid of a disposable tip when you're done with it.

Some things to keep in mind:

1. The plunger has two different down positions -- if you push it down, you can feel the first "stop" as a small amount of resistance just before the plunger is pushed all the way down. When you go to pick up sample, **only push the plunger down to that first "stop".** When you push out sample, **go down to the second "stop"** in order to get all of the sample out.
2. There are three different types of micropipette that have different ranges of volumes they can work with. On or near the plunger, there should be a label showing the range of volumes that particular micropipette can handle. DO NOT EVER TRY TO SET THE MICROMETER OUTSIDE OF THIS RANGE! For instance, if the label says "20-200 μL", never set the micrometer to less than 20 or more than 200. If you do, you can break the pipet, and they cost several hundred dollars to replace.
3. Micropipettes designed for smaller volumes often have micrometers that have resolution to the first or second decimal point. Values after the decimal point are usually shown in red numbers, or sometimes there is a black bar where the decimal point should be.
4. Don't push the plunger down too fast -- if you do you could "aerosolize" your sample, splashing droplets of it up into the air and potentially contaminating other samples or even your own body.
5. The vortex mixer is another major potential source of spills and aerosolized particles. Before using it, make sure that the cap of your test tube is securely fastened. Remember, if you make a spill, tell your TA immediately.

Basic micropipette procedure to move liquid from one solution to another:

1. Set the micrometer to the volume you intend to move.
2. Seat a fresh, sterile tip on the end of your pipette.
3. Push the plunger down **to the first stop**.
4. Insert the tip **just beneath the surface** of the fluid you want to move.
5. Slowly release the plunger. Keep your eye on the sample to make sure it is collected into the tip without any air bubbles.
6. Insert the tip **just above the surface** of the fluid you want to pipet into.
7. Slowly push the plunger down **to the second stop**. Keeping the plunger pushed down all the way, remove the tip from the fluid.
8. Eject the used tip into a waste container.
9. Use the vortex mixer to mix the contents of the tube you just added liquid to.

We will be using micropipettes A LOT in this course, so let's get comfortable with them early on. We will also be doing a good bit of statistical analysis of real data, which students often get really anxious about, so let's go ahead and get some of that out of the way early on as well. Here, you will practice using your micropipettes, and you will use a very basic statistical method called a **t-test** (Appendix 3) to see how good you are with the micropipette, and to try to determine if two solutions are actually different from each other. Check out this video for some useful pointers for this procedure:

https://www.youtube.com/watch?v=REViP2qinqQ&list=PLqTuWB3uliCzKB5aEaTePYq2ZL7E7tShu&index=2

YOUR TEAM NEEDS:

P1000, P200, and P20 micropipettes and tips
Vortex mixer
1 tube of 1% methylene blue
3 tubes of 10 mL water
2 tubes of "unknown solutions"
Mini-scale
1 weigh boat per team member
Dry erase board
Lab laptop computer

1. First, each team member should practice using a micropipette. One team member should transfer 15 μL of methylene blue solution into a tube of water using the P20 micropipette. Another team member should transfer 150 μL of methylene blue to another water tube with the P200, and the third member should place 750 μL of methylene blue into water with the P1000. Use the vortex mixer to blend each of the three tubes. Look at the four tubes and describe them to your teammates -- they represent a **dilution series** of the blue liquid. You will often make similar dilutions of bacterial cultures in this class.
2. Now, open Microsoft Excel on your laptop. One team member will pipet while another records data.
3. Place a weigh boat onto the scale and hit the "Tare" button.
4. Pipet 200 μL of unknown solution 1 into the weigh boat and record the mass.
5. Hit the tare button and repeat. Do this for 5 separate measurements.
6. Repeat for 5 measurements of unknown solution 2. Make sure to record the measurements of the different solutions in different columns.
7. Switch roles -- let the next team member do 5 replicate measurements of each solution. If your team has three members, make sure everyone does a set of measurements
8. Use the following video to help with the next three questions: https://www.youtube.com/watch?v=xCYO1u7G6vQ&list=PLqTuWB3uliCzKB5aEaTePYq2ZL7E7tShu&index=4. You will also find detailed explanations in **Appendix 2** and **Appendix 3**.
9. Use Excel to calculate the **means** and **95% confidence intervals** of your measurements for each solution (see Appendix 2). Compile all the measurements of a solution together, regardless of who did the pipetting.
10. Make a **bar graph with error bars** representing the data.
11. Write the "mean +/- 95% CI" for both samples on your dry erase board. Also on your board, write your guess as to whether your two unknown samples are the same or different.
12. Look at everybody's measurements. Which team has the most precise pipetters?
13. Now use Excel to conduct an **unpaired t-test** (see Appendix 3) on your two sets of measurements. Write the resulting **p-value** on your dry erase board and explain to the class what your conclusion is regarding your two samples.

Exercise B. ASEPTIC TECHNIQUE: Protecting your cultures from you, and you from your cultures

Aseptic technique is the art and science of keeping microbes where you want them. As a microbiologist, this is the most fundamental skill you have to develop. However, it's relevant to many other professions as well. For instance, as a health care professional, knowing how to avoid contaminating samples -- or worse yet, patients -- is probably a good thing. Here we will walk through the basics of setting up an aseptic work environment and performing four common culturing procedures: i) ***inoculating*** (putting bacteria into) a broth culture from a bacterial colony growing on an agar plate, ii) inoculating a broth culture from another broth culture, iii) streaking an agar plate to obtain isolated colonies, and iv) diluting a broth culture and spreading it onto an agar plate to obtain isolated colonies.

YOUR TEAM NEEDS:

Disinfectant
P20 and P200 micropipettes and tips
Inoculating loop
Cell spreader
Beaker of ethanol
Vortex mixer
Overnight broth culture of *Serratia marcescens*
Streaked BHI plate of *Serratia marcescens*
Per team member:
- 2 tubes of BHI broth
- 4 BHI plates
- 3 x 9.9 mL saline dilution blanks

MAKE SURE EACH TEAM MEMBER PRACTICES EACH OF THESE PROCEDURES!

DEMONSTRATION VIDEOS ARE AVAILABLE ON YOUTUBE FOR EACH PROCEDURE:

Streaking plates:

https://www.youtube.com/watch?v=xGGmCWHIl84&list=PLqTuWB3uliCzKB5aEaTePYq2ZL7E7tShu&index=2&t=1s

Inoculating broths:

https://www.youtube.com/watch?v=x65q24VSW-g&list=PLqTuWB3uliCzKB5aEaTePYq2ZL7E7tShu&index=3

i. SETTING UP THE WORKSPACE: THE BASICS (see Figure 3.2)

a. Squirt some disinfectant across your work surface and wipe it down with a paper towel.

b. Light your Bunsen burner. It should be on the side opposite your primary hand. So, if you are right-handed, your burner should be on the left side of your workspace. Note: the remaining instructions assume you are right-handed; if you are left-handed switch everything around.

c. Look at the flame -- imagine a magical bacteria-proof "force field" radiating out from it about a foot in every direction. Keep anything you want to be sterile within this force field.

d. If you have a paper protocol or a laptop you are working from, place it well to the right and back of your workspace. Make sure it is not close to the flame and not in a place where it will interfere with the movement of your hands.

e. Arrange whatever tools you will need -- micropipette, inoculating loop, cell spreader, etc. -- to the right of your workspace. Think about setting a place at a table -- these are your fork and spoon.

f. If you are using ethanol, put it on the right side of your workspace, behind the rest of your tools and well away from the flame.

g. If you are using a micropipette, make sure you've got a waste container for spent tips.

h. If you need them, put your box of pipet tips directly in front of you, just to the right of the flame. Never open the tip box unless it is close to the flame -- within its "force field".

i. If you need one, put your vortex mixer to your right. You don't want to have to reach over the flame to vortex a tube.

j. Put your "work" -- agar plates, test tube rack, or whatever -- directly in front of you. Put it as close to the flame as you can comfortably work. Never open a plate or a tube unless it is within the force field, and never leave it open any longer than you have to.

k. Do your work.

l. When you're done, turn off the flame and wipe down the work surface again with disinfectant.

ii. INOCULATING BROTH FROM A COLONY

a. Prepare a workspace with a loop and test tube rack as described above.

b. You will need an agar plate with the bacterium you want to inoculate. You can use any bacterial growth but it is best to start from an isolated **colony**, because (theoretically) a colony grew from a single isolated bacterial cell, so that you can be much more certain you're growing a pure culture.

c. Place the agar plate in front of the burner, to the left of the rack of tubes.

d. Loosen the cap of the test tube so that you can easily lift it off with one hand. Don't open it yet.

e. Hold your inoculating loop near the end of the handle and hold it at a 45º downward angle over the burner, with as much of the wire as possible in the flame. Hold it there until the loop is visibly red.

f. Remove the wire from the flame and count 10 seconds.

g. Moving quickly, lift up the lid of the petri dish with your left hand and lightly touch the loop to a single bacterial colony. YOU DON'T NEED MUCH MATERIAL so a light touch is sufficient.

h. Immediately after you have touched the colony with the loop, replace the lid of the petri dish. NEVER LEAVE THE PLATE OPEN even within the force field.

i. Grasp the cap of the tube between your middle and pointer fingers and lift it off. Grab the tube with your thumb and remaining fingers and pick it up.

j. CAREFULLY pass the mouth of the tube through the flame. Make sure not to set yourself on fire!

k. Hold the tube at a 45º angle facing toward your right side. It should be at about the same level as the flame and as close to the flame as you can comfortably get.

l. Insert the loop into the liquid, jiggle it a little bit, and remove it.

m. Pass the mouth of the tube through the flame again and replace it in the test tube rack. Put the cap back on as quickly as possible.

n. Flame the loop again. Place the tube into an appropriate incubator.

iii. INOCULATING A BROTH CULTURE FROM ANOTHER BROTH CULTURE

a. Prepare a workspace as described above with a test tube rack, a box of pipet tips, and a micropipette.

b. You will need two test tubes -- one with sterile broth and one with the "parent" culture you want to use to inoculate the sterile broth. Loosen both caps (but don't open either yet.

c. Set your micropipette to the volume you want to inoculate with. 100 μL is standard -- this is an approximately 100-fold dilution if you inoculate 10 mL of sterile broth.

d. Open the box of tips -- make sure it's accessible and inside the force field.

e. Flame the mouth of the parent culture tube as described above. Hold the tube at a 45º angle near the flame.

f. Being careful not to move the tube, seat a fresh tip onto your micropipette. Use the micropipette to collect the appropriate volume of the parent culture.

g. Remove the micropipette from the parent culture tube and hold it at a 45º downward angle inside the force field while you flame and re-cap the parent culture tube.

h. ALWAYS PAY ATTENTION TO WHAT YOUR OFF-HAND IS DOING! While you're flaming the tube, be aware of where the micropipette is -- while you're getting a fresh tip, be aware of where the test tube is.

i. Now open and flame the fresh tube, again being careful not to move the micropipette tip outside of the force field.

j. Holding the fresh tube at a 45º angle, insert the micropipette just beneath the level of the medium and inoculate the culture.

k. Flame and cap the fresh tube.

l. Eject the tip in to the waste container. Place the new tube into an appropriate incubator.

iv. STREAKING AN AGAR PLATE FOR ISOLATED COLONIES.

a. Set up a workspace as described above. You will need a loop, a fresh agar plate, and an agar plate or broth culture to inoculate from.

b. Using a sharpie, draw a capital "K" across the bottom of an agar plate. NOTE -- all writing should be done on the BOTTOM of a petri dish -- the part with the agar in it. It's possible to get lids switched up, but if the labels are on the bottom they will always be in the same place as the bacteria they represent.

c. Put the fresh plate directly in front of you and the parent culture to your left directly in front of the flame.

d. Flame your loop as described above.

e. If you are inoculating from an agar plate, collect some material from a colony as described above.

f. If you are inoculating from a broth culture, open and flame the tube, hold it at a 45º angle, and insert your loop just below the surface of the culture. Flame and cap the tube, and put it back in the rack.

g. Turn the fresh plate over. You should still be able to see the "K" through the agar -- turn it so the "K" is right-side-up.

h. Making sure it's in the force field, remove the lid from the fresh plate and place it to the left of the burner -- inside the force field -- face down.

i. Pick up the plate and hold it facing toward you near the flame. With your loop, "color in" or "streak" the box to the left of the "K".

j. Flame the loop and let it cool for 10 seconds.

k. Set the plate down and rotate it about 45º. Pick it back up and trace the loop lightly and ONLY ONCE through the first streak, to the right and into the box formed at the top of the "K". "Color in" this box as well.

l. Flame the loop again and repeat the last step, streaking from the top of the "K" into the box to the right of the K.

m. Flame the loop one more time and streak from the right of the K into the box at the bottom of the K.

n. Flame the loop once more and set it down. Put the lid back on the fresh agar plate.

o. **Invert** the plate by placing it lid-side down inside an appropriate incubator. Unless otherwise noted, agar plates should **always** be incubated inverted. This way, if condensation forms (as it often does), it will collect in the lid instead of on the surface of the agar where it would distort the growth of colonies.

v. SPREAD PLATING FOR ISOLATED COLONIES

This is perhaps the most challenging technique. Sometimes called "viable count plating", the goal is to take a dense broth culture of bacteria and dilute it until you reach a point where only a few cells per milliliter are left. Then, when you spread that dilute culture across an agar plate, only individual colonies form. If you count those colonies, as long as you know exactly how you diluted the original culture, you can back-calculate how dense the cells were in the original culture (see Appendix 1) -- or how many "Colony Forming Units" or "CFUs" were in every milliliter of the original culture.

In this exercise, we are going to perform a viable count on a dense overnight culture of *Serratia marcescens*. **As a rule of thumb, if a bacterial culture is so dense you can't see light through it anymore, it has somewhere between 10^8 and 10^{10} CFU/mL.**

a. Set up a workspace as described above with a P200 and a P20 micropipette, a box of tips for each, a cell spreader, a beaker of ethanol, a test tube rack with your parent culture and three 9.9 mL tubes of sterile saline ("dilution blanks"), and three fresh agar plates. Label the plates 1, 2, and 3.
b. Start with the rack of tubes directly in front of you. Position them left to right, with the parent tube on the left. Loosen all the caps. Position and open the box of P200 tips.
c. Using your micropipette as you would to inoculate a fresh broth culture (see above), transfer 100 μL from the parent culture into the first dilution blank. Close the blank well and vortex to mix.
d. Repeat, transferring 100 μL from the first blank into the second blank. Again, vortex.
e. Repeat one last time, transferring 100 μL from the second blank into the third. Vortex.
f. Move the rack of tubes to the left, in front of the burner. Place the agar plate labeled 3 in front of you. Exchange the P200 tips for P20 tips.
g. Use the P20 micropipette to collect 10 μL from the third dilution blank using proper aseptic technique.
h. Carefully lift the lid of plate #3. Lightly touch the pipet tip to the surface of the agar at an angle and slowly push the plunger down, leaving the 10 μL in the middle of the plate. DO NOT go down to the second plunger "stop", as this would disrupt the droplet of media on the agar surface.

i. Replace the petri dish lid and put down your micropipette. Insert the cell spreader into the beaker of ethanol and then place it at a 45º downward angle into the flame just long enough to ignite the ethanol.

j. Let the ethanol burn off and let the spreader cool for a count of ten.

k. Lift off the agar plate lid and set it aside, face down, inside the force field. Touch the cell spreader to the droplet of liquid in the center of the plate and slide the spreader back and forth. While doing this, use your left hand to rotate the plate. Try to cover the entire surface of the plate with the cell spreader.

l. Replace the petri dish lid and put the spreader back in the ethanol beaker.

m. Repeat steps h-l, but this time using 100 μL from the third dilution blank on plate #2.

n. Repeat steps h-l, using 10 μL from the second dilution blank on plate #1.

o. Invert the plates as described above and place them in the appropriate incubator.

p. After the plates have grown, have a look at them. At least one of them will have an undifferentiated mass of bacterial growth -- called a **lawn** -- which is not useful to us because it doesn't have individual colonies. Discard any such plates. It is possible that one plate will also have very few colonies; discard this one as well. Select only ONE plate of the three, ideally with between 50-300 colonies. Count these colonies by marking each with a sharpie while keeping count on a handheld "clicker".

q. Using the instructions in Appendix 1, determine the dilution factor of the plate you counted, and calculate the CFU/mL in the original culture. This is the **viable count** of that culture.

WASTE MANAGEMENT

Because we are working with potential hazardous microbes in this lab, it is important that we dispose of all waste materials appropriately.

1. *FIRST,* **nothing** *that comes into contact with microbes can leave the lab without being properly sanitized. This is why you are not allowed to use your phones or your own computers in lab, and why your lab coat and glasses have to stay in this room until they can be autoclaved at the end of the semester.*
2. *Any* disposable *plastic waste products that have touched microbes -- used petri dishes, plastic culture tubes, old tips, etc -- should be placed in one of the* ***biohazard bags*** *distributed around the lab. These will be sent off-site for incineration.*
3. *Any* reusable *glassware that has touched microbes must be autoclaved prior to re-use. Your TA will designate an area in each class session where these items should go.*
4. *Any* disposable *or* broken *glassware -- for instance, microscope slides -- must be sanitized and disposed of separately. Again, your TA will designate waste containers for this type of item.*

If you are in doubt about what to do with any waste item, ask your TA!

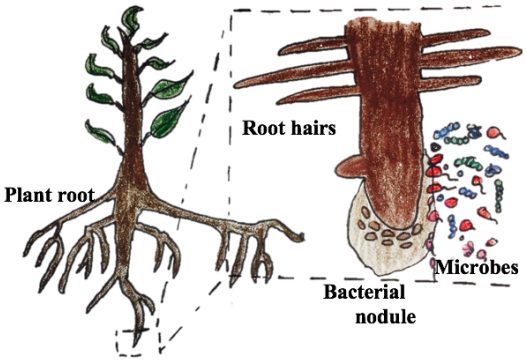
CHAPTER 4
BACTERIAL ISOLATION

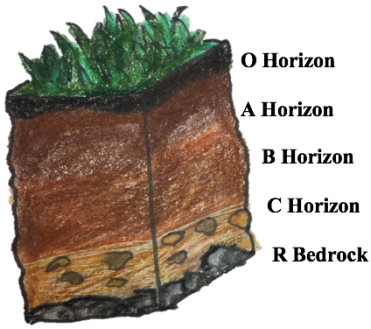
Part A. SOIL COLLECTION

UNEARTHING THE MICROBES IN YOUR COMMUNITY

You will learn how to sample microbial soil communities from dynamic ecosystems for isolation. You will begin to understand that microbes fill many different niches in different ecosystems.

Soil is the organic and inorganic material on Earth's surface. Microorganisms are abundant in soil -- in fact there are often more microbes in a teaspoon of soil than there are people on Earth. Sampling near a plant will include microbes that have important ecological interactions with plants, including both beneficial symbionts and pathogens. A sample taken from a small distance away from the base of the plant will likely have more diverse microbes because there are more different kinds of microbial interactions taking place near farther, younger roots. Leaf litter (plant matter that has yet to decay) is called the O horizon. Your sample will come from the layer below leaf litter where plants grow called the A horizon. The A horizon is surface soil that is rich with organic compounds. The layer below is the B horizon, which is full of accumulated minerals. Seated under these minerals is soil forming bedrock, or the C horizon. The C horizon helps create the hard bedrock below, or the R layer.





YOUR TEAM WILL NEED

- *Soil Collection Kit (sharpie, 1 Ziploc bag, gloves, 1 trowel)*
- *Temperature gun*
- *(Smartphone) camera*
- *pH probe*

DIRECTIONS

1. Find a sample site with your teammates.
2. **Draw a map** in your lab notebook showing where your sample site is. Also, **take a picture** of the site, making sure to show the plants growing nearby.
3. One partner will wear gloves and wipe away leaf litter. With a trowel, someone should dig into the soil site no more than 6 inches (about the length of your hand) down into the soil. Another will put a fist-sized sample of soil into the Ziploc bag. You should avoid surface litter, big rocks, and visible animals as much as possible. Label the bag with your names and the date.
4. Measure the temperature and pH at the direct center of your collection site with the temperature gun and pH probe.
5. Put all used materials aside and cover the area back with leaf litter. Take your gloves off and bring all materials with you back to the lab.

PART B. DILUTION AND SPREAD PLATING

Here, you will obtain pure cultures of environmental microbes in order to better understand their physiology and ecology. The first step in culturing microbes from environmental samples is to dilute the microbial soil communities in order to get isolated colonies (each of which arises from a single bacterium).

YOUR TEAM WILL NEED

- *Soil sample*
- *10mL sterile saline in 15mL Falcon Tube*
- *(3) 9.9mL saline DTs*
- *Sharpie*
- *10mL pipette and P200 pipette with tips*
- *(8) R2A plates and (8) BHI plates, both with cycloheximide*
- *Hockey stick spreader*
- *95% ethanol in a beaker*

DIRECTIONS:

1. Transfer your soil to a tube using the following methods:
   a. Label a 15mL Falcon tube with your team's name.
   b. Place approximately 1g (roughly the size of a Hershey kiss) of the bagged soil into the Falcon tube.
   c. Pipette sterile saline into the Falcon tube to the 10 mL mark.
   d. Vortex the Falcon tube at medium speed for one minute.

2. Wait for the soil to settle (this takes a few minutes).
3. Dilute your soil suspension using the following methods
   a. Label three 9.9mL saline blanks "DT 1", "DT 2", and "DT 3".
   b. Aseptically, transfer 100uL of soil suspension into DT 1 and mix by briefly vortexing.
   c. Aseptically, transfer 100uL from DT 1 into DT 2 and mix by briefly vortexing.
   d. Aseptically, transfer 100uL from DT 2 into DT 3 and vortex DT 3.
5. You should now have 3 finished DTs + your soil collection Falcon tube. Put your bagged soil in a dark area at room temperature.

At this stage you will use the spread plate method to grow your dilutions on agar media. You will use different media and different cultivation temperatures in order to isolate a wider variety of bacteria with a wider assortment of metabolic attributes. **R2A** is a relatively low-nutrient medium that contains yeast extract (amino acids, vitamins, coenzymes, growth factors, trace minerals), peptone (nitrogen, sulfur, carbon, energy), and has other organic compounds that help organisms with specific nutritional requirements grow (Casamino acids, glucose, pyruvate). **BHI** (brain-heart infusion) is a much simpler, high-nutrient medium that contains meat extracts. Both types of media also contain the antibiotic ***cycloheximide*** which kills fungi, greatly increasing the chances that you will isolate bacteria and not pesky eukaryotes.

SAFETY NOTICE: YOU ARE ALSO A EUKARYOTE, so cycloheximide is just as toxic to you as it is to soil fungi! Exercise caution when working with these plates, and always wear gloves.

1. Label the bottom (media side) of an R2A plate with "20 uL DT 1 RT" and your initials. "RT" stands for "room temperature"

2. Aseptically transfer 20 uL from DT 1 to the center of the R2A plate and spread using a flame-sterilized spreader.

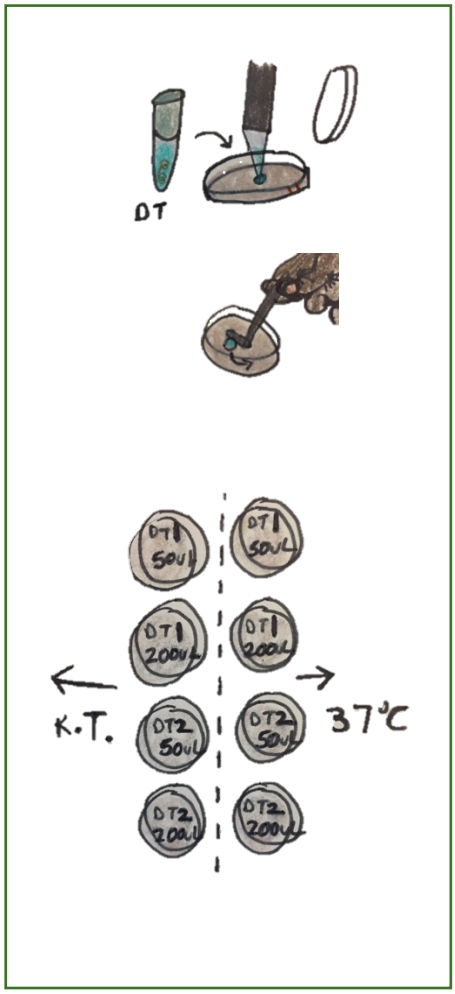
3. Invert the plate and set it aside.

4. Label the bottom of additional R2A plates with "200uL DT 1 RT", "20 μL DT 2 RT", and "200 μL DT 2 RT" and your initials. Repeat steps 2 through 6 for each of these plates, using the appropriate DT and plating volume for each.

5. Now repeat with the same volumes and DTs for 4 BHI plates.

6. Repeat these steps for the remaining R2A and nutrient agar plates, replacing "RT" with "37C".

7. Place all 8 RT plates inverted in the dark at room temperature. Place all 37C plates in the 37C incubator. Let them all incubate INVERTED (lid down) for at least 48 hours. Clean up your area as per your TA's instruction.



PART C. SOIL CHARACTERIZATION

At this stage you will also measure key chemical parameters of the soil environment. Organic matter and fertilizers are rich in Nitrogen, Phosphorus, and Potassium ("N-P-K") which are important for plant growth, and natural soils have widely varying levels of these key elements. Soil pH affects how easily surrounding plants take up nutrients from the soil and also has major effects on how microbes get energy and nutrients from their environment.

You will also quantify the moisture and organic matter content of the soil using a method called "Ash-Free Dry Mass". Here, you will weigh a soil sample while it is fresh, after it has been heated to 100C overnight to remove all water, and again after it has been "ashed" at 400C to oxidize all organic carbon to CO_2. Differing levels of water and organic matter content favor different kinds of microbial activity.

YOUR TEAM WILL NEED

- *Soil sample*
- *4 sterile saline dilution blanks*
- *N, P, K, and pH soil test reagent capsules*
- *N, P, K, and pH result color guides*
- *Foil packet*
- *Scale*
- *Sharpie*

DIRECTIONS:

1. For the NPK and pH samples, place approximately 1 g of soil into each of the 4 saline dilution blanks. Label the tubes "N", "P", "K", and "pH", and add the contents of the appropriate test capsule to each.
2. Vortex the tubes on medium-high speed for 30 seconds each. Place in a test tube rack and allow the soil to settle out.
3. Compare the color of the liquid after the soil has settled to the appropriate color guide and record your conclusions in your lab notebook.
4. For Ash-free Dry Mass, carefully weigh your **empty** foil packet and record this weight in your lab notebook.
5. Place about 10 g of soil into the packet (about the size of an Oreo cookie). Seal the packet and carefully weigh it again.
6. Label the packet by using the sharpie cap to make an indention in the foil with a number you will recognize later. Note: you can't use ink to label, because it will be destroyed by the ashing process!
7. Place the foil packet in the 100C drying oven overnight.

8. In the next lab session, re-weigh the packet and record the dry mass of the soil.
9. After lab, your TA will place your foil packet into a ***muffle furnace*** which will heat the soil to approximately 400 C for at least 3 hours. At this temperature, all organic matter is converted to CO_2 and "cooked out" of the sample, leaving behind only the inorganic (ash) portion of the soil.
10. In the next lab session, weigh the "ashed" packet and record this value in your notebook.
11. Calculate the percent moisture as dry mass divided by fresh mass.
12. Calculate the percent organic content as ashed mass divided by dry mass.

Part D. CLONAL ISOLATION

CHOOSING YOUR BACTERIAL FRIENDS

You will observe your plates to see their phenotypic diversity and observe patterns of microbial interaction. Based on your own aesthetic sensibilities, you will select isolates to work with for the remainder of the semester.

We often think of bacteria as extremely simple organisms, and in some ways they are. However, in other ways they are startlingly complex, and one of the ways this complexity manifests is in the way they grow in ***biofilms*** that become visible to the naked eye. Bacteria growing on agar surfaces can form many diverse colony morphologies; exhibit "helping" and "harming" interactions with other colonies; move around on the agar by swimming, sliding, or swarming; and make colorful pigments that serve a variety of purposes.

Here, you will pick isolates to work with for the remainder of the semester. You will make this selection based on your wild guess about which ones will be most fun to study. Look for organisms doing strange things on the plates, or that make funny shaped colonies. Definitely pick that funky pink thing that seems to have killed off everything around it. Or the green colony that might have been swimming away from the white cloudy patch. Anything that catches your eye probably did so for a reason.

Of particular interest here are bacteria that make pigments, since one of the activities we'll be doing is to make a painting with our isolates. Bacteria may create pigments for a variety of reasons, such as phototsynthesis, UV protection, antibiotic chemical warfare, storage of energy, stress resistance, virulence, and anti-freeze protection. Keep in mind that they cannot *see* their pigmentation (i.e., they cannot discern visual hues like our eyes can), and these pigments evolved long before anything on Earth had eyes -- meaning that the production of their pigmentation has evolved for non-visual purposes. Nevertheless, they look pretty neat to us, so if you see something brightly colored, by all means, pick it!

YOUR TEAM WILL NEED

- *(4) R2A or BHI plates*
- *4 x 5 mL tubes of R2A or BHI broth*
- *Dilution plates*
- *Camera*
- *Colored pencils*
- *Inoculating loop*
- *Parafilm*

DIRECTIONS

1. Observe your plates. First, identify plates where you can see isolated colonies. There should be at least one from each of your 4 sets of plates. In your notebook, sketch the overall plate, noting large-scale patterns where different kinds of growth interact with each other.
2. Next, as a team choose four particularly interesting colonies and mark their location on the back of the plate with a sharpie. Try to pick colonies without thinking too much about their "scientific" qualities -- just look for something weird, cool, pretty, or otherwise interesting to you.
3. Draw each of these four colonies in greater detail. Pay special attention to the edges of the colonies and their 3D textures. Make notes of the color and texture and label where each color came from (i.e. "*small goopy purple: DT 1 200*µL *BHI 37ºC*").
4. Now take photographs of the plates you sampled from. Make sure you can associate each photograph with the media type and temperature it was isolated from.
5. Seal the plates with parafilm and store them in the dark at room temperature alongside your soil sample.
6. Aseptically, use your inoculating loop to streak-isolate each of your chosen colonies onto fresh R2A plates (or BHI if that is what you isolated them from). Incubate your streak isolates at the same temperature that their original plate was incubated at (write "RT" or "37ºC" on the appropriate plates).
7. Also, use your loop to inoculate one tube of R2A/BHI broth for each organism. Incubate these at the appropriate temperature as well.
8. In the following class, look at your clonally isolated bacteria and **pick 2 that you like the most**. While you should prioritize "cool looking" microbes, make sure that the ones you pick grew well and appear to be "manageable" experimentally. Make sure to note whether or not your organisms grew well in the broth culture – some organisms don't like

Small goopy purple

DT 1 200uL BHI 37ºC

growing in liquid culture because they are adapted so specifically to biofilm conditions.

9. Name your two isolates and re-streak them onto fresh plates. Give them fun names that you will remember. **THESE ARE THE ISOLATES YOU WILL WORK WITH FOR THE REST OF THE SEMESTER**, so you're going to need to be friendly with them!
10. Parafilm all four of the streak isolation plates and store them with your soil plates and soil sample, just in case you need to go back to them later in the semester.
11. Your TA will **cryopreserve** your broth cultures by mixing them with glycerol (which prevents them from freezing solid) and placing them in a -80º C freezer. This will allow us to bring them back to life to do more experiments in the future if your work this semester discovers something new.

Part E. MAINTAINING YOUR ISOLATES

CARE AND FEEDING OF YOUR BACTERIAL FRIENDS

YOUR TEAM WILL NEED

- *(2) R2A/BHI plates*
- *2 x 5 mL tubes of R2A/BHI broth*
- *Inoculating loop*
- *P200 pipette and tips*
- *Parafilm*

DIRECTIONS

1. The following procedures need to be performed ONCE PER WEEK throughout the semester in order to maintain your cultures.
2. Inspect the previous week's broth and plate cultures of your isolates. Compare them to your notes about how they are supposed to look, and to the appearance of the previous week's cultures. If they look at all different, if you suspect contamination, or if they did not grow, tell your TA immediately.

3. Otherwise, streak each of your isolates for isolation on fresh R2A or BHI plates, and prepare new broth cultures by transferring 100 uL from the old culture into fresh broth.
4. Dispose of the old broth cultures.
5. Parafilm the old plates. Dispose of the previous week's streak plates and store the newer ones with your soil samples.
6. Incubate your cultures at the temperature they were isolated from. NOTE: after performing the Reaction Norms experiments later, you may discover that your organisms actually "prefer" a different temperature or medium than the one from which they were originally obtained. Always keep them maintained in their OPTIMUM environment, regardless of how they were isolated.

Note: in the subsequent experiments, team supply lists often mention "R2A/BHI" plates or broth. Experiments should be performed in the medium the organism grows most reliably in. Instructors should keep a tally of how many of each kind they need on a weekly basis and let laboratory prep staff know.

LAB WORKSHEET #1
"Isolation"

Your first experiments found you isolating bacteria from soil and characterizing their natural habitat. Use the data and observations you collected to complete this worksheet.

1. Provide a photograph of the environment surrounding the spot from which your soil was sampled.

2. Describe the plant life nearby – is it mostly grass, bare soil, are their herbs or trees, is it tended or long and wild, is it actively growing or dormant for the winter, etc.

3. What are the environmental characteristics of the soil environment from which you isolated your organism? Give temperature, pH, NPK levels, moisture content, and organic carbon content. For soils, moisture contents < 10% are low/dry and >40% are high/wet; for organic matter, <5% is low/C-poor and >20% is high/C-rich.

4. Provide pictures of the agar plates from which you isolated your organisms. Circle the colonies that were isolated. Indicate what type of medium and what temperature were used for each.

5. Comment on the diversity of microbes you observed on each medium and at each incubation temperature. Use the following rules of thumb:

Low Diversity	90% of colonies look the same
Medium Diversity	One type of colony is 30%+ of the total
High Diversity	No colony type is more than ~10% of the total

   Do you think the medium/incubation conditions influenced the diversity of cultured microbes? Why or why not?

6. Think about the colonies you isolated. Did you see colonies with similar appearances on other plates? Were they only on one type of medium, or found at one of the temperatures? Or were they found on most of the plates? **State a hypothesis** about at least one of your isolates that seeks an explanation for the pattern you observed.

7. Assume that you are writing a paper that involves the work you did in Chapter 4. **Write the "Introduction" section for that paper.**

Chapter 5
MICROBIAL GROWTH

MEASURING THE LIVES OF BACTERIA

In this chapter, you will learn how to use a spectrophotometer to estimate cell density of bacterial cultures, how to use spread plates to directly count bacteria in a culture, and how to use a "standard curve" to relate one type of measurement to the other. You will also use the growth rates of colonies on agar in different environments to construct "reaction norms" that describe the "niches" that microbes are adapted to live within.

Bacteria and mammals have very different modes of reproduction. Humans, for instance, grow to adult size and then reproduce sporadically over several decades. Bacteria, on the other hand, continually grow in size and periodically split in half when they become large enough in a process called binary fission. If you were to follow the fate of a single individual human and a single individual bacterium, these differences would be very apparent. For example, the long maturation time for humans means that reproduction is staggered into discrete generations, whereas bacteria grow and divide at a steady rate. Despite these differences, though, if we "zoom out" and look at millions of individuals at once, we see that both bacteria and humans (and everything else that grows) have similar growth dynamics. When resources are plentiful, population size increases exponentially. We can see this by plotting the logarithm of population size vs. time, which is a straight line. When resources start to dwindle, growth rate tapers off, eventually stagnating when resources are exhausted.

When we grow microorganisms in **batch cultures**, we can see all of these processes occurring (Fig. 5.1). When a culture is first inoculated, there is no growth for a certain amount of time. This is because the organisms are **acclimating** to their new environment by synthesizing new proteins. This period of no growth is called **lag phase**. Eventually, the organisms start to divide by binary fission. When they first start to do this, nutrients are plentiful and cells are few, so each generation of fission depletes the nutrients by only a small amount. Thus, over this time, the **growth rate** is fairly constant and the density of cells increases exponentially with time. We call this **exponential phase** or sometimes **log phase** because a plot of the logarithm of cell density vs. time is a straight line during this phase (between points B and C in Fig 5.1).

As cells become more concentrated and nutrients start to dwindle, the growth rate decreases, leading to a tapering-off of growth rate and eventually to a cessation of growth. This starvation period is called **stationary phase** and can last for hours to weeks depending on the type of bacteria and the medium in which they grow. After some amount of time, the cells will start to die, leading to a **death phase** that is initially rapid but, for many species, slows down after some period of time. Following the death phase, some cells can remain alive for weeks, months, or even years, and during this time a succession of mutants with a Growth Advantage in Stationary Phase **(GASP)** phenotype periodically "bloom" and grow by cannibalizing the cells that perished during the death phase.

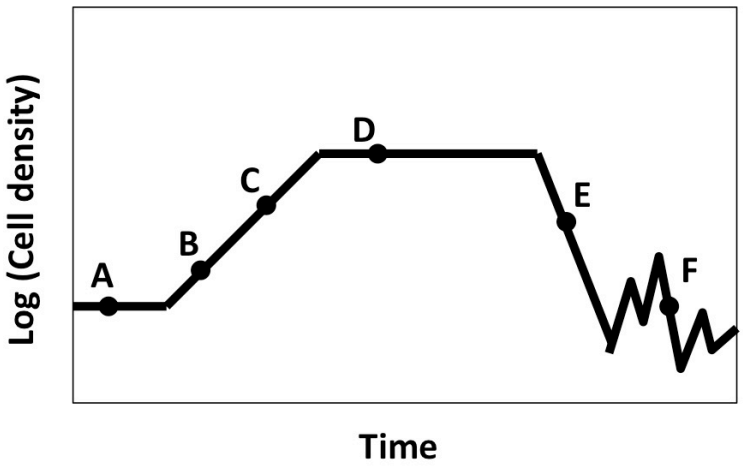


Figure 5.1. Example of a batch culture growth curve. ***A, lag phase; B-C, log phase; D, stationary phase; E, death phase; F, "GASP" phase***

There are several different ways to measure microbial growth. **Direct counts** actually look at individual cells and are usually the most accurate measures of growth. However, direct counts either require specialized, expensive equipment such as flow cytometers or time-consuming, laborious microscopic counts. Therefore we usually use **indirect counts**. The easiest type of indirect count uses the **optical density** or cloudiness of a culture to measure growth. The more bacteria there are in a liquid culture, the harder it is for light to get through the liquid without being absorbed by a bacterial cell, so we can use a **spectrophotometer** to measure the ability of a culture to absorb light to get a relative measure of growth. We can also use other methods, like the size of colonies on agar plates or the production of key, easily detected molecules such as chlorophyll.

A somewhat more involved method for counting cells is the **viable count** method, where we dilute and spread-plate a culture (similar to what we did with soil suspensions back in Chapter 4). By counting the colonies on the spread plate and back-calculating based on the **dilution factor** of the plate (see Appendix 1) we can determine how many living cells (or clusters of cells in some cases) were present in the undiluted culture. If we also took indirect measurements of the same cultures, we can produce a **standard curve** for predicting actual cell counts from optical density data (see Appendix 5).

EXPERIMENT A: GROWTH MEASUREMENTS AND STANDARD CURVES

YOUR TEAM WILL NEED

- *Spectrophotometer*
- *2 cuvettes*
- *Waste container*
- *R2A/BHI plates, 15*
- *9.9mL sterile saline dilution tubes, 30*
- *P200 pipetters and tips*
- *Sterile 1 mL disposable transfer pipets*
- *Overnight cultures of your isolates in 3 mL R2A broth*
- *3 mL R2A/BHI blank*
- *R2A/BHI dilution tubes (2 each): 4 mL, 5 mL, 6 mL, and 7 mL*
- *5 mL serological pipettes (x2)*
- *Pipet bulbs*
- *Clicker*
- *Sharpie*

DIRECTIONS (DAY 1):

1. Place 3 mL of sterile R2A/BHI broth into a cuvette. Record the optical density at 660 nm. This is your blank.
2. As described in the table to the right, prepare a series of dilutions of each of your unknowns in sterile broth. Tubes with the indicated amount of broth have been prepared for you. Vortex the overnight cultures before making these dilutions, and vortex the dilution tubes after preparing them.

Dilution	R2A	Culture
1/2	4 mL	4 mL
3/8	5 mL	3 mL
1/4	6 mL	2 mL
1/8	7 mL	1 mL

3. Using a clean 5 mL serological pipette, transfer 3 mL of the 1/8 dilution of one of your isolates to a cuvette using a transfer pipette. Wipe down the sides of the cuvette and measure optical density at 660 nm. Record this value, then pour the culture into the waste container.
4. Repeat with another 3 mL of the same dilution. This is a **technical replicate** which helps minimize the influence of measurement error on your standard curve.
5. Measure the remaining dilutions of that isolate, **moving to increasingly less dilute** cultures. Always record each dilution's optical density **using**

two technical replicates (i.e., don't just read the same cuvette twice, get two different 3 mL samples).
6. Dispose of the serological pipette in the biohazard waste.
7. Use a new clean serological pipette to repeat steps 3-6 with the second isolate.
8. Pipet 100 μL of the original overnight culture as well as each diluted culture (1/2 through 1/8, for each isolate) into a 9.9 mL dilution tube. Vortex, then transfer 100 μL of this dilution tube to another 9.9 mL dilution tube. Vortex, and repeat with a third tube. Vortex the last tube. This should result in 5 sets of 3 tubes for each isolate.
9. Pipet 50 μL of the 3rd dilution tube, 5 μL of the 2nd dilution tube, and 50 μL of the 2nd dilution tube onto separate agar plates (make sure the plates are properly labeled ahead of time). Spread with a flame-sterilized cell spreader. Invert the plates and incubate at whatever temperature you normally incubate your isolates until the next class period. This should result in 15 plates.

DIRECTIONS (DAY 2):

1. Take your viable count plates from day 1. For each time point, only one or two of the three will be countable, while the others will either be empty or covered in an uncountable mess of colonies.
2. Count the colonies on the *best plate only* (i.e., one with between 20-300 clearly separated colonies). Use a sharpie to mark each colony as you count it, and use a tally count "clicker" to keep up with the count. When you are done, write the number of colonies you counted on the plate.
3. Repeat with the "best" plate for each dilution of each isolate. Record this count, and the dilution factor of the plate (see Appendix 1 for how to calculate this value), in your lab notebook next to the measurement of OD from the culture the plate was made from.
4. Compute the viable concentration of bacteria in CFU/mL by dividing the colony count by the dilution factor for each plate and record this value in your lab notebook, again next to the original OD measurement and colony count.
5. Make a plot in Excel of CFU/mL (Y-axis) vs. Optical Density (X-axis). There should be a set of dilutions where this plot is a straight line -- use ONLY THESE POINTS in step 7.
6. Use **linear regression** (Appendix 5) of CFU/mL (Y-axis) vs. Optical Density (X-axis) for the points that are in a straight line in your plot to create your **standard curve**. **Write the slope and intercept of this regression down in your lab notebook where you will be able to find it later** -- this will let you calculate CFU/mL based on a quick OD measurement for future experiments.

EXPERIMENT B. REACTION NORMS

You will test your environmental isolates to try to estimate their response curves to the key environmental parameters *temperature*, *pH*, **and** *salinity*. **Additionally, you will determine whether or not they are able to grow in the absence of oxygen.**

Microbes inhabit virtually every surface on earth. They can be found in brine channels between ice crystals at the South Pole, growing (slowly) at temperatures below -20º C in liquid 10 times saltier than the ocean and with a pH as low as battery acid. Other microbes can be found at hydrothermal vents, growing happily at temperatures greater than 120º C under millions of pounds per square inch of pressure, eating methane and breathing iron. Some microbes live deep beneath the earth, using radioactive decay as a source of energy in isolated communities, and others have survived months of exposure to the vacuum and radiation of outer space. Microbes also flourish in between these extremes as well.

A variety of adaptations are available to microbes (and other types of organisms) that allow growth in different environments. Some, like elevated GC content in DNA as a response to high temperature or compatible solute synthesis as a response to high salt, can fine-tune the cell to a particular type of environmental condition and help to define the ***optimum*** condition for the organism, or the environment where they are "happiest". Other adaptations increase stress tolerance, thus helping organisms tolerate a wider ***range*** of conditions. Together, these adaptations determine an organism's ***reaction norm*** to changes in a given environmental parameter (Fig. 5.2). The combination of reaction norms to many different environmental parameters is a major determinant of an organism's ***fundamental niche***, or the range of environments where an organism is capable of growing. In practice, however, there are many combinations of parameters where any given organism is *capable* of growth, but does so at such low growth rates that it will be outcompeted by better adapted organisms, limiting its ***realized niche***.

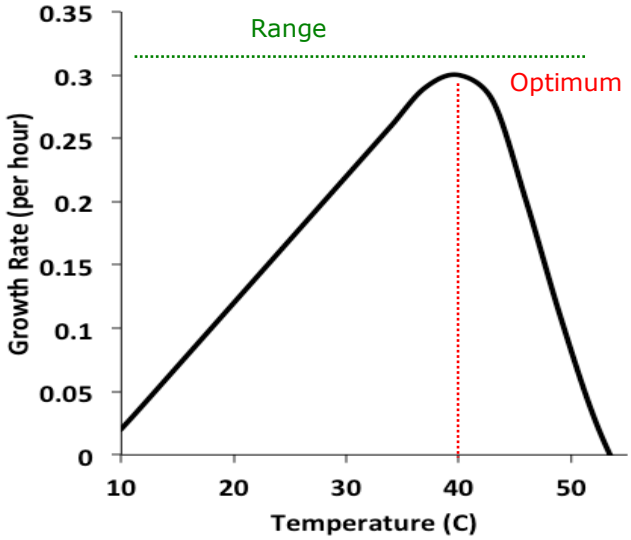


Figure 5.2. Reaction norm of an organism to changes in temperature.

Additionally, you will be using an anaerobe jar (Fig. 5.3) to see how your isolates respond to changes in oxygen levels. This device has an airtight seal, allowing you to manipulate the internal atmosphere. In one jar, you will include a packet containing a palladium catalyst that, when activated, will react with atmospheric oxygen to convert it into water, resulting in an anaerobic environment inside the jar. In another jar, you will place a small candle that will burn until the oxygen is very low, producing a **microaerophilic** environment.

Additionally, you will put an indicator strip into the anaerobic jar that changes color based on the presence or absence of oxygen, which will let us know whether or not the packet was effective in eliminating oxygen from the jar.

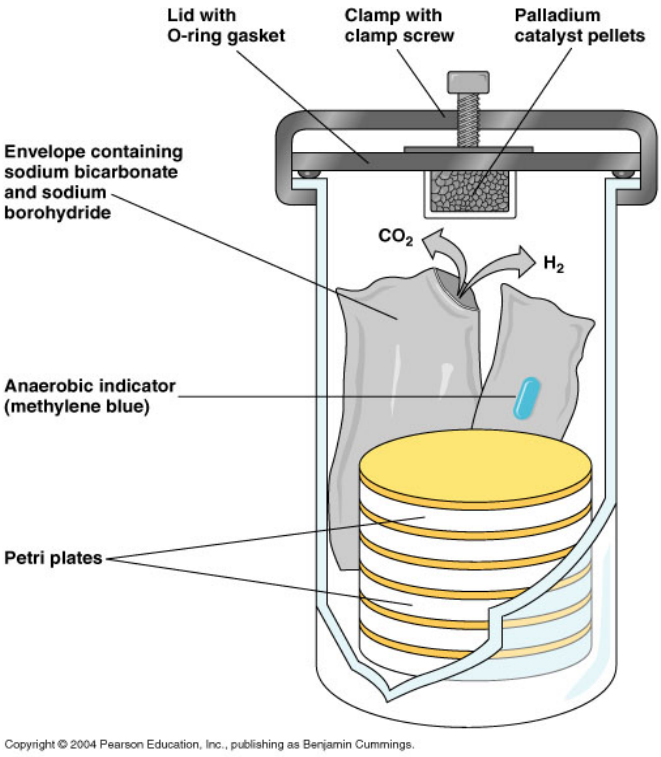


Figure 5.3. A GasPak Anaerobe Jar system. The packet releases H_2 gas into the airtight jar, which reacts with O_2 in the presence of a palladium catalyst to yield water, producing an anaerobic environment.

YOUR TEAM WILL NEED

- *Unknown isolates growing on R2A or BHI plates*
- *2 plates each of R2A/BHI set to pH 3, pH 5, pH 7, and pH 9*
- *2 R2A/BHI plates each at 0.5%, 3.5%, 10%, or 15% NaCl concentration*
- *20 R2A/BHI plates*
- *2 1/10 R2A plates*
- *2 R2A/BHI plates (whatever the opposite is of what is usually used)*
- *2 Ziploc bags and parafilm*
- *Digital calipers*
- *Anaerobe jar (x2)*
- *GasPak*
- *Tealight candle*

DIRECTIONS (DAY 1):

1. Label each pH plate with its pH value and one of your isolates' names.
2. Label each NaCl plate with its NaCl concentration and one of your isolates' names.
3. Label 2 R2A/BHI plates each either 4C, 15C, RT, 30C, 37C, 42C, or 55C and one of your isolates' names.
4. Label 2 R2A/BHI plates "Anaerobic" along with your isolates' names. Label 2 other plates "Microaerophilic" along with your isolates' names.
5. If you normally use R2A, label 2 BHI plates "High nutrient". If you normally use BHI, label two R2A plates "Med. Nutrient". Label 2 1/10 R2A plates "Low Nutrient".
6. If you haven't already re-streaked your isolates this week, do it now, and **work from the old plate** today.
7. Streak each plate for isolated colonies using the appropriate organism. Be very careful not to streak with too hot of a loop.
8. Incubate pH and salt plates at whatever temperature you normally incubate your unknowns.
9. Place the anaerobe plates and microaerophilic plates in separate anaerobe jars that will be incubated at an appropriate temperature. Your TA will prepare the GasPak and candle for you.
10. Incubate temperature plates in the appropriate incubator. Plates to be incubated at 42C or 55C must be wrapped in parafilm and sealed in Ziploc bags to avoid drying out.

DIRECTIONS (DAYS 2-4):

11. During the following lab session, check the plates. Separate plates with visible growth from those without, and return the plates without growth to the appropriate incubator. Make sure that high temperature plates go back in their Ziploc bags!
12. If you see evidence of contamination, or if a plate in the middle of a growth series didn't grow (e.g., 15C and 30C grew but RT didn't), restreak a new plate to replace the bad one(s).
13. For the plates with detectable growth, use your digital calipers to measure the diameter of 10 colonies. Record these values in your notebook.
14. Make notes of any phenotypic differences you observe and then dispose of the plates.
15. Repeat 10-13 in lab periods 3 and 4 for a total of 2 weeks of observations. If no growth is observed on the 4th day of observation, record a value of "0" for the plate.

ANALYSIS:

1. Transcribe your data into Excel in a spreadsheet with five columns: "Organism", "Condition", "Days", "Diameter", and "Growth Rate".
   a. Each row in the spreadsheet will record ONE SINGLE COLONY MEASUREMENT. You will have very many rows.
   b. "Organism" is the name of the organism whose colony you measured.
   c. You should have the following conditions: Temperature (4C, 15C, 23C, 30C, 37C, 42C, 55C), pH (3, 5, 7, 9), Salt (0, 0.5, 5, 10, 15), Oxygen (high, low, none), and Nutrients (low, medium, high).
   d. "Days" is the number of days between when you streaked the plate and when you measured the colony. So if you streaked on Monday and measured the following Monday, put "7" in the "Days" column.
   e. "Diameter" is the measurement of ONE SINGLE COLONY. Thus, you should have 10 rows for each condition.
   f. Note that some different conditions are filled in using the same set of measurements. For instance, if you normally incubate your organism on BHI agar at 37C, then your "0 Salt" plate is the same as your 37C plate. Make sure to fill in data for all conditions!
2. Calculate the growth rate in the last column as "Diameter" divided by "Days". The units for this growth rate are "mm per day". Importantly, this is different from the **exponential growth rate** you will learn to calculate in lecture, but it is a useful, experimentally viable measurement for us to use here.
3. Calculate the **MEAN** and **95% Confidence Interval** for each organism under each condition (see Appendix 2).
4. Use your calculations to **plot reaction norms** for pH, salinity, and temperature for each of your unknowns as bar graphs with error bars showing the 95% Confidence Interval.
5. For each environmental condition (pH, salinity, temperature, oxygen, nutrient), **predict the optimal condition and range** and classify the organism appropriately (e.g. thermophile, neutrophile, halophile, facultative anaerobe, oligotroph/copiotroph, etc).

LAB WORKSHEET #2
"Adaptation"

Using the results of these experiments, compare the environmental conditions of the soil from which you isolated your organisms to their actual reaction norms to predict if each is well-adapted to its environment. Use the data you have collected to complete this worksheet.

1. Provide reaction norms for temperature, pH, salinity, nutrient concentration, and O2 availability for each of your isolates. Do this by plotting your calculate growth rates for each environmental series as bar graphs with error bars representing the 95% confidence intervals. For each parameter, indicate the **optimum** and **range**. Make sure to include all of the following conditions:
    a. Temperature: 4C, 15C, 23C (RT), 30C, 37C, 42C, 55C
    b. pH: 3, 5, 7, 9
    c. Salinity: 0%, 0.5%, 3.5%, 10%, 15%
    d. Nutrients: Low (1/10 R2A), Medium (R2A), High (BHI)
    e. O_2: None (anaerobe jar), Low (candle jar), High (atmospheric growth)

2. Use one or more of the following terms to describe each of your isolates: psychrophile, mesophile, thermophile, hyperacidophile, acidophile, neutrophile, alkaliphile, halophile, extreme halophile, oligotroph, copiotroph, obligate anaerobe, facultative anaerobe, microaerophile, aerobe

3. Compare your isolates' optimum environment to the actual environment. If they are different, estimate how much slower your organism would grow in the soil than in its optimum environment.

4. At the actual conditions of their environment, do either of your isolates have significantly different growth rates? (use one or more **unpaired t-tests** to answer this question)

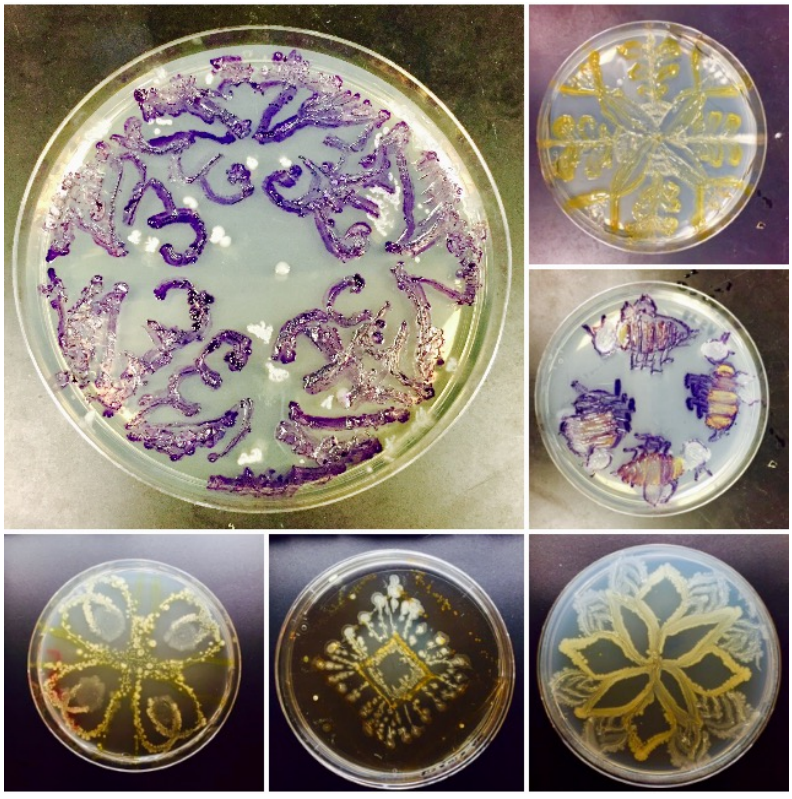
5. Are your isolates well-adapted to their environment? If not, why do you think their optimal environment is different than the one in which you found them?

6. **State a hypothesis about the ecology of your two isolates.** They were both found growing in the same small amount of soil. What prevents one of them outcompeting the other and driving it to extinction?

7. Assume that you are writing a manuscript that uses these experiments. **Write the "Results" section of that manuscript.**

Chapter 6
AGAR ART

Since at least the early 20th century, microbiologists have enjoyed the unusual pastime of "painting" with bacteria. Alexander Fleming, the discoverer of penicillin, was also an accomplished microbial artist, and it's plausible that his artistic efforts led to his fortuitous discovery. Later, Selman Waksman used art-like "streak plates" to systematically search for antibiotic-producing soil bacteria, leading to the discovery of streptomycin and other drugs (you will try these in a subsequent exercise). For the past few years, the American Society for Microbiology has sponsored an international Agar Art competition, and every year the entries become more numerous and more ambitious.



Here, you'll follow in these illustrious footsteps to create your own microbial masterpiece. On the one hand, you should have fun and try to make something personal and striking that you can show off to your friends and family. On the other hand, you'll get to see your isolates interacting with each other, and with other pigmented bacteria, in unique, unplanned settings, and you might learn something about their ecological relationships in the process.

PART A. TEMPLATE DESIGN

CREATING A PERSONALIZED DESIGN

The internet is exploding with colorful works of agar art from the ASM Agar Art contest, many of which began with simple sketches. We have made sketches very similar to what some of the ASM Agar Art winners would have used for their winning designs.

We know that agar art calls upon contemporary tools, but due to the circular constraints, also pays homage to Mandalas. Mandala means "circle" in Sanskrit, an ancient language in India. People across African, Aztec, Chinese, Indian, Japanese, Tibetan and other cultures use mandalas for meditation and expression, using radial symmetry to balance the artwork and represent their place in the universe. Mandalas (top left) may be an option for you if you want to play around with overlapping shapes rather than a figurative image.

YOU WILL NEED

- *1 blank template; Glue; Scissors; Pencil, Colored pencils; 1 empty petri dish*

DIRECTIONS

1. Design images on your blank templates. ***Ensure there are places in your design where lines cross, because those are the places where microbial interactions will occur*.** Fill up one sheet with different designs (up to six designs).
2. Cut out your one favorite circle design. Either take a photo of it or scan it.
3. Take the lid of an empty petri dish and place the lid so its surface area is on the table. Glue/Tape your template on the inside of a blank empty petri dish lid, facing upwards. This lid with template inside can now be used again and again!

PART B. AGAR ART

CREATING A MICROBIAL MASTERPIECE

You will observe your plates to see phenotypic variation based on the abiotic (temperature/media) and biotic (other organisms) environment. You will then combine the techniques of plating bacteria with the art of drawing. Painting with the bacteria or "invisible ink" will show you the role materials have in guiding artistic processes.

Here you will use your named isolates as well as 2 previously cultured colored microbes to translate your mandala into an actual living painting. You're going to replicate the same painting, with the same microbes in the same patterns, on 3 different petri dishes. You have a choice: either use 3 different types of media at the same temperature, or the same type of medium (R2A) at 3 different temperatures. Use your observations from your original isolation plates to decide which environmental changes make the biggest differences in the phenotypes you think are most interesting.

The 3 media choices differ from each other in the amount of nutrition they provide. As we've discussed earlier R2A is a relatively low-nutrient medium. It is particularly useful for growing environmental bacteria because they are used to low nutrient concentrations in their native environment, and can suffer oxidative stress due to imbalanced metabolism when transferred to rich media. R2A also contains pyruvate, which eliminates hydrogen peroxide from the medium and provides additional protection from oxidative stress. "Brain-Heart Infusion" or BHI medium is an even higher nutrient medium, known to maximize growth rates and biomass yields for many types of bacteria, but can be difficult for slower growing environmental bacteria to tolerate. PLAG medium is a **defined medium** that contains only inorganic salts and four commonly-used carbon substrates -- pyruvate, lactate, acetate, and glycerol. It is the least nutritious of the three options.

If you choose temperature, you can opt for 4º C (a refrigerator), 15º C (similar to the native soil temperature, probably), room temperature (about 23º C), 30º C (a warm summer day), or 37º C (human body temperature). Microbes tend to grow faster at higher temperatures, but at some point the temperature starts to stress them out and will eventually kill them. Stress also happens at low temperature, but generally it is a gradual effect, whereas at high temperature cells often go quickly from "happily growing" to "stone dead" over a few degrees.

So, both medium composition and temperature are capable of increasing growth rate, but also causing stress. Both of these conditions are likely to influence the phenotypes and interactions of the organisms you use to make your artwork, and will help you gain insights into "who your isolates are".

EACH STUDENT WILL NEED

- *Streak isolation plates for both isolates*
- *Additional colored bacteria*
- *Template dish*
- *Glue*
- *Camera*
- *Pencil, Colored pencils*
- *Sterile wooden sticks*
- *Parafilm and scissors*
- *EITHER:*
    - *1 each R2A, PLAG, and BHI plate, or*
    - *3 R2A plates*

DIRECTIONS

1. First, re-streak your isolates onto fresh agar plates and return the new plates to the incubator.
2. Take a couple of minutes to look at your classmates' isolates and show them yours. Get to know their names, their colors and shapes, and any weird quirks they exhibit.
3. You will create art using BOTH of your team's isolates. Also, you will select two other isolates to use, choosing either from the pigmented bacteria provided by your TA or from other isolates obtained by your classmates (make sure to ask first!)
4. Fit your first media plate snuggly into your template lid on the bottom (keep the media lid on). Draw a "registration mark" on your template and on the media plate to account for any wiggle.
5. You used four different colors in your mandala templates. To make your art, each color in your template must correspond to one of your bacterial isolates. The color in the mandala doesn't need to resemble the color of the bacteria. Assign a color to each bacterium and write it down in your notebook so you don't forget. Also number the isolates 1-4.
6. Aseptically, take the top empty lid off. Dab a colony from Isolate 1 with a stick and carefully trace all the respective colored lines in your mandala onto your agar, starting in the upper left corner. Make sure the registration mark on your plate and the template plate stay lined up.
7. Throw away the stick. Use a new stick to apply Isolate 2. Make sure to NEVER touch the original isolation plate with a stick after the stick has touched your artwork -- always use a fresh stick.
8. Continue with Isolates 3 and 4 until all lines have been traced.
9. To make sure all the lines get covered, rotate your plate 180º and repeat steps 6-8.

10. Repeat steps 4-9 with your other 2 art plates.
11. Seal the art plates with Parafilm and place them INVERTED in the appropriate temperature condition.
12. After 1 week of incubation, photograph all of your plates.

NOTE: It's perfectly acceptable to experiment with other painting techniques. For instance, you could use an inoculating loop or needle instead of the wooden sticks; you could experiment with burning/melting the agar with a hot loop; or even carving into the agar using a sterilized instrument.

LAB WORKSHEET #3
"Interactions" (Chapter 6)

You have now become an agar artist. Use your observations of your artwork to answer the following questions.

1. Attach a picture of your template. Directly beneath it, attach pictures of your actual artwork. Also attach pictures of your teammates' art plates and, if any other teams used your isolates in their art, attach pictures of those plates as well. Indicate which medium and what temperature were used for each plate.

2. List three things that are different in your artwork than you expected.

3. List at least two things that are different between your two replicate paintings. Do you see similar differences when you compare your paintings to your teammate's paintings (or other teams' paintings) who used the same isolates? Are any of the differences associated with differences in growth conditions?

4. State a **hypothesis** about why your two paintings look different from each other, and why both look different than your intention. Try to take your findings from Worksheets 1 and 2 into consideration.

5. Assume you are writing a professional manuscript that focuses on this art project. **Write an Abstract** that includes your motivation to make the design you chose, the results you observed, and your conclusions/hypotheses about why your plates looked the way they did. **DO NOT EXCEED 200 WORDS.**

Chapter 7

MOLECULAR IDENTIFICATION

Part A. 16S PCR

AMPLIFICATION OF SMALL SUBUNIT RIBOSOMAL RNA GENE

Traditionally, microbes were identified based on observable phenotypes and physiological characteristics. For instance, gram-negative rods that can't grow on lactose were considered to be "a thing". In some cases, this was useful -- for instance, many important human pathogens such as *Shigella* and *Escherichia coli* are lactose-negative gram-negative rods. However, there are thousands of other lactose-negative gram-negative rods in nature that have no close relationship to *E. coli.* Indeed, when scientists first started trying to classify bacteria along the same lines as macroscopic organisms, they were prone to throwing up their hands in defeat because there seemed to be no rhyme or reason to the evolutionary relationships between bacteria. Carl Linnaeus, the Swedish biologist who invented the genus/species Latin binomial naming system used for all organisms today, lumped all microbes into a group called "Chaos" and essentially washed his hands of classifying them.

Fast-forward 2 centuries to the 1980's and the lab of Carl Woese at the University of Illinois Champaign. Woese studied the structure of the ribosome, and was one of the first to sequence the DNA that encoded ribosomal RNA. He discovered that there were parts of the sequence that were nearly identical across all organism he looked at, from bacteria to humans to oak trees, but in between these "highly conserved" regions other regions were quite variable. Woese proposed that you could determine evolutionary relationships between organisms based on the sequences of these variable regions, and he and his graduate students proceeded to re-classify the microbial world based on the sequence of the small subunit, or 16S, rRNA. Today 16S and other DNA sequences are the primary way that researchers classify microbes and understand the relationships between different groups.

In this lab, you will use the polymerase chain reaction (PCR) to amplify a portion of the 16S gene that is about 1100 base pairs long and sits in between 2 sequences that are almost universally conserved in bacteria. Those conserved regions are complementary to two "universal primers" which are short pieces of single-strand DNA you will add to your PCR mixture that will target the 16S gene for amplification. You will also add a "template" to your mixture, which is the DNA you

want to amplify. In this experiment, that template will come from a portion of a bacterial colony suspended in sterile water.

After the PCR runs, you will look at the product to make sure it worked right using **gel electrophoresis**. Once you have pure DNA, you will quantify how much DNA you have using **spectrophotometry**. DNA absorbs UV light at 260 nm, and you can estimate how much DNA you have in a sample by how strongly the sample absorbs light at that wavelength.

Finally, you will send your purified DNA, along with a small amount of primer, to the UAB DNA sequencing facility, where it will be sequenced using the Sanger method. Shortly after, we'll learn how to analyze that sequence data and use it to identify your microbial isolates.

YOUR TEAM WILL NEED

- *A PCR rack*
- *6 sterile PCR tubes*
- *1 tube of GoTaq Master Mix (232.5 μL)*
- *1 tube of Primers U341F and UA1406R (10 μM e., 15 μL)*
- *1 positive control tube of purified E. coli DNA (5 μL)*
- *1 negative control tube of sterile deionized water (5 μL)*
- *1 box each of P20 and P200 pipet tips*
- *A P20 and a P200 pipetter*
- *A sharpie*
- *Some sterile toothpicks*
- *Bucket of ice*
- *Plates with your unknowns, streaked for isolation*

DIRECTIONS

1. Keep your reagents on ice until you're ready to use them.
2. Put your PCR tubes in the PCR rack. Label them with a sharpie: 1, 2, 1C, 2C, pos, and neg.
3. Pipet 50 μL of sterile water into tubes 1C and 2C.
4. Using a sterile toothpick, aseptically collect some material from a colony of unknown isolate #1 and suspend it in the water in tube 1C by swirling the toothpick around in the water. Make sure to get enough material that the water looks somewhat cloudy. Repeat with unknown #2 in tube 2C.
5. Add 12.5 μL of primers to your GoTaq master mix. Mix by vortexing.
6. Add 49 μL of master mix + primers to each of the tubes 1, 2, pos, and neg.
7. Add 1 μl of tube 1C to tube 1. Repeat with tube 2C and tube 2.
8. Add 1 μL of E. coli DNA to tube "pos". This is your positive control; if it fails to amplify something is wrong with your reagent or your technique.
9. Add 1 μL of sterile water to tube "neg". This is your negative control; if it amplifies, you contaminated your master mix somehow.
10. Put your PCR rack on ice and tell your TA you're done.

11. When everyone finishes their tubes, they will place them all into a thermal cycler, which will subject them to the following program:
    a. 10 minutes at 95º C -- this lyses the resuspended cells.
    b. 30 seconds at 95º C -- this denatures the DNA, causing the strands to separate.
    c. 30 seconds at 50º C -- this temperature is low enough to allow the primers to anneal to the single-stranded DNA, but high enough that they can only bind to exact sequence matches.
    d. 90 seconds at 72º C -- this is the optimal temperature for Taq DNA polymerase, which means it makes new double-stranded DNA fastest at this temperature. During this step, DNA is elongated from the primers, amplifying the target V4 region.
    e. Go back to step b 34 more times -- steps b, c, and d are cycled 35 times, resulting (ideally) in an increase in copy number of the target region of 2^{35} (or 2.4×10^{10}) times.
12. The PCR will run overnight. Your TA will put your plate in the fridge tomorrow morning.

Part B. GEL ELECTROPHORESIS
SEPARATION OF MACROMOLECULES (DNA)

You will separate any DNA molecules in the PCR tube based on their size (in base pairs) using gel electrophoresis. Electrophoresis is the movement of charged particles suspended in a gel exposed to an electric current. Because larger molecules move slower through the gel, electrophoresis can be used to estimate the size of DNA. You will add a dye solution to your sample that will cause the DNA to become fluorescent, letting you see any bands by shining an ultraviolet light on the gel. If your PCR worked correctly, you will then purify the products to remove the PCR enzymes, unused primer DNA, and salts. To do this you will use a resin column that binds DNA but lets everything else pass through.

YOUR TEAM WILL NEED

- *Ice bucket*
- *PCR products from previous experiment*
- *Gel*
- *1X TAE Buffer*
- *Parafilm*
- *6X GelRed solution*
- *DNA ladder*
- *Electrophoresis chamber and power supply (at your bench)*

DIRECTIONS

1. Keep your reagents on ice until you're ready to use them. Place your gel into your electrophoresis chamber. The wells need to be closest to the black (negative) terminal since DNA will move toward the red (positive) terminal.
2. Add TAE buffer to the electrophoresis chamber until it is JUST above the level of the gel.
3. On a small piece of parafilm, pipet 5 x 2 uL "spots" of 6X GelRed solution. This is the fluorescent dye that will make your sample visible.
4. Carefully pipet 10 μL of DNA ladder onto one of the GelRed spots. ONLY GO DOWN TO THE FIRST STOP on the pipet plunger; pipet up and down to mix. Using the same pipet tip, move the mixture to the first well of your gel.
5. Repeat with each of your PCR products and your positive and negative controls, putting each product in a separate well. Use a different pipet tip for each product. MAKE SURE TO RECORD WHAT SAMPLE YOU PUT IN EACH WELL in your lab notebook!
6. Put the lid on the chamber and hook the electrodes up to your power supply. Make sure the black wire goes to the black electrode and the red wire goes to the red electrode!
7. Turn the power supply on. Set to "constant voltage" and dial in to 100 V. You should see bubbles rising through the buffer at the ends of the electrophoresis chamber.
8. You should see two colored bands moving across the gel, one yellow and one blue. The yellow band corresponds to a product of about 10 bp in size and the blue band to a product about 1000 bp in size (about the size of your product). Run the gel until the yellow band is about 3/4 of the way down the length of the gel (between 15 and 30 minutes)
9. When the gel has finished running, turn off the power supply, open the chamber, and take out the gel. Place the gel on the transilluminator to visualize the bands. MAKE SURE TO PROTECT YOUR EYES.
10. **Take a picture** of your gel and/or **record the position** of each band (and any lanes that don't have bands) in your notebook.
11. Assuming your results resemble the output in Figure 7.1, proceed to the next step. Otherwise, you may need to re-do one or more of the reactions; consult your TA.

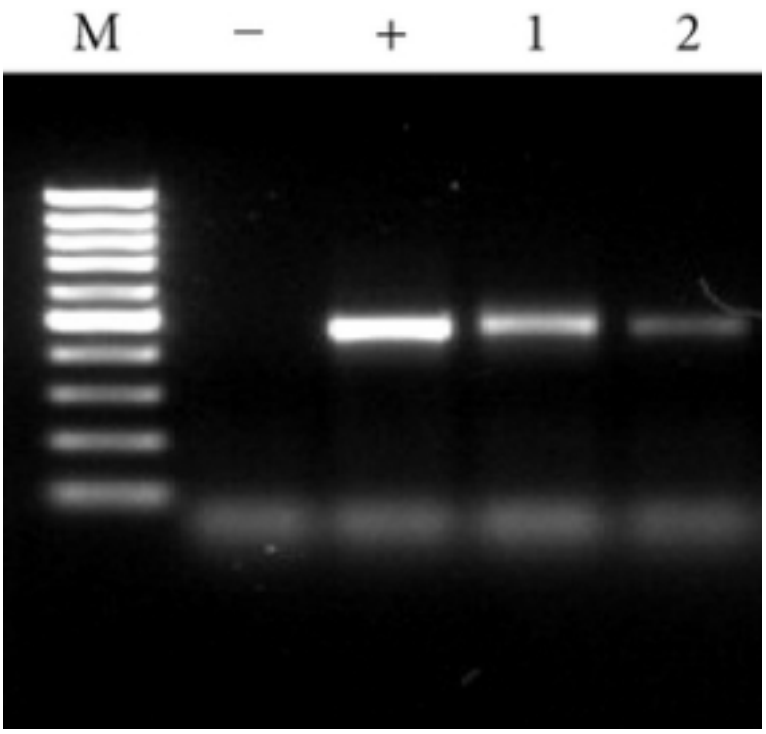


Figure 7.1. If your reactions worked, your gel should look about like this.

- *The molecular weight marker ladder is on the left side (M).*
- *The negative control (-) shows no product*
- *The positive control (+) and both unknowns show products of about the same size.*
- *The different brightness of the unknowns indicates that one started out with more DNA than the other.*
- *The hazy bands at the bottom of the gel are unreacted primers. Remember that* smaller fragments move faster than larger ones!

Part C. PURIFICATION AND QUANTIFICATION

SPECTROPHOTOMETRY AND SPECTROPHOTOMETRIC QUANTIFICATION

One way of studying substances suspended or dissolved in water is to see how strongly they absorb light at various wavelengths, a technique called "spectrophotometry". When you shine a light through a liquid sample, the farther it goes through the sample, the more it interacts with whatever is in the liquid. Every compound has a particular spectrum: in other words each compound absorbs light at different wavelengths with a unique pattern. DNA's spectrum is shown in **Figure 7.2** (below).

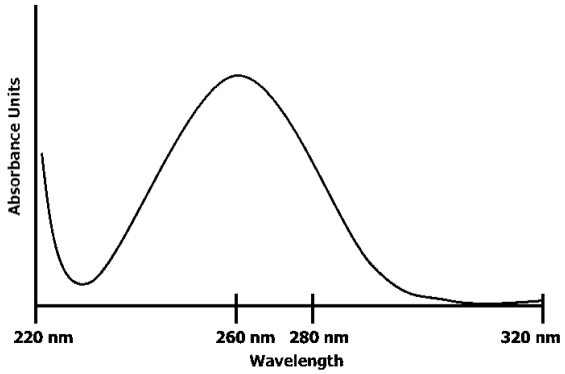


Figure 7.2. Ideal spectrophotometric scan profile for purified DNA. Notice the large peak at 260nm.

You can tell that there's a peak in absorbance at 260 nm, and it's almost non-existent at 230 nm. That means if you shine a light through a DNA solution, it will come out the other end relatively depleted in photons with wavelengths of 260 nm, changing the light's color. The more of a substance is in a solution, the stronger the absorbance of light becomes. If we know how much of a given wavelength is absorbed per mole of the compound (the "molar extinction coefficient" or ε) and how far the light has to travel through the solution (the path length or l), we can use the difference in intensity of light at that wavelength between the light's source and a detector on the opposite side of the sample (the absorbance, A) to calculate the concentration c of the compound in the solution. This is the Beer-Lambert Law:

$$c = \frac{A}{\varepsilon l}$$

The value of ε for double-stranded DNA is 0.02 (ng/μL)-1 cm-1. So, if you measured A = 0.2, DNA concentration would be calculated as 100 ng/μL.

Protein has a peak absorptivity at 280 nm, and any amount of protein contamination can result in overestimates of DNA concentration. Completely pure DNA should have a ratio of A260:A280 of about 1.8, if this is lower, it indicates protein contamination and means that the concentration estimate is probably high.

In this exercise, you will use a **spin column kit** that will allow you to separate your DNA from proteins, salts, and primers in your PCR mix, resulting in a pure double-stranded DNA mixture. You will then use spectrophotometry to quantify the concentration and purity of your DNA sample.

YOUR TEAM WILL NEED

- *2 PCR purification spin columns*
- *PCR products from your 2 unknowns*
- *1 tube each of column binding (Buffer PB), column wash (Buffer PE), and elution (Buffer EB) solutions*
- *5 sterile eppendorf tubes*
- *P200 and P1000 pipetters and tips*
- *A microcentrifuge*
- *Take 3 DNA Quantification Plate or NanoDrop*

DIRECTIONS

1. Label 2 eppendorf tubes each with the names of your unknowns. Also label your spin columns.
2. Add 250 μL Buffer PB to 1 eppendorf tube per organism.
3. Add your PCR products to the PB. Pipet up and down to mix.
4. Pipet the PB/DNA mixture into the spin column. Centrifuge at 14,000 g for 1 minute. MAKE SURE THE CENTRIFUGE IS BALANCED AND THE LID IS ON!
5. Pour off the liquid in the collection tube. Add 750 μL Buffer PE to the spin column. Spin at 14,000 g for 1 minute.
6. Pour off liquid in collection tube and centrifuge again (dry) for 1 minute.
7. Carefully remove the spin column and put in the clean eppendorf tube.
8. Add 30 μL Buffer EB to the center of the resin at the bottom of the spin column, wait 1 minute, then spin at 14,000 g for 1 minute.
9. Discard spin column. Centrifuge remaining product for 5 min at 14,000 g.
10. Being careful not to agitate the tube or contact the bottom of the tube with the pipet tip, remove 25 μL of liquid to a clean eppendorf tube.
11. Label this tube with your team name/number and isolate name/number.
12. After everyone in the class has finished purifying their products, each team will pipet 2 uL of each DNA prep onto one of the spots on the BioTek Take 3 quartz slide at the front of the class.
13. Your TA will pipet a 2 μL spot of very pure water as a Blank control onto a free spot on the slide.
14. Your TA will analyze the sample using a BioTek plate reader.
15. Alternative: a NanoDrop spectrophotometer can also be used if available.
16. Your TA will send you the quantification information and 260/280 ratio, as well as the spectrophotometric profile (as in Figure 7.2) from your isolates.
17. Make sure your DNA is clearly labeled and place it in the box for freezing. Your TA will send it to the sequencing facility, where it will be sequenced by the Sanger method using an AB1 sequencer.

Part D. BIOINFORMATIC IDENTIFICATION

BACTERIAL IDENTIFICATION AND ANALYSIS

You have amplified the 16S rRNA gene from your two environmental isolates and sent the PCR products off to be sequenced. Today, you're getting those sequences back. However, you can't do much with the raw data -- we have to process it in a number of ways prior to analysis. Here, we'll walk through the steps required to make sense of your sequence data. First you'll *quality control* the raw read information, then we'll *align* it using information from the thousands of other 16S rRNA sequences stored in online databases. With the alignment, you can *identify* your microbes.

Step 1: Quality Control. You'll recall that we used the Sanger method to sequence your PCR products. Sanger sequencing takes a DNA template and extends it from a single primer (your forward PCR primer) with Taq polymerase using cycling conditions similar to PCR. However, in addition to the standard dNTPs, a Sanger sequencing mix includes 4 dideoxy NTPs as well. These ddNTPs are missing the crucial O atom necessary to form the next phosphodiester bond and so they *terminate* the extension of the DNA molecule whenever they are incorporated. Therefore, a Sanger mixture will include many fragments of different lengths, each terminated wherever a ddNTP was incorporated. Modern Sanger sequencing uses ddNTPs that are labeled with fluorescent molecules, such that molecules ending in an A glow one color, those ending in a C glow a different color, and so on for G and T as well. We separate these fragments by size on an acrylamide gel and detect fluorescence as the various fragments pass the end of the gel, starting with the smallest fragments (the 5' end of the product being

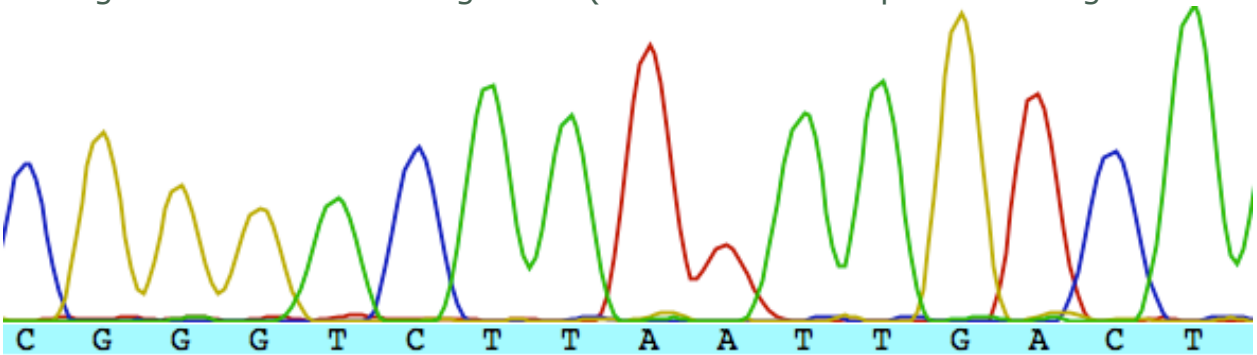


Fig. 7.3. A chromatogram. The ddCTPs were labeled blue, ddGTPs were yellow, ddTTPs were green, and ddATPs were red.

sequenced) and ending with the largest fragments (the 3' end of the product being sequenced).

However, interpretation of a Sanger reaction isn't THAT straightforward -- you don't necessarily get an unambiguous sequence. What you *actually* get is a *chromatogram*, or a graph with 4 separate "traces" showing light intensity at the 4 wavelengths emitted by the different fluorescent ddNTPs (Fig. 7.3). Ideally, this will result in a pattern of clearly separated "peaks" cleanly representing fragments of different sizes. However, this isn't always the case, and often the smallest and largest fragments have overlapping color traces that make them more or less unreliable. We therefore have to do a "quality control" step to eliminate the worst offenders in terms of ambiguous peaks, prior to "calling the base pairs" and outputting a sequence.

To do this, we will use a program called **SeqTrace**. SeqTrace lets us visualize the chromatogram itself, and then apply various quality criteria used to trim the sequences. The program scans the chromatogram and makes "base calls" based on detection of peaks in the 4 traces. It then assigns each call a "quality score" that defines how likely it is that the base call is correct. A number of factors influence the quality score, including the width of the peak and the height of the peak relative to the output at that location from all 3 other traces. The quality score Q is related to the probability P of a mistaken call by the equation:

$$Q = -10 \log_{10} P$$

Here, we will trim base pairs from the beginning and end of each sequence by looking for the point when the percentage of base calls exceeding a critical Q threshold passes a given point.

DIRECTIONS – QUALITY CONTROL

1. Download your organisms' AB1 files from Canvas onto your lab computer.
2. Find and open SeqTrace on your computer.
3. Click the "sheet" icon (Create New Project) at the top left of the SeqTrace window.
4. Click "Choose" and then navigate to the folder containing your sequences. NOTE: **select the folder**, don't actually go into it by double-clicking.
5. Click "Sequence Processing" and set "Min. confidence score" to 10 and "Trim until at least 9 out of 10 bases are correctly called". This will cut 5' and 3' regions where less than 90% of bases are called at a 90% accuracy level or better.

6. Click the "+" icon and select the .ab1 files that correspond to your isolates. Click "Add".
7. Click "Traces" and "View selected traces" to see what the chromatograms look like.
8. You'll see four different colored lines representing the 4 different ddNTPs. Above these traces are the quality scores for each peak. Below the traces are the "called bases", or which of the 4 colors is highest at a given position. Note that these bases are not evenly spaced horizontally, because the space between peaks varies over the length of the chromatogram.
9. Below the window containing the chromatogram and the called bases are two sequences. The top one is the "raw sequence" and the bottom one is the "working sequence", which is what is left over after the quality controls have been applied. How much of the raw sequence was eliminated by quality control? Note how many base calls have become "N"s in the working sequence. Why do you think SeqTrace has done this?
10. When you're done examining your sequence, click "Sequences", "Generate Finished Sequences", and "For all trace files". SeqTrace will go through all the files in the project and apply your quality control parameters to convert trace files to simple sequences of A, C, G, T, and N.
11. Then click "Sequences", "Export Sequences", and "From all trace files". Give your file a name and save it to the same folder with your team's AB1 files.

At this point you have generated a "FASTA" file which is a standard "flat format" text file for saving lists of sequences. Each sequence in a FASTA file has two lines. The first line starts with a ">" and gives the sequence a name and can also list attributes of various kinds. The second line is a simple string of nucleotide letter codes. For instance, here is one possibility:

>Awesomebacterium morrisii, isolated from soil on 3/25/2016

ACTAGCGTACGTGTGGTGCNAACTGGTTAATATAACAGAATCGAGGGGTAC

Let's look at your FASTA file. Open the folder where you saved it, find the file, and right-click it. Click on "Open with" and navigate to "Notepad" (in your Windows directory). Because a FASTA file is just a text file, you can view it in any text editing or word processing software. Scroll down through the file and note how the sequences are stored, 2 lines each.

Step 2: Alignment and Identification Many tools exist to identify environmental DNA sequences by comparison with online databases. These databases are truly gargantuan, with hundreds of billions of base pairs collected from many thousands of samples of all kinds from all over (and under) the world. One strategy is to just take your string of nucleotides and find other strings of nucleotides that are most like it. However, given the vastness of the databases, there is a good chance that even long strings of nucleotides will find spurious "hits" by random chance. A superior method for identifying close matches to sequences is to use an evolutionarily-informed method. However, this requires us to know more than just the sequence of nucleotides -- we also have to know how the nucleotides from sequence A match up to those in sequence B. To do this, we can *align* an unknown sequence against a group of sequences from well-studied organisms. You can get a rough idea about how this process works (and possibly help out medical science!) by playing the game at the following website:

http://phylo.cs.mcgill.ca

Seriously, go back and click the link. It's fun and you'll have a much better idea of what's going on in this experiment after you've played it for a few minutes.

Here, we are going to use a very common algorithm called the "Basic Local Alignment Search Tool" or "BLAST", operated from the US government's NCBI servers.

DIRECTIONS – ALIGNMENT AND IDENTIFICATION

1. Open an internet browser and navigate to *https://blast.ncbi.nlm.nih.gov/Blast.cgi*
2. Click "Nucleotide BLAST" on the left side of the screen.
3. Under "Enter Query Sequence", click "Choose File" and select your FASTA file.
4. Make sure "Standard databases (nr etc)" and "Highly similar sequences (megablast)" are checked. Also check the box by "Uncultured/environmental sample sequences" to make sure whatever you get has a name.
5. Click "BLAST" and wait
6. On the Results screen (Figure 7.4) you'll be able to view the closest matches in the NCBI database to your organism. The ones at the top of the "Sequences Producing Significant Alignments" list are the most likely IDs for your organism.
7. Click the "Results for" box to cycle between your two isolates.
8. Note that there are several metrics for the quality of the alignment. For instance, "Query Coverage" shows how much of your submitted sequence

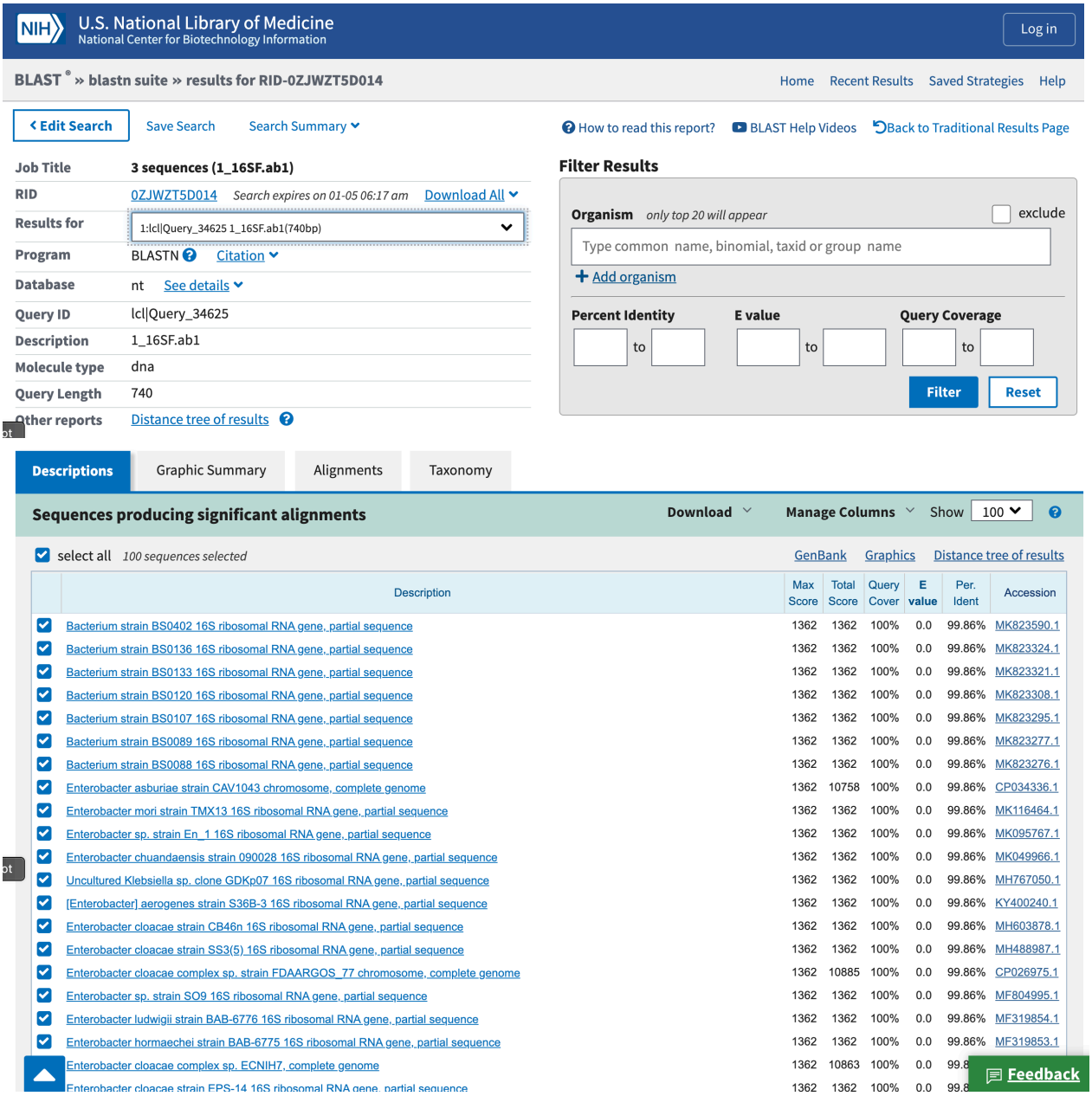


Figure 7. 4. BLAST Output from the NCBI server

actually aligned to the database sequence. The "E value" is the probability that a randomly generated sequence could generate an equally good alignment. The "Per. Ident" is how many base pairs are identical in your organism's sequence with the database match. The "Total Score" takes all of these values plus other information (e.g. the quality of the database sequence) into account. For the pictured organism, the ID is most likely *Enterobacter cloacae*.

9. In some cases you can't distinguish a top match. Even here, it's not completely clear that the sequence matches *Enterobacter cloacae* better than *Enterobacter asburiae*. That's okay – 16S evolves slowly, which makes it a good phylogenetic marker for separating distantly related organisms, but not so

great for more closely related organisms. Also, the naming customs for bacterial species have changed often over the years, making it even more complex. Do your best – do an Internet search for the various organism names and see if any of them match the other characteristics your organism expresses.

10. It's also possible that you won't get any really strong matches like the one shown in Figure 7.4. Try to figure out why. Was your PCR product relatively low quality, resulting in a short sequence? Or is your organism truly different than things in the database?

CHAPTER 8
CLASSICAL IDENTIFICATION

In this chapter you will conduct a suite of experiments with your isolates that have been used for decades to identify and characterize bacteria. None of them are as reliable as modern molecular techniques like 16S PCR, but they are still useful for several reasons. First, they are fast and relatively cheap, and therefore remain the most common methods for clinical identification of infectious organisms in hospitals around the world. Second, they give you important information about the metabolic abilities of your organisms that you can't learn from rRNA sequences alone. In some cases, you may be able to distinguish between closely related bacterial species that share identical 16S sequences, but have very different characteristics.

8A. MICROSCOPY AND GRAM STAIN

Use and Care of the Microscope

The most important discoveries of the laws, methods and progress of nature have nearly always sprung from the examination of the smallest objects which she contains.

JEAN BAPTISTE LAMARCK

Objectives

After completing this exercise, you should be able to:

1. Demonstrate the correct use of a compound light microscope.
2. Diagram the path of light through a compound microscope.
3. Name the major parts of a compound microscope.
4. Identify the three basic morphologies of bacteria.

Background

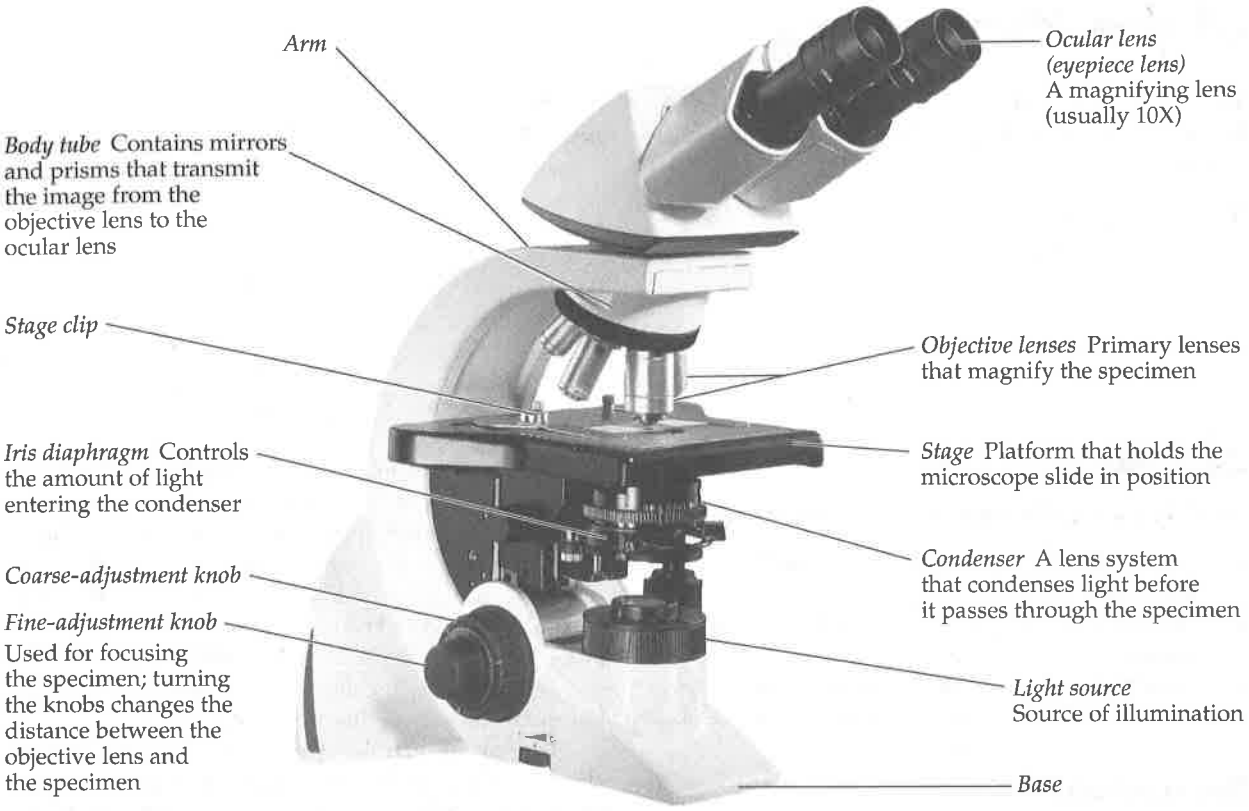
Virtually all organisms studied in microbiology cannot be seen with the naked eye but require the use of optical systems for magnification. The microscope was invented shortly before 1600 by Zacharias Janssen of the Netherlands. The microscope was not used to examine microorganisms until the 1680s, when a clerk in a dry-goods store, Antoni van Leeuwenhoek, examined scrapings of his teeth and any other substances he could find. The early microscopes, called **simple microscopes,** consisted of biconvex lenses and were essentially magnifying glasses. To see microbes, a compound microscope, which has two lenses between the eye and the object, is required. This optical system magnifies the object, and an illumination system (sun and mirror or lamp) ensures that adequate light is available for viewing. A **brightfield compound microscope,** which shows dark objects in a bright field, is used most often.

You will be using a brightfield compound microscope similar to the one shown in Figure 1a. The basic frame of the microscope consists of a **base,** a **stage** to hold the slide, an **arm** for carrying the microscope, and a **body tube** for transmitting the magnified image. The stage may have two clips or a movable mechanical stage to hold the slide. The light source is in the base. Above the light source is a **condenser,** which consists of several lenses that concentrate light on the slide by focusing it into a cone, as shown in Figure 1b. The condenser has an **iris diaphragm,** which controls the angle and size of the cone of light. This ability to control the *amount* of light ensures that optimal light will reach the slide. Above the stage, on one end of the body tube, is a revolving nosepiece holding three or four **objective lenses.** At the upper end of the tube is an **ocular** or **eyepiece lens** (10× to 12.5×). If a microscope has only one ocular lens, it is called a **monocular** microscope; a **binocular** microscope has two ocular lenses.

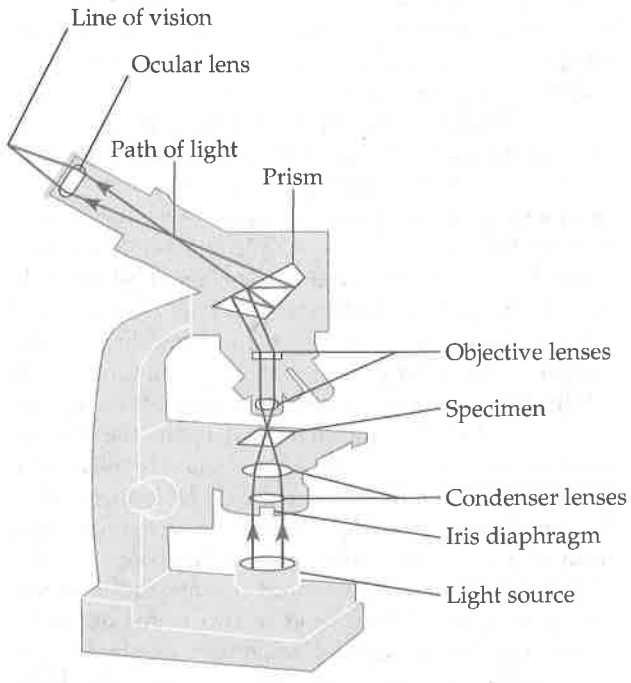
By moving the tube closer to the slide or the stage closer to the objective lens, using the coarse- or fine-adjustment knobs, one can focus the image. The larger knob, the **coarse adjustment,** is used for focusing with the low-power objectives (4× and 10×), and the smaller knob, the **fine adjustment,** is used for focusing with the high-power and oil immersion lenses. The coarse-adjustment knob moves the lenses or the stage longer distances. The area seen through a microscope is called the **field of vision.**

The **magnification** of a microscope depends on the type of objective lens used with the ocular. Compound microscopes have three or four objective lenses mounted on a nosepiece: scanning (4×), low-power (10×), high-dry (40× to 45×), and oil immersion (97× to 100×). The magnification provided by each lens is stamped on the barrel. The total magnification of the object is calculated by multiplying the magnification of the ocular (usually 10×) by the magnification of the objective lens. The most important lens in microbiology is the **oil immersion lens;** it has the highest magnification (97× to 100×) and must be used with immersion oil. Optical systems could be built to magnify much more than the 1000× magnification of your microscope, but the resolution would be poor.

Resolution or **resolving power** refers to the ability of lenses to reveal fine detail or two points distinctly separated. An example of resolution involves a car approaching you at night. At first only one light appears, but as the car nears, you can distinguish two



(a) Principal parts and functions



(b) Arrows show the path of light (bottom to top)

Figure 1

The compound light microscope. **(a)** Its principal parts and their functions. **(b)** Lines from the light source through the ocular lens illustrate the path of light.

headlights. The resolving power is a function of the wavelength of light used and a characteristic of the lens system called **numerical aperture.** Resolving power is best when two objects are seen as distinct even though they are very close together. Resolving power is expressed in units of length; the smaller the distance, the better the resolving power.

$$\text{Resolving power} = \frac{\text{Wavelength of light used}}{2 \times \text{numerical aperture}}$$

Smaller wavelengths of light improve resolving power. The effect of decreasing the wavelength can be seen in electron microscopes, which use electrons as a source of "light." The electrons have an extremely short wavelength and result in excellent resolving power. A light microscope has a resolving power of about 200 nanometers (nm), whereas an electron microscope has a resolving power of less than 0.2 nm. The numerical aperture is engraved on the side of each objective lens (usually abbreviated N.A.). If the numerical aperture increases—for example, from 0.65 to 1.25—the resolving power is improved. The numerical aperture is dependent on the maximum angle of the light entering the objective lens and on the **refractive index** (the amount the light bends) of the material (usually air) between the objective lens and the slide. This relationship is defined by the following:

N.A. = $N \sin \theta$
N = Refractive index of medium
θ = Angle between the most divergent light ray gathered by the lens and the center of the lens

As shown in Figure 2, light is refracted when it emerges from the slide because of the change in media as the light passes from glass to air. When immersion oil is placed between the slide and the oil immersion lens, the light ray continues without refraction because immersion oil has the same refractive index ($N = 1.52$) as glass ($N = 1.52$). This can be seen easily. When you look through a bottle of immersion oil, you cannot see the glass rod in it because of the identical N values of the glass and immersion oil. The result of using oil is that light loss is minimized, and the lens focuses very close to the slide.

As light rays pass through a lens, they are bent to converge at the **focal point,** where an image is formed (Figure 3a). When you bring the center of a microscope field into focus, the periphery may be fuzzy due to the curvature of the lens, resulting in multiple focal points. This is called **spherical aberration** (Figure 3b). Spherical aberrations can be minimized by the use of the iris diaphragm, which eliminates light rays to the periphery of the lens, or by a series of lenses resulting in essentially a flat optical system. Sometimes a multitude of colors, or **chromatic aberration,** is seen in the field (Figure 3c). This is caused by the prismlike effect of the lens as various wavelengths of white light pass through to a different focal point for each wavelength. Chromatic aberrations can be minimized by the use of filters (usually blue); or by lens systems corrected for red and blue light, called *achromatic lenses;* or by lenses corrected for red, blue, and other wavelengths, called *apochromatic lenses*. The most logical, but most expensive, method of eliminating chromatic aberrations is to use a light source of one wavelength, or **monochromatic light.**

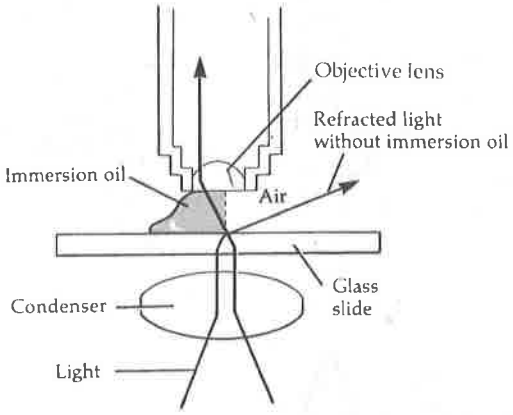


Figure 2
Refractive index. Because the refractive indexes of the glass microscope slide and immersion oil are the same, the oil keeps the light rays from refracting.

Compound microscopes require a light source. The light may be reflected to the condenser by a mirror under the stage. If your microscope has a mirror, the sun or a lamp may be used as the light source. Most compound microscopes have a built-in illuminator in the base. The *intensity* of the light can often be adjusted with a rheostat.

The microscope is a very important tool in microbiology, and it must be used carefully and correctly. Follow these guidelines *every* time you use a microscope.

General Guidelines

1. Carry the microscope with both hands: one hand beneath the base and one hand on the arm.
2. Do not tilt the microscope; instead, adjust your stool so you can comfortably use the instrument.
3. Observe the slide with both eyes open, to avoid eyestrain.
4. Always focus by moving the lens away from the slide.
5. Always focus slowly and carefully.

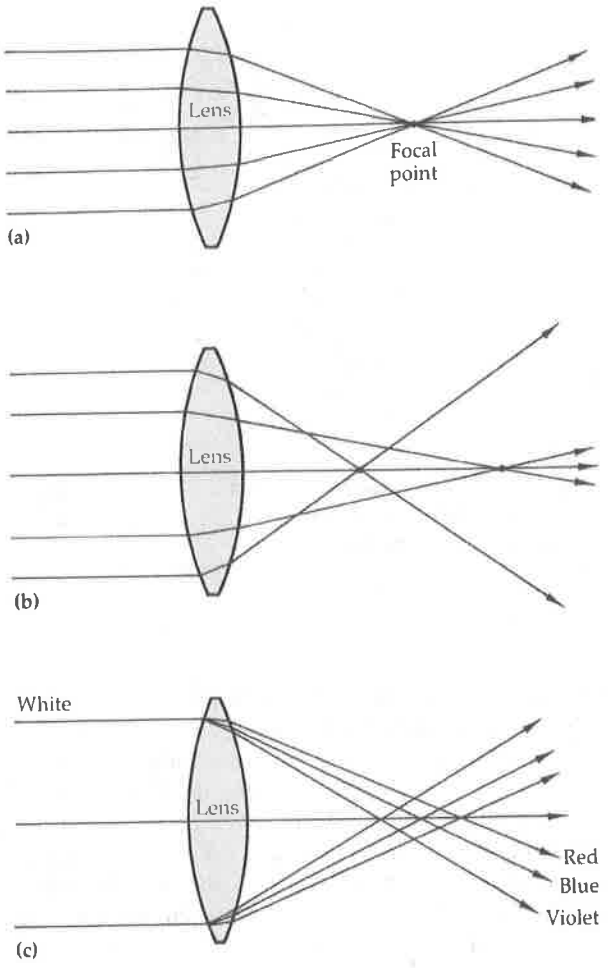


Figure 3

Focal point. **(a)** An image is formed when light converges at one point, called the focal point. **(b)** Spherical aberration. Curved lenses result in light passing through one region of the lens having a different focal point than light passing through another part of the lens. **(c)** Chromatic aberration. Each wavelength of light may be given a different focal point by the lens.

6. When using the low-power lens, the iris diaphragm should be barely open so that good contrast is achieved. More light is needed with higher magnification.
7. Before using the oil immersion lens, have your slide in focus under high power. *Always focus with low power first.*
8. Keep the stage clean and free of oil. Keep all lenses except the oil immersion lens free of oil.
9. Keep all lenses clean. Use *only* lens paper to clean them. Wipe oil off the oil immersion lens before putting your microscope away. Do not touch the lenses with your hands.
10. Clean the ocular lens carefully with lens paper. If dust is present, it will rotate as you turn the lens.
11. After use, remove the slide, wipe oil off it, put the dust cover on the microscope, and return it to the designated area.
12. When a problem does arise with the microscope, obtain help from the instructor. Do not use another microscope unless yours is declared "out of action."

Materials

Compound light microscope

Immersion oil

Lens paper

Prepared slides of algae, fungi, protozoa, and bacteria

Procedure

1. Place the microscope on the bench squarely in front of you.
2. Obtain a slide of algae or fungi and place it in the side clips on the stage.
3. Adjust the eyepieces on a binocular microscope to your own personal measurements.
   a. Look through the eyepieces and, using the thumb wheel, adjust the distance between the eyepieces until one circle of light appears.
   b. With the low-power (10×) objective in place, cover the left eyepiece with a small card and focus the microscope on the slide. When the right eyepiece has been focused, remove your hand from the focusing knobs and cover the right eyepiece. Looking through the microscope with your left eye, focus the left eyepiece by turning the eyepiece adjustment. Make a note of the number at which you focused the left eyepiece so you can adjust any binocular microscope for your eyes.
4. Raise the condenser up to the stage. On some microscopes, the condenser can be focused by the following procedure:
   a. Focus with the 10× objective.
   b. Close the iris diaphragm so only a minimum of light enters the objective lens.

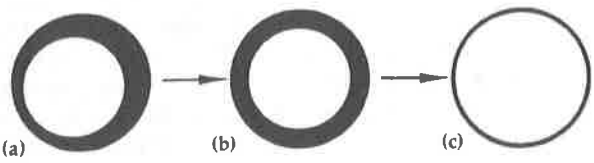


Figure 4

Using low power, lower the condenser until a distinct circle of light is visible **(a)**. Center the circle of light using the centering screws **(b)**. Open the iris diaphragm until the light just fills the field **(c)**.

c. Lower the condenser until the light is seen as a circle in the center of the field. On some microscopes the circle of light may be centered (Figure 4) using the centering screws found on the condenser.
d. Raise the condenser up to the slide, lower it, and stop when the color on the periphery changes from pink to blue (usually 1 or 2 mm below the stage).
e. Open the iris diaphragm until the light just fills the field.

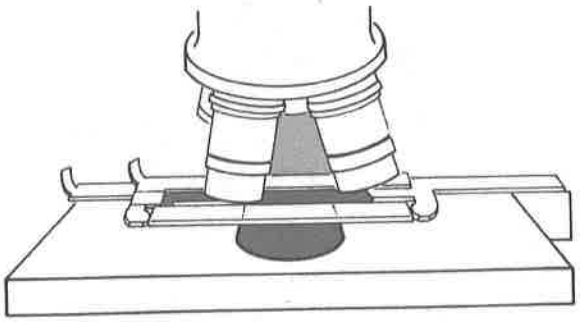
5. Diagram some of the cells on the slide under low power. Use a minimum of light by adjusting the _____.

6. When an image has been brought into focus with low power, rotate the turret to the next lens, and the subject will remain almost in focus. All of the objectives (with the possible exception of the 4×) are **parfocal;** that is, when a subject is in focus with one lens, it will be in focus with all of the lenses. When you have completed your observations under low power, swing the high-dry objective into position and focus. Use the fine adjustment. Only a slight adjustment should be required. Why? _____

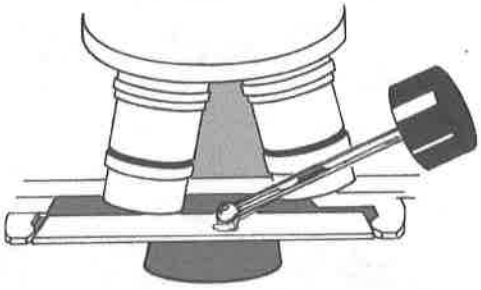
More light is usually needed. Again, draw the general size and shape of some cells.

7. Move the high-dry lens out of position, and place a drop of immersion oil on the area of the slide you are observing. Carefully click the oil immersion lens into position. It should now be immersed in the oil (Figure 5). Careful use of the fine-adjustment knob should bring the object into focus. Note the shape and size of the cells. Did the color of the cells change with the different lenses? _____ Did the size of the field change? _____

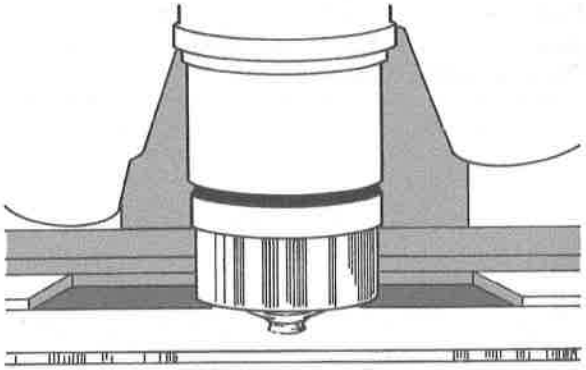
8. Record your observations and note the magnifications.



(a) Move the high-dry lens out of position.



(b) Place a drop of immersion oil in the center of the slide.



(c) Move the oil immersion lens into position.

Figure 5

Using the oil immersion lens.

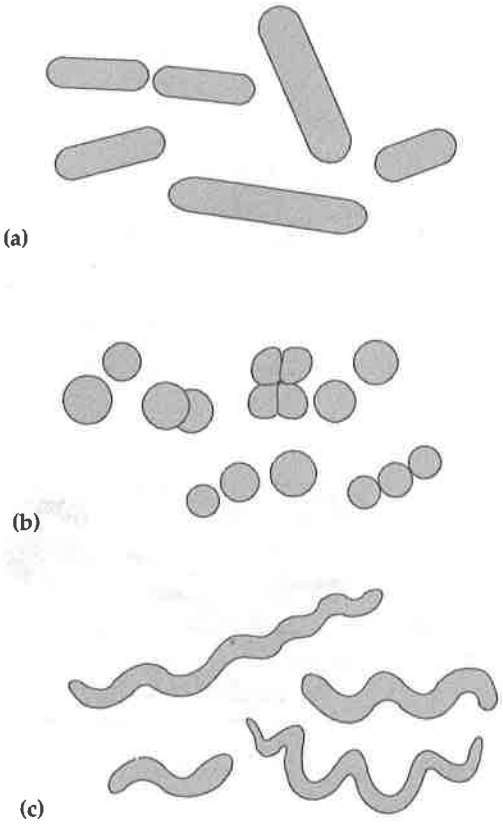


Figure 6

Basic shapes of bacteria. **(a)** Bacillus (plural: bacilli), or rod. **(b)** Coccus (plural: cocci). **(c)** Spiral.

9. When your observations are completed, move the turret to bring a low-power objective into position. *Do not* rotate the high-dry (40×) objective through the immersion oil. Clean the oil off the objective lens with lens paper, and clean off the slide with tissue paper or a paper towel. Remove the slide. Repeat this procedure with all the available slides. When observing the bacteria, note the three different morphologies, or shapes, shown in Figure 6.

Preparation of Smears and Simple Staining

Objectives

After completing this exercise, you should be able to:

1. Prepare and fix a smear.
2. List the advantages of staining microorganisms.
3. Explain the basic mechanism of staining.
4. Perform a simple direct stain.

Background

Most stains used in microbiology are synthetic aniline (coal tar derivative) dyes derived from benzene. The dyes are usually salts, although a few are acids or bases, composed of charged colored ions. The ion that is colored is referred to as a **chromophore.** For example,

$$\text{Methylene blue chloride} \rightleftharpoons \underset{\text{(Chromophore)}}{\text{Methylene blue}^+} + Cl^-$$

If the chromophore is a positive ion like the methylene blue in the equation shown, the stain is considered a **basic stain;** if it is a negative ion, it is an **acidic stain.** Most bacteria are stained when a basic stain permeates the cell wall and adheres by weak ionic bonds to the negative charges of the bacterial cell.

Staining procedures that use only one stain are called **simple stains.** A simple stain that stains the bacteria is a **direct stain,** and a simple stain that stains the background but leaves the bacteria unstained is a **negative stain.** Simple stains can be used to determine cell morphology, size, and arrangement.

Before bacteria can be stained, a thin film of bacterial cells, called a **smear,** must be placed on a slide. A smear is made by spreading a bacterial suspension on a clean slide and allowing it to air-dry. The smear must be **fixed** to kill the bacteria; coagulated proteins from the cells will cause cells to stick to the slide. The dry smear is passed through a Bunsen burner flame several times to **heat-fix** the bacteria. Heat fixing may not kill all the bacteria. Alternatively, the dry smear can be placed on a 60°C slide warmer for 10 minutes or until chemically fixed. To **chemically fix** the bacteria, cover the smear with 95% methyl alcohol for 1 minute. Fixing denatures bacterial enzymes, preventing them from digesting cell parts, which causes the cell to break, a process called *autolysis*. Fixing also enhances the adherence of bacterial cells to the microscope slide.

Materials

Methylene blue

Wash bottle of distilled water

Slide

Inoculating loop

Cultures

Staphylococcus epidermidis slant

Bacillus megaterium broth

Techniques Required

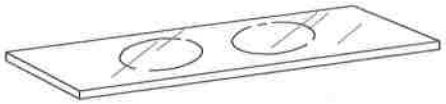
Compound light microscopy

Inoculating loop

Procedure

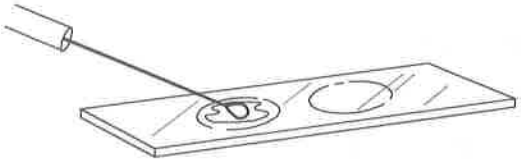
1. Clean your slide well with abrasive soap or cleanser; rinse and dry. Handle clean slides by the end or edge. Use a marker to make two dime-sized circles on the bottom of each slide so they will not wash off. Label each circle according to the bacterial culture used.
2. Sterilize your inoculating loop by holding it in the hottest part of the flame (at the edge of the inner blue area) or the electric incinerator until it is red-hot. The entire wire should get red. Allow the loop to cool so that bacteria picked up with the loop won't be killed. Allow the loop to cool without touching it or setting it down. Cooling takes about 30 seconds. You will determine the appropriate time with a little practice.

The loop must be cool before inserting it into a medium. A hot loop will spatter the medium and move bacteria into the air.

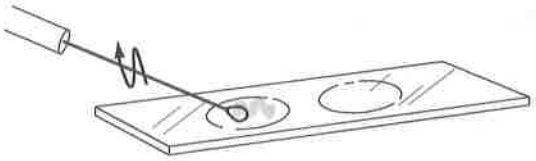


(a) Mark the smear areas with a marking pencil on the underside of a clean slide.

FROM SOLID MEDIUM

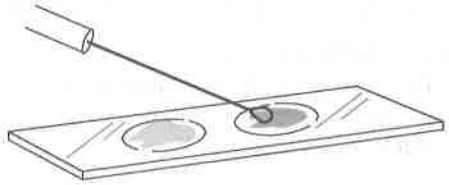


(b) Place 1 or 2 loopfuls of water on the slide.

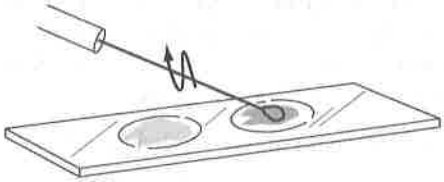


(c) Transfer a very small amount of the culture with a sterile loop. Mix with the water on the slide.

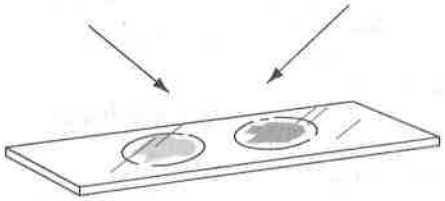
FROM LIQUID MEDIUM



(d) Place 2 or 3 loopfuls of the liquid culture on the slide with a sterile loop.

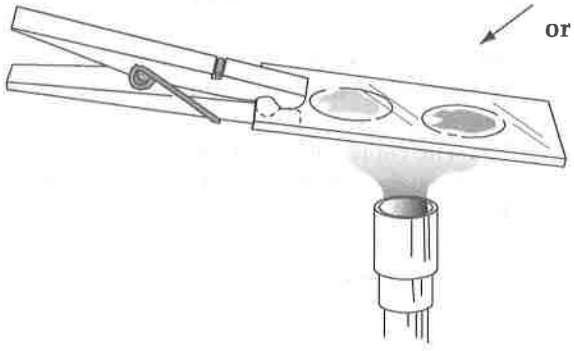


(e) Spread the bacteria within the circle.

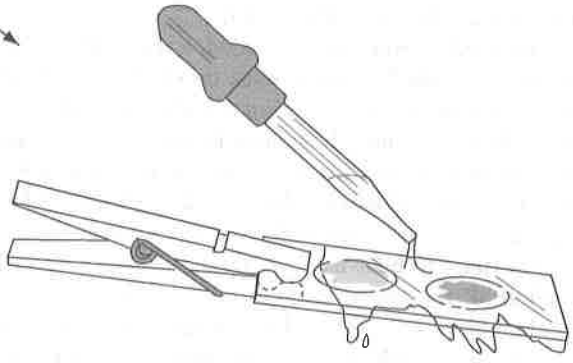


(f) Allow the smears to air-dry at room temperature.

or



(g) Pass the slide through the flame of a burner two or three times.



(h) Cover the smears with 95% methyl alcohol for 1 minute, and then let the smears air-dry.

Figure 1

Preparing a bacterial smear.

3. Prepare smears (Figure 1).
   a. Make a smear of bacteria from the broth culture in the center of one circle. Flick the tube of broth culture lightly with your finger to resuspend sedimented bacteria, and place 2 or 3 loopfuls of the culture in the circle. Sterilize your loop between each loopful. Spread the culture within the circle.
   b. Sterilize your loop.

Always sterilize your loop after using it and before setting it down.

   c. For the bacterial culture on solid media, place 1 or 2 loopfuls of distilled water in the center of the other circle, using the sterile inoculating loop. Which bacterium is on a solid medium? _____
   Sterilize your loop.
   d. Using the cooled loop, scrape a *small* amount of the culture off the slant—do not take the agar (Figure 2). If you hear the sizzle of boiling water when you touch the agar with the loop, resterilize your loop and begin again. Why? _____
   Try not to gouge the agar. Emulsify (to a milky suspension) the cells in the drop of water, and spread the suspension to fill a majority of the circle. The smear should look like diluted skim milk. Sterilize your loop again.
   e. Let the smears dry. *Do not* blow on the slide because this will move the bacterial suspension. *Do not* flame the slide because flaming will distort the cells' shapes.
   f. Hold the slide with a clothespin and fix the smears by one of the following methods (Figure 1g or h):
   (1) Pass the slide quickly through the blue flame two or three times or place it on a 60° slide warmer for 10 minutes.
   (2) Cover the smear with 95% methyl alcohol for 1 minute. Tip the slide to let the alcohol run off, and let the slide air-dry before staining. Do not fix until the smears are completely dry. Why? _____

5. Stain smears (Figure 3).
   a. Use a clothespin to hold the slide, or place it on a staining rack.
   b. Cover the smear with methylene blue and leave it for 30 to 60 seconds (Figure 3a).

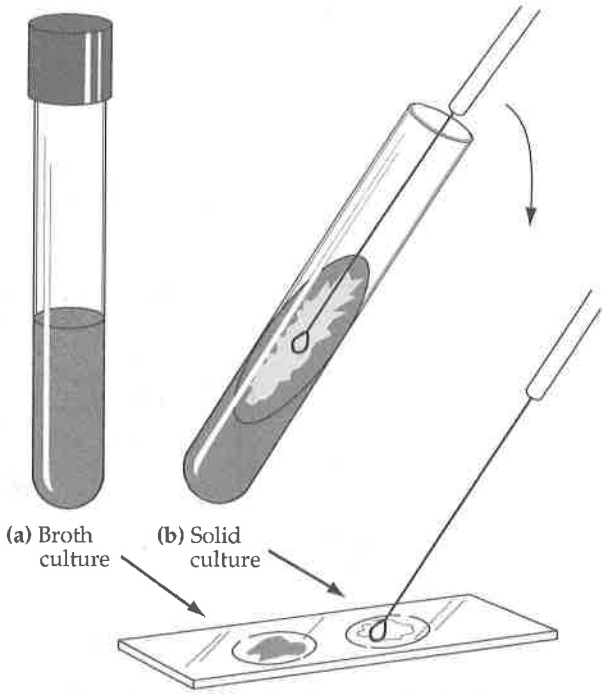
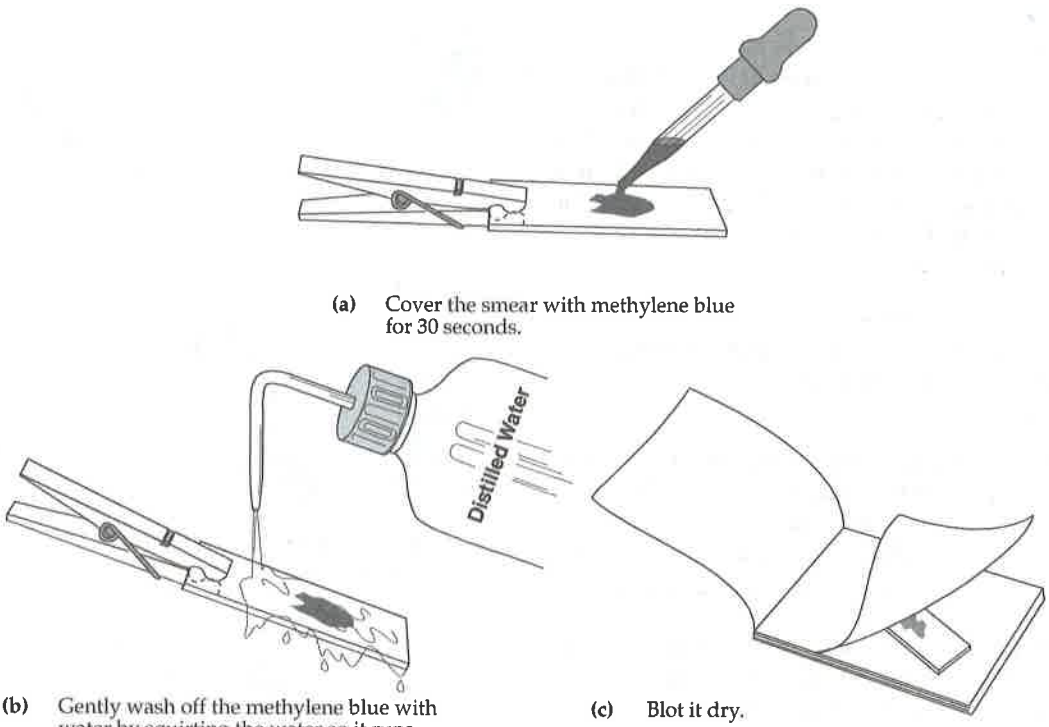


Figure 2
Transferring bacteria. **(a)** Transfer 2 or 3 loopfuls of microbial suspension to a slide. **(b)** Gently scrape bacteria from the agar surface and transfer the bacteria to a loopful of water on a slide. Be careful to avoid gouging into the agar.

   c. Carefully wash the excess stain off with distilled water from a wash bottle. Let the water run down the tilted slide (Figure 3b).
   d. Gently blot the smear with a paper towel or absorbent paper and let it dry (Figure 3c).
6. Examine your stained smears microscopically using the low, high-dry, and oil immersion objectives. Put the oil *directly* on the smear; coverslips are not needed. Record your observations with labeled drawings.
7. Blot the oil from the objective lens with lens paper, and return your microscope to its proper location. Clean your slides well, or save them as described in step 8.
8. Stained bacterial slides can be stored in a slide box. Remove the oil from the slide by blotting it with a paper towel. Any residual oil won't matter.



(a) Cover the smear with methylene blue for 30 seconds.

(b) Gently wash off the methylene blue with water by squirting the water so it runs through the smear.

(c) Blot it dry.

Figure 3
Simple staining.

Gram Staining

Objectives

After completing this exercise, you should be able to:

1. Explain the rationale and procedure for the Gram stain.
2. Perform and interpret Gram stains.

Background

The Gram stain is a useful stain for identifying and classifying bacteria. The **Gram stain** is a differential stain that allows you to classify bacteria as either gram-positive or gram-negative. The Gram-staining technique was discovered by Hans Christian Gram in 1884, when he attempted to stain cells and found that some lost their color when excess stain was washed off.

The staining technique consists of the following steps:

1. Apply **primary stain** (crystal violet). All bacteria are stained purple by this basic dye.
2. Apply **mordant** (Gram's iodine). The iodine combines with the crystal violet in the cell to form a crystal violet–iodine complex (CV–I).
3. Apply **decolorizing agent** (ethyl alcohol or ethyl alcohol–acetone). The primary stain is washed out (decolorized) of some bacteria, while others are unaffected.
4. Apply **secondary stain** or **counterstain** (safranin). This basic dye stains the decolorized bacteria red.

The most important determining factor in the procedure is that bacteria differ in their *rate* of decolorization. Those that decolorize easily are referred to as **gram-negative,** whereas those that decolorize slowly and retain the primary stain are called **gram-positive.**

Bacteria stain differently because of chemical and physical differences in their cell walls. Crystal violet is picked up by the cell. Iodine reacts with the dye in the cytoplasm to form a CV–I that is larger than the crystal violet that entered the cell. The CV–I cannot be washed out of gram-positive cells. In gram-negative cells, the decolorizing agent dissolves the outer lipopolysaccharide layer, and the CV–I washes out through the thin layer of peptidoglycan.

The Gram stain is most consistent when done on young cultures of bacteria (less than 24 hours old). When bacteria die, their cell walls degrade and may not retain the primary stain, giving inaccurate results. Because Gram staining is usually the first step in identifying bacteria, the procedure should be memorized.

Materials

Gram-staining reagents:

- Crystal violet
- Gram's iodine
- Ethyl alcohol
- Safranin

Wash bottle of distilled water

Slides (3)

Cultures

Staphylococcus epidermidis

Escherichia coli

Bacillus subtilis

Techniques Required

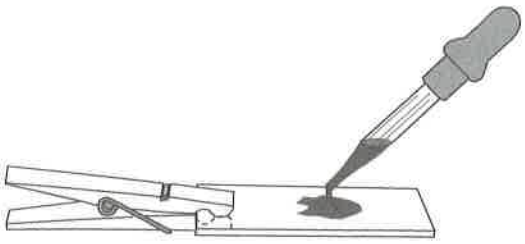
Compound light microscopy

Smear preparation

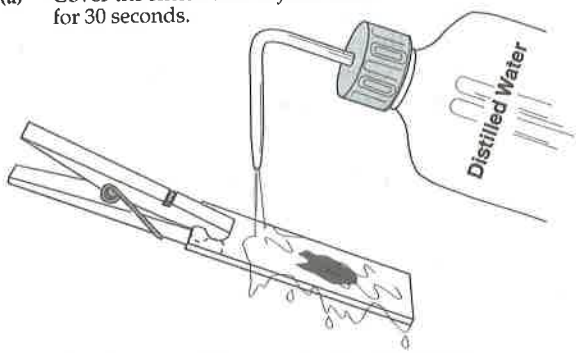
Simple staining

Procedure (Figure 1)

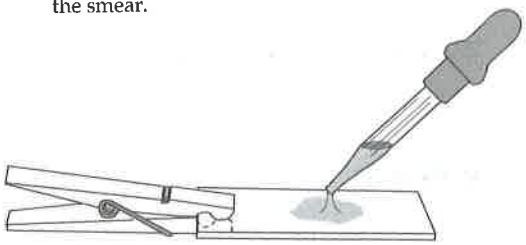
1. Prepare and fix smears. Clean the slides well, and make a circle on each slide with a marker. Label each slide for one of the cultures.
2. Prepare a Gram stain of one smear. Use a clothespin or slide rack to hold the slides.
   a. Cover the smear with crystal violet and leave it for 30 seconds (Figure 1a).
   b. Wash the slide carefully with distilled water from a wash bottle. Do not squirt water directly onto the smear (Figure 1b).
   c. Cover the smear with Gram's iodine for 10 seconds (Figure 1c).



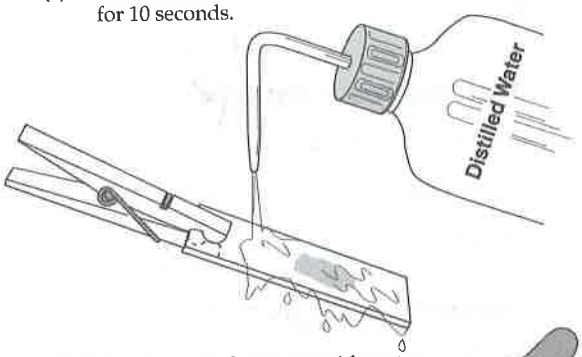
(a) Cover the smear with crystal violet for 30 seconds.



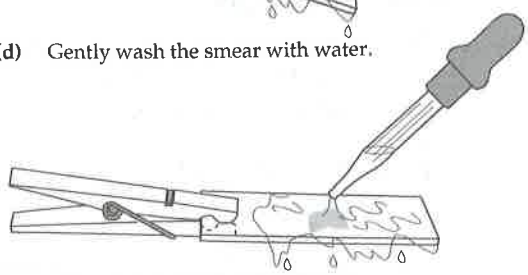
(b) Gently wash off the crystal violet with water by squirting the water so it runs through the smear.



(c) Cover the smear with Gram's iodine for 10 seconds.

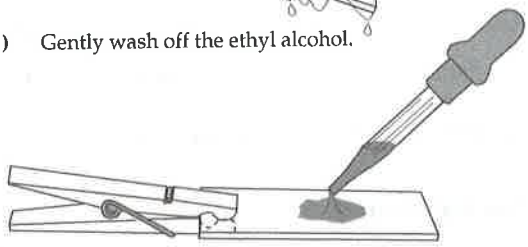


(d) Gently wash the smear with water.

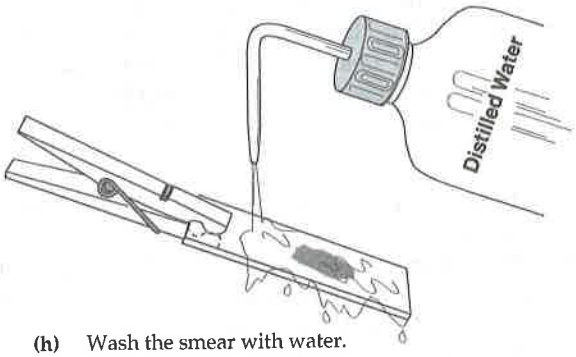


(e) Decolorize it with ethyl alcohol.

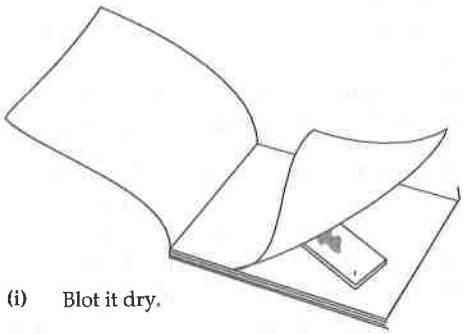
(f) Gently wash off the ethyl alcohol.



(g) Cover the smear with safranin for 30 seconds.



(h) Wash the smear with water.



(i) Blot it dry.

Figure 1
The Gram stain.

d. Wash off the iodine by tilting the slide and squirting water above the smear so that the water runs over the smear (Figure 1d).

e. Decolorize it with 95% ethyl alcohol (Figure 1e). Let the alcohol run through the smear until no large amounts of purple wash out (usually 10 to 20 seconds). The degree of decolorizing depends on the thickness of the smear. This is a critical step. *Do not overdecolorize*. However, experience is the only way you will be able to determine how long to decolorize. Very thick smears will give inaccurate results. Why? _____

_____

f. Immediately wash gently with distilled water (Figure 1f). Why? _____

g. Add safranin for 30 seconds (Figure 1g).

h. Wash the slide with distilled water and blot it dry with a paper towel or absorbent paper (Figure 1h and i).

3. Repeat step 2 to stain your remaining slides.

4. Examine the stained slides microscopically, using the low, high-dry, and oil immersion objectives. Put the oil directly on the smear. Record your observations. Do they agree with those given in your textbook? _____

If not, try to determine why. Some common sources of Gram-staining errors are the following:

a. The loop was too hot.
b. Excessive heat was applied during heat fixing.
c. The decolorizing agent (ethyl alcohol) was left on the smear too long.
d. The culture was too old.
e. The smear was too thick.

8B. RESPIRATION, CATABOLISM, and CHEMOTAXIS

In these exercises, you will learn about how your isolates make the energy they need to grow. You will test whether your isolates express an aerobic cytochrome oxidase, as well as whether they can anaerobically respire nitrate (i.e., perform denitrification) or sulfate. Additionally, you will test whether they can grow on a number of common carbon compounds. Finally, you will investigate whether your isolates are motile, and if so, if they are capable of chemotaxis toward simple carbon substrates.

Millions of different carbon compounds exist in nature, and each one is structurally unique. This presents a challenge to organisms that want to use those compounds either for energy (catabolism) or to build biomass (anabolism), because enzymes only recognize particular molecular shapes. Thus, microbes are limited in the range of compounds they can metabolize by the presence or absence of particular genes and gene pathways (i.e. ***operons***) in their genomes. Some pathways are quite common (e.g. glucose utilization) whereas others are rare enough to be useful in identifying particular taxonomic groups of microbes (e.g. lactose utilization).

Carbon catabolism has three primary steps. First, the compound has to be transported into the cell by a transmembrane transporter protein. In some cases (e.g. involving polymeric catabolites such as DNA and protein) this requires the substrate to first be broken down extracellularly by ***exoenzymes*** into its component parts. Second, the compound has to be chemically converted into a form (usually glucose) that can be shunted into one of the central carbon catabolism pathways (such as glycolysis). Finally, the reducing equivalents (e.g., NADH) produced by catabolism must be regenerated, either by funneling their electrons to a terminal electron acceptor (***respiration***) or by depositing them onto an intermediate carbon metabolite (***fermentation***).

In these experiments, we will discover which carbon compounds your organisms can grow on, what (if any) terminal electron acceptors they can use, and whether or not they are able to search for growth substrates in their environment through motility.

8.B.i. RESPIRATION and FERMENTATION

The most familiar type of respiration, ***aerobic*** respiration, uses oxygen as the terminal electron acceptor. However, many bacteria are capable of "breathing" other oxidized inorganic chemicals. Nitrate in particular is commonly used for respiration, in a process called ***denitrification*** which is very important for natural biogeochemical cycling of nitrogen. Many enteric bacteria such as *E. coli* are capable of simply reducing nitrate to nitrite. Other "environmental" organisms are capable of further reducing nitrite to nitrous oxide and eventually back to dinitrogen gas. Still other important organisms are capable of **reducing sulfate** or even carbon dioxide with very low energy yields – but they are capable of continuing to grow even in very resource-poor environments.

Respiration generates large amounts of energy via oxidative phosphorylation. However, terminal electron acceptors aren't always available, and in that case fermentation is necessary to regenerate NAD^+ and keep catabolic processes going. Like respiration, fermentation also takes several forms. The simplest fermentation pathway results in the formation of lactic acid. Unfortunately, lactic acid is highly toxic: because it is a small polar molecule, it doesn't easily escape the cell and must be transported out of the cell (possibly actively, using ATP) or else the cytoplasm pH will drop precipitously, causing stress. Additional pathways can produce a variety of end products in addition to lactic acid, including formate, succinate, ethanol, and hydrogen gas (***mixed acid fermentation***) or neutral carbohydrates (such as ***acetoin***).

YOUR TEAM WILL NEED

- *Plates and broth cultures of unknown isolates growing at their optimum temperature*
- *2 tubes of nitrate broth*
- *Nitrate reagent a (dimethyl-α-naphthylamine)*
- *Nitrate reagent B (sulfanilic acid)*
- *Zinc dust*
- *Oxidase strips (2)*
- *Sterile petri dish*
- *Sterile saline*
- *2 Peptone Iron deeps*
- *4 MRVP tubes*
- *Methyl red*
- *V-P reagent 1 (α-naphthol)*
- *V-P reagent II (40% KOH)*
- *Sterile toothpicks*
- *Dropper of 3% hydrogen peroxide*
- *BHI plate containing isolated colonies of* Staphylococcus epidermidis

OXIDASE TEST

A cytochrome oxidase is necessary for using oxygen as a terminal electron acceptor in aerobic respiration. This test identifies the presence of a cytochrome *c* oxidase like the one found in eukaryotic mitochondria as well as many bacteria. Note that some bacteria (particularly the Enterobacteriaceae) are capable of aerobic growth because they produce a *different* cytochrome oxidase (not detected by this test).

$$2\ \text{Cytochrome} + O_2 + 4\ H^+ \xrightarrow{\textit{Cytochrome oxidase}} 2\ \text{Cytochrome}^+ + 2\ H_2O$$

OXIDASE TEST DIRECTIONS

1. Place two oxidase test strips in a sterile petri dish. Moisten each with water.
2. Using sterile toothpicks, smear several colonies of one isolate onto the moistened area of one test strip; repeat with the other isolate and the other test strip.
3. If your isolate makes a hard colony, place as much growth as possible on the oxidase strip and then add a loopful of sterile saline.
4. If the strip turns blue-black within 30 seconds, it is oxidase positive.

NITRATE REDUCTASE TEST

Nitrate is one of the best alternative electron acceptors for anaerobic respiration and many microbes will use it first when the oxygen runs out by reducing it to nitrite. Some microbes are further capable of using nitrite as a terminal electron acceptor, resulting in the production of nitrous oxide gas, and these gases can be further reduced to dinitrogen gas. The use of nitrate as a terminal electron acceptor is called *denitrification* and is one of the 'legs' of the 'nitrogen triangle' that controls the cycling of nitrogen in nature. The other two legs are *nitrogen fixation* (used for assimilatory nitrogen metabolism) and *nitrification* (performed by lithotrophs that use ammonium as an electron donor).

Nitrate reductase

$$\text{Cytochrome} + NO_3^- \longrightarrow \text{Cytochrome}^+ + NO_2^- + H_2O$$

Denitrification

$$NO_3^- \rightarrow NO_2^- \rightarrow N_2O\ (g) + NO\ (g) \rightarrow N_2\ (g)$$

NITRATE REDUCTASE TEST DIRECTIONS

1. Label each nitrate tube with your organism's name. If your organism grows well in broth, inoculate the appropriate tube with a single colony. If not, re-suspend as much growth as you can from a plate into 100 μL of sterile saline, and use this to inoculate the nitrate broth.
2. Incubate the nitrate tubes at the organism's optimum temperature.
3. As soon as you can see growth in the tubes, add 5 drops of nitrate reagent A and 5 drops of nitrate reagent B to the tube and shake gently to mix. If the broth turns red within 30 seconds, this indicates the presence of nitrite.
4. If no red color develops, carefully (it's toxic!) add a small pinch of zinc dust. If it turns red after 20-30 seconds, this indicates that nitrate is still present and that the organism cannot respire nitrate. If no color forms, this means that the nitrite was further reduced to volatile nitrous oxide or nitrogen gas (full denitrification).
5. If growth isn't obvious the first-class period after inoculation, keep incubating the culture for 2 more class periods. At the end of this period, perform steps 3-4 regardless of whether growth is visible or not. In this case, you cannot trust negative results (because lack of nitrate reduction could indicate either that the organism can't reduce nitrate, or that it just didn't grow) but you can trust any positive result.

THIOSULFATE REDUCTION TEST

Sulfate (including thiosulfate and other oxidized forms of sulfur) can be used by some *sulfur reducing bacteria* as a terminal electron acceptor. There are several pathways through which this can occur; the one used by *Desulfovibrio* is shown below. The hydrogen gas is a by-product of fermentation reactions; sulfate reduction is important because it allows growth to continue in even the most energy-poor environments.

$$SO_4^{2-} + H_2\,(g) + 8\,H^+ \rightarrow H_2S\,(g) + 4\,H_2O$$

THIOSULFATE REDUCTION TEST DIRECTIONS

1. Using an inoculating needle, jab one of your isolates into a Peptone Iron deep. Repeat with the other isolate and the other Peptone Iron tube.

2. Incubate the Peptone Iron tubes at your isolates' optimum temperatures.
3. If the medium turns black, this means that hydrogen sulfide was produced and reacted with the iron to form a black precipitate, indicating that the organism is a sulfur reducer.
4. If bubbles or cracks appear in the medium, this means that gas was produced during fermentation. H_2 is more disruptive than H_2S or CO_2, so this usually indicates the production of hydrogen (common for sulfur reducers).

METHYL RED/VOGES-PROSKAUER (MRVP) TEST

As described above, organisms must be able to ferment in order to continue growing after all terminal electron acceptors are exhausted. While many different patterns of fermentation exist, they can broadly be classed into those that produce acidic end products and those that do not. One major non-acidic fermentative pathway is the 2,3-butanediol pathway, which produces the compound acetoin (one of the primary flavor molecules in butter) as a waste product. The MRVP test simultaneously tests for the presence of low pH (with the indicator methyl red) and acetoin in organisms that growing on glucose in poorly-buffered media. A positive VP test is a traditional diagnostic test for *Enterobacter* species. Along with the Indole and Citrate tests in the next section, MRVP forms the classical IMViC diagnostic series.

DIRECTIONS

1. Inoculate two MRVP tubes with each of your unknown organisms.
2. Incubate at each organism's optimum temperature until growth is observed. If no growth is observed after three class periods, proceed with the following steps anyway.
3. To one tube of each organism, add 5 drops of methyl red. If the medium turns red, this indicates that the pH of the medium is < 4.4 and most of the glucose has been converted into acid.
4. To the other set of tubes, add 12 drops of VP reagent I and 3 drops of VP reagent 2.
5. Cover each tube with a parafilm square and gently mix. Leave open to the air for 15-30 minutes to oxidize any acetoin present. A positive VP test will develop a pinkish-red color and indicates that the cells are converting acidic fermentation products into neutral acetoin.

CATALASE TEST

Organisms that are able to tolerate the presence of oxygen have to also be able to detoxify toxic **reactive oxygen species** that always form when oxygen gas is present. One of these toxic compounds is hydrogen peroxide, and most aerobic organisms produce an enzyme called catalase that **disproportionates** peroxide into water and oxygen gas:

$$H_2O_2 \xrightarrow{\textit{Catalase}} H_2O + O_2$$

Catalase is easily detected because, in the presence of hydrogen peroxide, it produces vigorous bubbling due to the release of oxygen gas. If you have ever observed bubbles after applying hydrogen peroxide to a cut, you have seen catalase (from your red blood cells) at work.

DIRECTIONS

1. To perform the catalase test, place a single drop of hydrogen peroxide on an isolated colony of *Staphylococcus epidermidis*. You should see vigorous production of bubbles as *S. epidermidis* converts the peroxide to oxygen gas. This is your positive control – *S. epidermidis* is a strong catalase producer.
2. Perform the catalase test on isolated colonies of your unknown organisms. Record the level of catalase activity as follows:
    a. "-" if you haven't seen any bubbles form after 60 seconds
    b. "+/-" if you see a very few bubbles after 60 seconds
    c. "+" if you see significant bubble formation within 10 seconds
    d. "++" if the colony immediately foams up like *S. epidermidis*.
3. Record the catalase result in your notebook.

8.B.ii. CARBON CATABOLISM

YOUR TEAM WILL NEED

- *Your unknown organisms growing* **as lawns** *on agar plates*
    - *NOTE: these plates must be prepared specially one lab session before performing this experiment.*
- *Your original soil sample*
- *3 x 9.9 mL sterile saline blank*
- *1 Biolog EcoPlate*
- *P200 and P1000 pipets and tips for each*
- *Cell spreader and ethanol*
- *2 Simmons Citrate slants*
- *2 MIO deeps*
- *Kovacs' Reagent*

The Biolog EcoPlate contains 32 different carbon sources, many of which are common in natural environments. Each well contains a single carbon source (see Figure 8.1), inorganic salts and trace metals, and a tetrazolium dye that will develop a rich purple color if an organism inoculated into the well is able

BIOLOG Microbial Community Analysis

EcoPlate™

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

FIGURE 1. Carbon Sources in EcoPlate

Figure 8.1. Pattern of carbon substrates on the EcoPlate.

to metabolize the carbon source. Each plate has 3 replicate wells of each carbon substrate; you will inoculate a plate with each of your isolates as well as a suspension made from the soil you isolated your organism from.

You will also be testing for the ability to use tryptophan and citrate as a sole carbon source, as these are major diagnostic features for commonly isolated microorganisms. Simmons citrate agar contains a pH indicator that turns blue under high pH; metabolism of citrate releases carbonates that alkalinize the medium. The MIO (Motility/Indole/Ornithine) deep scores three phenotypes: motility, tryptophan catabolism, and amino acid decarboxylation. Motility will be observed as growth extending outward from the stab point. **Indole** is produced as a byproduct of catabolizing the aromatic amino acid tryptophan and is detected by a cherry red color after addition of Kovacs reagent. **Ornithine** is a simple amino acid, but not one of the 20 found in organisms. Nevertheless, the same enzymes that catabolize the carboxyl groups of amino acids can also catabolize ornithine, leaving putrescine as a by-product. Because putrescine is basic, this causes the pH of the medium to increase and the pH indicator bromocresol purple turns purple. Beware! Putrescine smells as bad as you think it would with a name like that…

DIRECTIONS

1. The lab session prior to performing this experiment, make an extra agar plate for each of your organisms. These plates should be streaked as **lawns**.
    a. Using your loop, streak an *entire* plate with some growth of the organism
    b. Rotating the plate 90 degrees, repeat, streak the entire plate again with some more growth.
    c. Do this two more times, rotating the plate 90 degrees each time. The goal is to cover the entire plate with growth instead of selecting for isolated colonies.
2. The day you will perform this experiment, check your lawn plates to make sure they are free of contamination. If you see any evidence of multiple organisms on the plate, repeat step 1 and try again next class period.
3. Place 2 mL of sterile saline from one of the saline tubes directly onto one of your plates. Using the cell spreader, "scrape" as much growth as you can off of the agar and into the saline.
4. Using the P1000, remove as much saline + culture as you can back into the saline tube.
5. Repeat steps 2-4 with the other organism.

6. Place approximately 1 g of soil into the third saline blank. Mix by vortexing for 1 minute, then allow the soil to settle out of suspension.
7. Using a fresh tip for each well, inoculate wells A1-H4 in your EcoPlate with 200 μL of the saline suspension for your first isolate. Repeat with the second isolate in wells A5-H8. Inoculate wells A9-H12 with 100 μL of your soil suspension.
8. Incubate the EcoPlate at room temperature.
9. Streak each of your organisms on a Simmons citrate slant and incubate at the organism's optimum temperature.
10. Use an inoculating needle to inoculate a MIO deep with each of your organisms by stabbing it deeply into the agar. Incubate at your organism's optimum temperature.
11. In the next lab session, examine your Simmons citrate and MIO tubes. A blue color in the citrate tube indicates use of citrate, and a purple color in the MIO deep indicates amino acid decarboxylation.
12. Add 4-5 drops of Kovacs' Reagent to the MIO deep. The formation of a cherry red color indicates that the culture was able to metabolize tryptophan and produce indole.
13. Inspect your EcoPlate after 1 week. For each carbon substrate, classify each isolate (and the complete soil community) as either a non-user (no color), a slow user (weak purple color) or a strong user (rich purple color).

8.B.iii. MOTILITY and CHEMOTAXIS

Some bacteria sit still and let dinner come to them -- or more precisely, rely on physical forces like water currents, wind, or the movement of animals to get them from point A to point B. However, many bacteria are pretty good at moving around on their own. There are several modes of motility in nature, although here we are mostly concerned with the fastest: flagellar swimming (unicellular) and swarming (social). Microbes use their flagella and sensor proteins in their membranes to navigate chemical gradients, swimming toward things they like (food, light, relatives) and away from things they don't like (poisons, high temperatures, competing organisms) in a process called ***chemotaxis***. At its heart, the genetic and enzymatic mechanisms of motility are relatively simple, but they can be "tweaked" in many ways, yielding a bewildering variety of behaviors that are fine-tuned to the particular organism, its unique metabolic requirements, and its social relationships to the other members of its community. Here we will look at the type of motility (if any) that your isolates have as well as whether they like to swim toward or away from particular carbon sources, and also how they respond to other organisms in their environment.

YOUR TEAM WILL NEED

- *Your unknown organisms growing on plates*
- *Sterile saline*
- *Sterile toothpicks*
- *Sterile filter paper discs*
- *Sterile empty petri dish*
- *2 low-carbon swim agar plates*
- *3 R2A or BHI swim agar plates*
- *Forceps*
- *Broth cultures or saline suspensions (see previous experiment) of your isolates*
- *Eppendorf tubes containing 50 μL each of:*
    - *1% glucose*
    - *1% serine*
    - *1% mannose*
    - *1% phenol (hazardous, use caution)*
- *P20 pipetter and tips*

DIRECTIONS

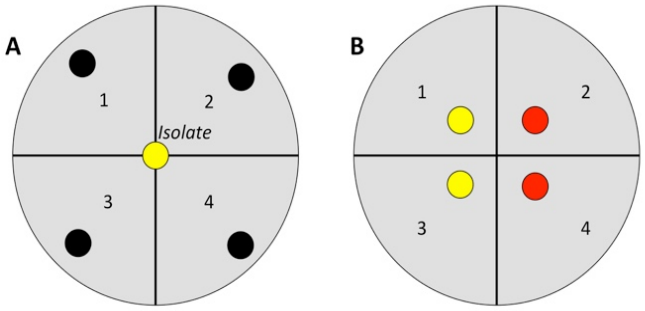


Figure 8.2. Inoculation patterns for swim agar plates.

1. BE CAREFUL WITH THE SWIM AGAR PLATES! The agar is very soft and will break if you jostle the plates too much. Also, they cannot be inverted like normal plates.
2. CAREFULLY divide each plate into quadrants with a sharpie. Hold it over your head and mark on it without turning it upside down. Label the quadrants 1, 2, 3, and 4.
3. Immerse the tips of your forceps in ethanol and flame them like you would a cell spreader. Place 14 sterile filter paper disks into the empty petri dish using the forceps. Make sure they are cleanly separated from each other.
4. Place 10 μL of glucose directly onto two of these disks. Repeat with the other carbon substrates and different disks.
5. Flame your forceps again. As shown in **Figure 8.2A**, place a disk of glucose near the outer edge of quadrant 1 on one low-carbon plate for each isolate. Repeat with serine in quadrant 2, mannose in quadrant 3, and phenol in quadrant 4.
6. Now put 10 μL of each of your isolates onto 4 sterile discs.
7. Flame your forceps, then place one of these discs in the exact center of the low-carbon plate. Place another in the exact center of an R2A (or BHI) swim agar plate. Repeat with the other isolate.
8. For the second R2A swim agar plate, place discs of your isolates in each quadrant as shown in **Figure 8.2B** (yellow dots indicate isolate 1, red dots indicate isolate 2). Make sure each spot is facing the same organism in one direction and the opposite organism in the other direction. The spots should be relatively close to the center of the plate.
9. Place the plates **lid-up** and incubate at the optimum temperature for each isolate. If the two isolates on the mixed plate have different temperature optima, place the plate at the lower of those two optima.
10. Observe the plates over the next couple of weeks. Record whether they are motile, and if so, what macroscopic form the motility takes (see Fig. 8.3 for example).

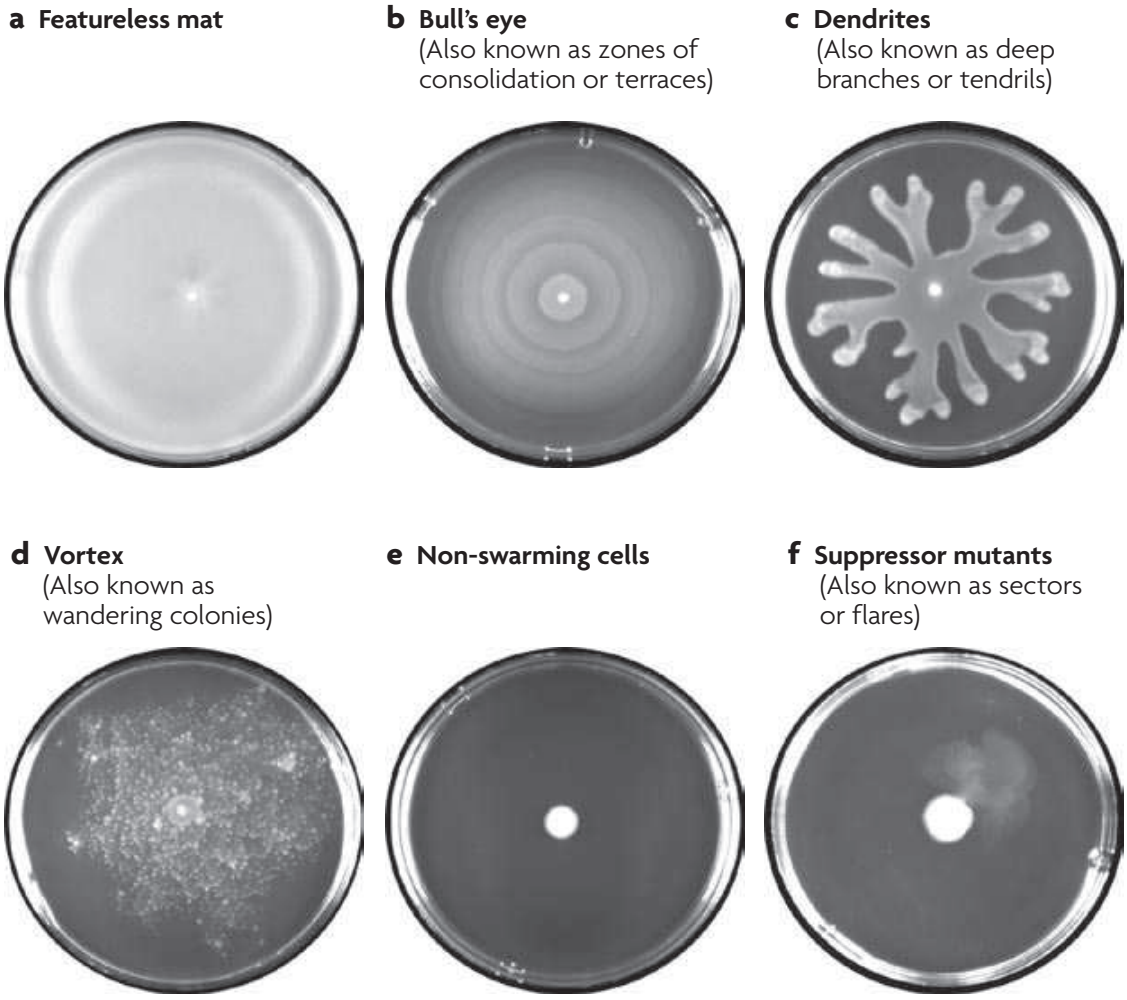


Figure 8.3. Some patterns of motility on swim agar. From Kearns 2010, Nature Reviews Microbiology

Test	Positive	Negative
Oxidase	*Bacillus subtilis*	*Enterobacter aerogenes*
Nitrate	*Enterobacter aerogenes, Pseudomonas aeruginosa*	*Lactococcus lactis*
Peptone Iron (H_2S Production)	*Proteus vulgaris, Escherichia coli*	*Enterobacter aerogenes*
Methyl Red	*Escherichia coli*	*Enterobacter aerogenes*
Voges-Proskauer	*Enterobacter aerogenes*	*Escherichia coli*
Simmons Citrate	*Citrobacter freundii*	*Escherichia coli*
MIO Indole	*Proteus vulgaris*	*Enterobacter aerogenes*
MIO Ornithine Decarboxylase	*Enterobacter aerogenes*	*Proteus vulgaris*
Swim agar	*Bacillus subtilis, Pseudomonas aeruginosa, Proteus vulgaris*	*Staphylococcus aureus*

Controls: The TA should inoculate these cultures as controls so the students can see different test results.

Required cultures:

Bacillus subtilis

Citrobacter freundii

Enterobacter aerogenes

Escherichia coli

Proteus vulgaris

Pseudomonas aeruginosa

Lactococcus lactis

Staphylococcus aureus

8.C. CLINICAL IDENTIFICATION

For the first two centuries that microbiology was practiced, there were no molecular methods for identifying microbes. Instead, researchers and clinicians identified them with a battery of tests that, while not as precise or as phylogenetically broad as PCR, became extremely useful for differentiating the important pathogens that infected humans, other animals, and plants. Here you will get a taste for what clinical microbiologists have done for decades – and still do today in most hospital labs.

YOUR TEAM WILL NEED

- The collected results from all of the experiments in Chapter 8
- *Bergey's Manual of Determinative Bacteriology, Ninth Edition*

DIRECTIONS:

1. *Bergey's Manual* is a compendium of most of the well-characterized prokaryotic taxa that contains the guidelines necessary to distinguish different groups. Early chapters distinguish between the major divisions of microbial life. In Chapter IV, *Bergey's* presents "The Four Major Categories of Bacteria" which will be your first decision. The Categories are:
    a. Gram-negative bacteria with cell walls
    b. Gram-positive bacteria with cell walls
    c. Bacteria without cell walls (the mycoplasmas)
    d. Archaea

    Based on the methods we used to isolate your organisms, you almost certainly didn't isolate a mycoplasma. You probably didn't isolate an archaeon, and if you got a positive 16S PCR product, you definitely didn't (the primers we used only work on bacteria). Therefore your first division is based on your Gram stain. Keep in mind, however, that many organisms are Gram-variable.

2. Chapter V divides these major groups into their many sub-groups based mostly on their morphology and ability to use/tolerate oxygen. Since we culture our isolates in normal atmospheric air (with oxygen) and in pure cultures (i.e. without any eukaryotic host cells), we know you don't have any obligately anaerobic bacteria or obligate intracellular parasites, which eliminates many of these groups (Groups 6-9). Assuming we don't have archaea or mycoplasmas, we can also eliminate Groups 30-35. Among the remaining groups, Groups 1-5 and Groups 10-16 are Gram Negative,

and Groups 17-29 are Gram Positive. Read the descriptions (pp 17-20) to see which group fits your organisms the best, and go to the indicated page to read more. Some key characteristics that are likely to be important:

   a. Was your organism able to grow in the anaerobe jar? If so you can exclude Groups 2, 4, 12, 14, 16, and 21.
   b. Does your organism produce endospores? Then it is a Gram Positive bacterium in Group 18 (regardless of what your Gram stain result told you).
   c. What shape does your organism have? Cell shape as well as the presence of multicellular structures are major keys to the various Groups.
   d. The most common isolates from soil come from Groups 4-5, 17-18, 20, and 22-29 (the Actinomycetes).
   e. In particular, the Actinomycetes are very common in soil, very diverse, and often hard to diagnose. It's worth investigating the chapter on these organisms (p. 605) to see if any of them fit your isolate, especially if you are having difficulty pinning down what you have.

3. In the sections corresponding to each group, there are a variety of charts showing how different genera/species respond to many of the tests you have done. In some cases (e.g. MRVP, catalase) you have done the exact test indicated. In others (e.g. your Biolog plates) you may have done tests that are equivalent; if in doubt ask your TA or use the Internet. Use your results to find the closest match in *Bergey's.* Does it correspond to your 16S ID?
4. Are there other tests you could do that would possibly differentiate between multiple groups? It is possible that we have the necessary reagents – ask your TA!

LAB WORKSHEET #4
"Who Am I? Why Am I Here?"

In these experiments, you collected information that allowed you to identify your isolates and gain some more insight into what ecological niches they occupy in nature. The information you have is: environmental tolerance ranges and optima (from Worksheet 2); carbon compounds utilized; motility patterns; respiratory TEAs and fermentation pathways used; and the taxonomic identification of your organism. Use that information to complete this worksheet.

1. Attach your gel photo. Label the lanes.

2. Report the 260/280 ratio of your purified PCR DNA and use it to estimate how pure your sample was.

3. How long was your quality-controlled sequence? What was its closest match in the NCBI Database? Was it unambiguous or were there numerous different species that couldn't be distinguished from each other with the information you collected?

4. What carbon compounds can your organisms metabolize? What type of molecules are these (e.g. lipids, carbohydrates, amino acids, etc)? What are some natural sources of those carbon compounds? Are you likely to find them in the soil?

5. Attach a photo of your gram-stained organisms. Identify them by their Gram result and cellular morphology.

6. Attach photos of your swim agar plates. Do your organisms swim, and if so how fast, and toward (or away from) what? How do you think this might affect their ecological role in the soil community?

7. What was your organism identified as by 16S? What was the closest match in the Bergey's manual? Do they agree? If not, what do you think caused the discrepancy?

8. What are some characteristics of your organism according to Bergey's manual and/or the Internet?

9. What terminal electron acceptors can your organism use? What kind of fermentation (if any) do they perform? Rank the respiratory and fermentative pathways for metabolizing glucose available to each isolate from most energy gained to least energy gained per mole of substrate.

10. Does your organism produce catalase? Does this phenotype fit with your observations of oxygen preferences from Worksheet 2?

11. Use one or more of the following terms to describe your organisms (mix and match prefixes and suffixes as needed): heterotroph, phototroph, chemotroph, organotroph, lithotroph, autotroph, nitrate reducer, sulfate reducer

12. Using as much of the above information as possible as well as information from the previous Worksheets, **state a hypothesis** about what your organisms' niches are. Some possibilities are things like "plant roots", "dead plant decomposer", "animal guts", "plant leaves", "human skin", etc etc etc. Explain your choice using the data you have presented, and **suggest an experiment** that would allow you to test your hypothesis.

13. Assume that you have prepared a professional manuscript about experiments designed to test what niche your organisms occupied. Write a "Discussion" section and a conclusion for that manuscript based on the results described above.

CHAPTER 9
ANTIMICROBIALS

Humans use a wide variety of antimicrobial compounds to try to restrict microbial growth. These range from chemical toxins like bleach and hydrogen peroxide to complex antibiotics like penicillin. These can all also occur as pollutants from human activity, and can also be produced by microbes. Overuse of some of these has been shown to favor the evolution of resistance in the population, and some strains are naturally more resistant than others for a variety of reasons. Here, we will assess levels of resistance to common and clinically important antimicrobials in your isolates using a test called a **disk-diffusion assay** (or, in the case of antibiotic disk-diffusion assays, a **Kirby-Bauer test**). We will also measure the resistance of your isolates to heavy metal solutions, since organisms that can resist these are also often resistant to antibiotics. Basically, small pieces of sterile filter paper are soaked with a defined concentration solution of the indicated antimicrobial compound. The disk is then placed on an agar plate that has been completely covered with bacteria to produce a **confluent lawn** of growth. Because the antimicrobial diffuses outward into the agar, the farther away a cell is, the lower concentration of the compound it encounters, and beyond a certain distance growth is essentially unaffected. After the bacteria have grown, resistance is measured by the diameter of this **zone of inhibition**, where no growth occurred, around the disk (Figure 9.1). **Broader** zones of inhibition indicate **lower** levels of resistance.

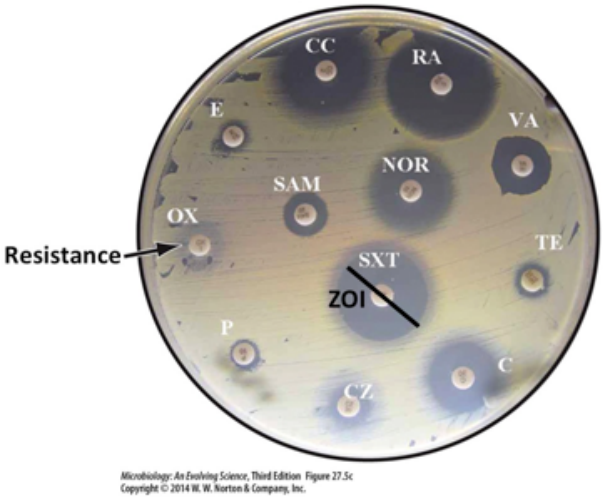


Figure 9.1. Kirby-Bauer disk diffusion assay. ZOI indicates Zone of Inhibition.

Microbes also *produce* antimicrobials. Indeed, the source of most of our clinically useful antibiotics is microbes themselves – especially the soil microbes of the genus *Streptomyces*. Selman Waksman, who discovered streptomycin and other antibiotics, used a technique called "cross-streaking" to determine if one bacterial strain was able to inhibit the growth of another. You will replicate his method here.

i. ANTIMICROBIAL RESISTANCE

YOUR TEAM WILL NEED

1. *Fresh plates of your isolates*
2. *18 R2A or BHI plates*
3. *Inoculating loop*
4. *Forceps*
5. *Antibiotic disc dispensers*
6. *Solutions of manganese, lead, and zinc (careful, these are toxic)*
7. *Sterile filter paper discs*
8. *Empty, sterile petri dishes*
9. *Digital caliper*

DIRECTIONS:

1. Label agar plates appropriately: 9 plates for each of your isolates. For 3 plates for each isolate, use a sharpie to divide the plate into three sectors and label these "Zn", "Mn", and "Pb".
2. Using your inoculating loop, completely cover the plates with growth from one of your isolates. Rotate the plates 90 degrees and repeat. Repeat twice more, rotating 90 degrees each time.
3. Using ethanol-flamed forceps, place disks containing the 8 antibiotics (Table 9.1) evenly across the surface of your plates, using 4 disks per plate. Put replicate disks of the same antibiotic on different plates.
4. Place 6 sterile filter paper discs into a clean petri dish. Place 10 uL of Zn solution on each, and then place them on the appropriate sectors of your plates. Repeat with the other metals.
5. Incubate each plate at the appropriate temperature for your isolate.
6. In the following class period, examine your plates. Measure the diameter of each zone of inhibition using digital calipers and record it in your notebook.

ANALYSIS:

a. Calculate the mean and 95% confidence interval for the zone of inhibition of each isolate for each compound.
b. Report these values for your two isolates in a table as mean +/- confidence interval.
c. Compute **unpaired t-tests** for each of your isolates vs. each other for each antimicrobial. Are your isolates significantly different from each other? Is one conspicuously more resistant than the other?

d. Share your data with the other teams in your section. Are there any antimicrobials that either of your isolates are conspicuously more resistant to than the other isolates? If so, why do you think that might be?
e. Consult the "ZOI" column in Table 1. Use **one-sample t-tests** to determine if either of your isolates are clinically resistant to any antibiotics. If so, look in the literature to see if you can find information on whether other closely-related organisms are also resistant. Are your isolates unusual?

Table 9.1. Antibiotics used in this experiment.

Antibiotic	Class	ZOI (mm)[1]	Mechanism of Action
Penicillin	β-lactam	<28/<14*	Cell wall synthesis
Ampicillin	β-lactam	<13/<28**	Cell wall synthesis
Cephalothin	Cephalosporin	<14	Cell wall synthesis
Chloramphenicol	Unique	<12	Protein synthesis
Erythromycin	Macrolide	<13	Protein synthesis
Gentamicin	Aminoglycoside	<12	Protein synthesis
Streptomycin	Aminoglycoside	<11	Ribosome proofreading
Vancomycin	Non-ribosomal peptide	<9	Cell wall synthesis

* Staphylococci/all other bacteria; ** Gram negative/Gram positive

[1]ZOI means Zone of Inhibition; any ZOI with diameter less than this value in considered clinically resistant.

ii. ANTIMICROBIAL PRODUCTION

YOUR TEAM WILL NEED

1. *Fresh plates of your isolates*
2. *6 R2A or BHI plates*
3. *Inoculating loop*

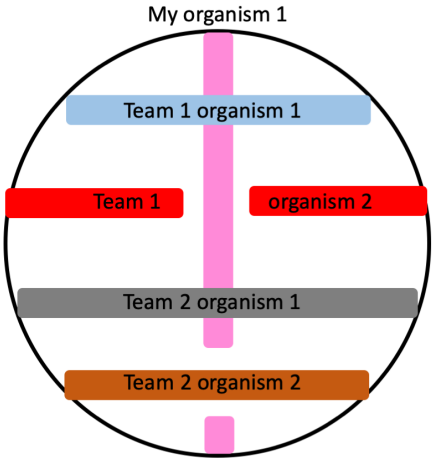


Figure 9.2. A streak plate testing your organism against the isolates from two other teams. Based on these results, your isolate inhibited Team 1's isolate 2, and Team 2's isolate 2 inhibited your organism.

DIRECTIONS:

1. You will make cross-streak plates to test your organisms out against each other and against the other isolates in your lab section (see Fig. 9.2). Make sure to label all plates appropriately.
2. On 3 plates, "paint" one of your isolates along a single line at the middle of the plate as shown. Repeat with the other isolate and the remaining 3 plates.
3. On one plate for each isolate, draw 4 lines of the opposite isolate perpendicularly across main line as shown. Flame your loop in between lines.
4. On the second and third plates, have the members of the other groups in your section draw perpendicular lines with their isolates. Make sure to record the names of each isolate on the appropriate plate so you know "who is who".
5. Incubate at an appropriate temperature (ideally one where all of the organisms can grow).
6. Inspect the plates the next lab period. Note any zones of clearance where two streaks interact. Also note any other interesting phenotypic changes.
7. Obtain the species IDs of any isolates that your organism inhibits, or that inhibit your organism. Are there any trends? Is your organism easy to inhibit, or does it inhibit many others?

LAB WORKSHEET #5
"Offense and Defense"

In these experiments, you measured your isolates' resistance to a variety of toxic compound, including antibiotics and heavy metal ions. Use the data you collected to answer the following questions. Make sure you also complete **either** Worksheet 5A (Stress Tolerance) or 5B (Competition) depending on which other set of experiments you performed.

1. For each antibiotic and toxic metal, make a bar graph comparing the average zone of inhibition for each of your isolates, with error bars representing the 95% confidence intervals. For each toxin, indicate if one isolate is significantly more resistant than the other (use **unpaired t-tests** to compare replicate measurements of the two isolates). Place asterisks above pairs that are significantly different: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

2. Is one of your isolates generally more resistant to antibiotics than the other? If so, is it also more resistant to heavy metals? Explain your answer.

3. For each isolate and each antibiotic, use a **one-sample t-test** (page 117) vs. the appropriate ZOI value for clinical resistance. Make a table with one row for each antibiotic and one column for each isolate; fill the cells with the p-values from these tests. Are any of your isolates **clinically** resistant to any of the antibiotics? If so, which ones?

4. Make a table with all of the class' isolates (including yours) in one column and two more columns with representing your isolates. For each pairwise interaction, indicate "++" if both organisms grew, "+-" if your isolate inhibited the other isolate, "-+" if the other isolate inhibited your organism, or "- -" if neither organism grew (they inhibited each other). In the bottom two rows, enter the total number of organisms that inhibited each of your isolates, and the total number of organisms each of your isolates inhibited.

Test Streak	Isolate 1	Isolate 2
Isolate 1	++	-+
Isolate 2	+-	++
Other team Isolate 1	+-	-+
Other team Isolate 2	+-	-+
Other team Isolate 3	++	-+
# Inhibited	3	0
# Inhibited by	0	4

5. Are either of your isolates antibiotic producers? If so, are they also resistant to antibiotics produced by other organisms? If in doubt as to which of your classmates' isolates are antibiotic producers, ask around.

Chapter 10
STRESS TOLERANCE

WHAT CAN YOUR BACTERIA WITHSTAND?

So far, we've put a lot of effort into understanding what your microbes "like" -- their favorite temperature and pH, what they like to eat, whether or not they like oxygen, and so forth. But like all living things, microbes spend a great deal of their time in sub-optimal conditions and even in environments so stressful they are unable to grow and face the real possibility of death. In many ways, microbes are more vulnerable to stress than larger organisms. For example, their high surface area to volume ratio means that toxic chemicals diffuse more rapidly from the environment into their cells; their slow swimming speeds make it hard for them to get away from adverse environments; and their unicellularity means they don't have the option to jettison damaged cells via apoptosis the way that multicellular animals and plants can. On the other hand, microbes also have distinct advantages that make them much more tolerant of some stresses. For instance, the relative simplicity of their genetics and metabolism make them less vulnerable to radiation and other mutagens; their tiny size helps them survive freezing; and their enormous metabolic flexibility relative to multicellular eukaryotes means that many microbes can rapidly adapt to radically changed environmental conditions that would be lethal to nearly all multicellular life.

Abiotic stresses like the above aren't the only stresses that microbes face – they also have to deal with other microbes (and macroscopic organisms) in their environment that want to outcompete them. Sometimes this direct competition takes the form of the two strains simply trying to grow faster than the other – but it often occurs that one or both attempt to directly kill the competitor, using antibiotics or other strategies. In environments where antibiotics are being used, organisms also often develop resistance adaptations, leading to an evolutionary arms race between different strains.

As with any other trait, stress tolerance and antibiotic production/resistance varies between microbial strains. It should not surprise you to learn that more stressful environments tend to select for organisms with higher stress tolerance. For instance, cyanobacteria from the ultra-cold and dry Antarctic Dry Valleys have world-record levels of tolerance to desiccation and freezing. However, it might surprise you to learn that they also have exceptionally high tolerance to many other stresses, including oxidative stress and UV radiation, despite these not being particularly important in their natural habitat. In many cases, resistance to one type of stress leads to cross-resistance to many other kinds, because the stress response mechanisms are related. Whether stress comes from heat, cold, radiation, osmotic shock, or antibiotics, microbial survival is enhanced by very

similar adaptations, including antioxidants, chaperone proteins, and DNA repair machinery.

Understanding microbial stress tolerance and competition is of great importance both to our understanding of the biogeochemical role of microbes in the environment and to our engineering of human environments to minimize disease. How does microbial activity change in frozen lakes, or in globally warming oceans? How quickly does a germicidal UV lamp sanitize a medical work surface? How much penicillin does it take to reliably kill a pathogen? These questions will have different answers for different strains. In these experiments we will see how tough *your* isolates are, and consider how that might affect their role and competitive ability in their natural environment.

NOTE: These experiments require ***liquid cultures*** of your isolates. If your organisms grow poorly in liquid media, you will need to resuspend colonies in saline to achieve a liquid culture (see experiment 8.B.ii above).

SPOT TITER PLATING

You've done many viable-count spread plates this semester. Some of the experiments here will use a similar technique, but designed to look at *many* dilutions on a *single* plate. The reason for this is that we will not have a very good idea how many living bacteria are in a sample, because we are actively trying to kill them off. The "spot titer" plates you will make here are not as *accurate* as full spread plates because you often can't count individual colonies, but you can see growth over a much wider range of dilutions. Whenever an experiment calls for you to set up a spot titer plate, use the following protocol, which is demonstrated graphically in Figures 10.1 and 10.2.

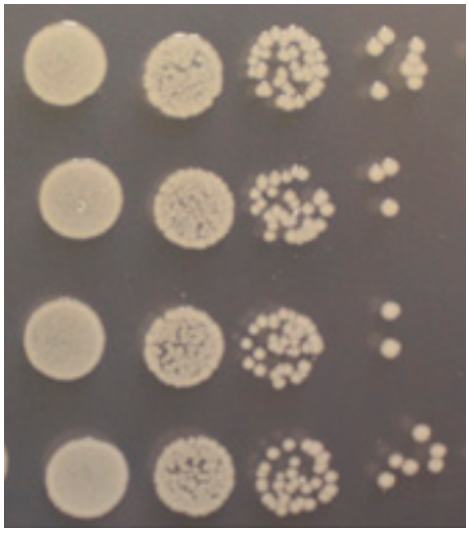


Figure 10.1. Spot titer rows.

DIRECTIONS

1. You can put up to 4 rows of 4 spots each onto a single plate. Each row represents a single series of 4 dilutions for one culture. See **Figure 10.1** for an example.
2. You will titrate cultures into sterile saline droplets placed inside empty, sterile petri dishes. For each plate you wish to make, take one petri dish (bottom or

lid) and make 4 rows containing 4 90 uL droplets each of sterile saline. The droplets will hold together because of surface tension.

3. If you are titrating from a dense overnight culture, you will need to first dilute the culture by placing 100 uL into a 9.9 mL sterile saline dilution blank and vortexing (Fig. 10.2A). If you are starting from a non-opaque culture, you do not need to perform this pre-dilution step.
4. Make sure to label your agar plates such that you know which row corresponds to which culture/treatment.
5. For the first culture to be diluted, place 10 uL from the culture (or the pre-dilution tube) into the top left saline droplet (Fig. 10.2B). Only press down to the first stop on your pipet to avoid splattering liquid. Pipet up and down to mix.
6. Change tips and then pipet 10 uL from the first droplet to the droplet immediately to its right (Fig. 10.2C).
7. Repeat step 6 for the final two droplets in the row. Using the last tip and moving right to left, place 10 uL from each droplet in a row on the agar plate (Fig. 10.2D). Again, the surface tension should hold the droplets together, but be careful not to jostle the plate until the drops have absorbed into the agar.
8. Proceed with the second culture and the second row of droplets; continue until all four cultures have been diluted and placed on the agar plate. Let the plate sit lid-up until the droplets absorb into the agar, then invert and place in the appropriate incubator.

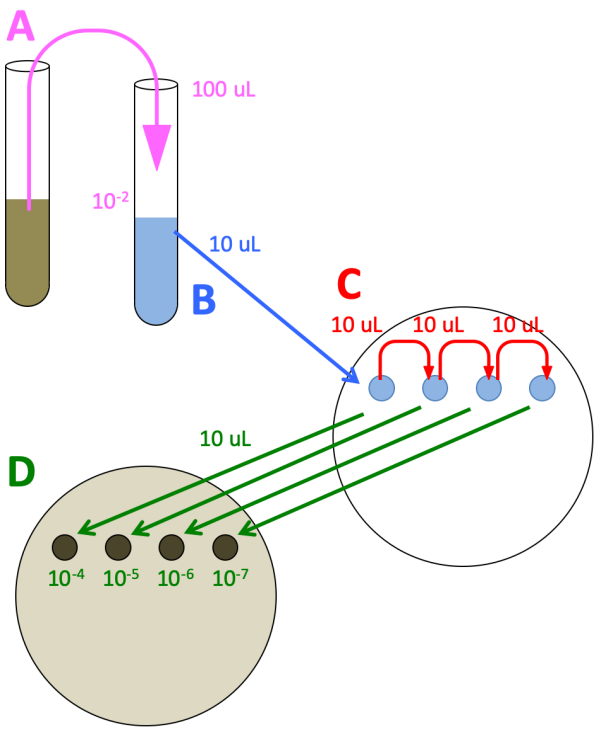


Figure 10.2. Spot titer plating. A culture is first diluted 100-fold into sterile saline (A), then sequentially diluted 4 times in a 96-well plate (B and C) before being "spotted" onto an agar plate (D).

i. HEAT SHOCK

All organisms have an optimal temperature, where growth and metabolism are maximal and stress is minimal. However, it is common for organisms to spend some time in environments that deviate from this optimum. When temperatures change gradually, many organisms can continue to grow across a very wide range of temperatures. However, sudden shifts in temperature are much more difficult to accommodate, and this is especially true for very small organisms whose internal temperatures equilibrate rapidly with the external environment. As an example of problems that "heat shock" can cause, *E. coli* can be transformed with foreign DNA by heat shocking it, because the sudden temperature change actually causes holes to open up in its cell membrane! Here, we will test your organisms to see how tolerant they are to heat shock.

YOUR TEAM WILL NEED

1. Your isolates in sterile saline at approximately 10^7 CFU/mL
2. P1000, P200, and P20 pipets and tips
3. Thermal cycler
4. Sterile PCR tubes x 8
5. Ice bucket
6. Sterile saline and sterile petri dishes for spot-titering
7. 2 R2A or BHI plates

DIRECTIONS

1. Place 50 μL of each of your isolates into 4 separate sterile PCR tubes.
2. Place 3 of the tubes in the thermal cycler. Put the other tube in an ice bucket.
3. Set the PCR thermal cycler to run the "heat shock" program. Program it to use a maximum temperature 10 C warmer than the optimal temperature for your organism. The program will start with a 15 minute incubation at 4 C, then rapidly raise the temperature rapidly to the heat shock temperature and hold it for 3 minutes before returning to 4 C.
4. If necessary, run the cycle separately for your two organisms.
5. After the cycle runs, take all of your tubes and make spot titer plates with all 3 replicates and the control for one isolate onto a single plate.
6. Incubate the titer plates at the appropriate temperature for your isolate.
7. In the next class period, quantify each dilution series. Pick the most dilute spot titer that has growth and attempt to count the colonies (best guess is okay if they are overgrown). Convert each count to CFU/mL.

ii. FREEZE/THAW TOLERANCE

Freezing is almost always lethal for multicellular organisms, and even for larger, eukaryotic microbes. This is because the microscopic structure of ice works like spears to disrupt membranes, killing cells. In order to survive freezing temperatures, cells have three options. First, they can depress the freezing temperature in their local environment, for instance by secreting solutes -- analogous to how humans salt the roads when it snows. Second, they can control the structure of the ice, using ice-nucleating proteins to channel the ice crystals around critical membranes and prevent ice damage. Bacteria that generate these types of proteins are common in the atmosphere and in many cases form the core of hailstones. They are also found growing *very* slowly in brine channels a mile beneath the surface of Antarctic glaciers. Here, we will measure your isolates' ability to survive being frozen.

YOUR TEAM WILL NEED

1. Your isolates in sterile saline at approximately 10^7 CFU/mL
2. P1000, P200, and P20 pipets and tips
3. Sterile Eppendorf tubes x 8
4. Room temperature water bath
5. Floating tube rack
6. Liquid nitrogen
7. R2A/BHI plates x 4
8. Sterile saline and petri dishes for spot titers

DIRECTIONS

1. Place 100 μL of each of your isolates into four separate sterile Eppendorf tubes.
2. Place three tubes of each organism in the floating tube rack. Give to your TA to immerse in liquid nitrogen. Leave the fourth tube on the bench.
3. Remove the tubes from liquid nitrogen and immerse them in room temperature water to quickly unfreeze them.
4. Do spot titer plates for each tube.
5. Repeat steps 2-4.
6. Incubate the titer plates at the appropriate temperature for your isolate.
7. In the next class period, quantify each dilution series. Pick the most dilute spot titer that has growth and attempt to count the colonies (best guess is okay if they are overgrown). Convert each count to CFU/mL.

iii. ULTRAVIOLET RESISTANCE

The majority of life on earth depends on light for its existence. Plants, algae, and photosynthetic bacteria use pigments to extract energy from light and use it for chemical work, including the fixation of carbon by photosynthesis. However, the same energetic properties of light that drive metabolism can also cause cellular damage, and the higher energy, shorter wavelengths of ultraviolet light are much more destructive than visible light. UV can cause damage directly, for instance by modifying DNA bases (Figure 10.3). It can also cause indirect damage by ***photo-oxidizing*** small carbon compounds, producing free radicals that can non-specifically attack most biological molecules.

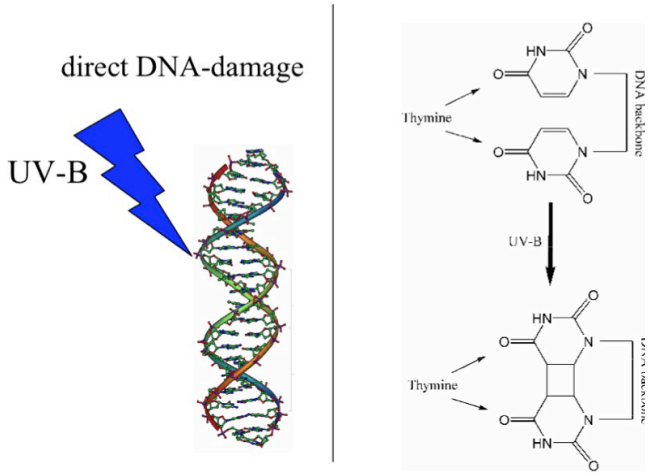


Figure 10.3. Thymine dimers caused by UV radiation

Microbes (and other living things) have a number of defenses against UV damage. First, they can use pigments and other antioxidant chemicals to intercept high-energy photons and trap their energy in non-reactive forms. As an example, the orange pigment β-carotene found in plants is capable of directly absorbing UV photons and dispersing them as harmless heat, and can also detoxify light-produced free radicals. Second, organisms can counter UV damage with general stress resistance enzymes such as catalase and chaperones. Third, many organisms express DNA repair enzymes that specifically target the types of damage caused by UV. A key example found in many bacteria is ***photolyase***, which uses energy from visible light to break apart thymine dimers. We will be specifically testing for the activity of photolyase in this experiment.

YOUR TEAM WILL NEED

1. *Your isolates in sterile saline at approximately 10^7 CFU/mL*
2. *P1000, P200, and P20 pipets and tips*
3. *Handheld UV lamp*
4. *Face shield*
5. *Empty petri dishes x 4*
6. *Sterile saline and petri dishes for spot titers*
7. *Aluminum foil*
8. *6 R2A/BHI plates*

DIRECTIONS

1. Take four sterile, empty petri dishes. Pipet 100 μL of each of your organisms into well-separated spots in each. The surface tension of the spot should hold the drops together. Make sure you know which spot corresponds to which organism.
2. Take a piece of aluminum foil and cover one of the petri dishes as thoroughly as you can without disturbing the drops.
3. Wearing a face shield and being cautious not to expose any of your classmates, use the hand-held UV lamp to expose one of the uncovered dishes to 10 seconds of UV light. Hold the lamp approximately 6 inches above the plate. Have a teammate time the exposure with a stopwatch.
4. Repeat with one of the covered plates. Immediately replace the aluminum foil as soon as the exposure is finished.
5. The remaining two plates are light and dark controls; do not expose them to UV.
6. Make spot-titer plates for each droplet as described above. Do titer rows for all 4 treatments of the same organism on the same plate.
7. After 10 minutes, repeat steps 3-6 but increase the exposure time to 30 seconds. Make sure to re-expose the SAME two plates, leaving two unexposed plates as controls, and make sure to replace the foil on the dark-incubated plates.
8. Repeat once more, increasing exposure time to 90 seconds.
9. Incubate all plates at the appropriate temperature for the isolates.
10. In the next class period, quantify each dilution series. Pick the most dilute spot titer that has growth and attempt to count the colonies (best guess is okay if they are overgrown). Convert to CFU/mL.

iv. OSMOTIC SHOCK

Like temperature, all organisms that live in liquid environments have an optimal solute concentration where metabolism and growth are maximal, and respond to sudden changes in their osmotic environment as stresses. The media we have been growing your organisms in has a low solute concentration; we will test how they respond to being suddenly introduced to either a VERY low solute environment, or a very high solute environment.

YOUR TEAM WILL NEED

1. Your isolates in liquid medium at > 10^7 CFU/mL
2. P1000, P200, and P20 pipets and tips
3. *2 x 1 mL Eppendorf tubes of sterile R2A, ultra-pure water, artificial seawater, and 30% NaCl*
4. *6 R2A/BHI plates*
5. *Petri dishes and sterile saline for spot titers*

DIRECTIONS

1. Place ~10 μL of each of your organisms into one tube of each salt concentration. Vortex to mix.
2. Incubate at room temperature for 15 minutes, then perform spot-titers for each tube, placing all 4 treatments for the same organism on a single plate.
3. After 45 minutes, do another set of spot-titers.
4. Incubate the titer plates and the tubes at the appropriate temperature for your isolate.
5. In the following class period, perform a final series of spot-titers for each tube and incubate the resulting plate at the appropriate temperature.
6. Quantify each dilution series. Pick the most dilute spot titer that has growth and attempt to count the colonies (best guess is okay if they are overgrown). Convert to CFU/mL.

ANALYSES for experiments i-iv:

1. For UV and osmotic shock experiments:
   a. For each of your isolates and each treatment, calculate the slope of the regression line for log (CFU/mL) vs. time of exposure using the **`linest`** function in Excel (Appendix 5).
   b. For each isolate, make a bar graph of UV killing efficiency showing the negative of the slope for each treatment (light and dark control, light and dark UV exposure) along with the 95% confidence interval of the slope as error bars. Are the slopes significantly different (i.e., do the error bars overlap)? Does UV have an effect on your isolates? Is one isolate more resistant than the other?
   c. For each isolate, make a bar graph of osmotic shock killing efficiency showing the slope for the control and each salt level along with the 95% confidence interval of the slope as error bars. Are the slopes significantly different? Is one of your isolates more resistant to osmotic shock than the other?
   d. Use the UV graph to predict whether your isolates express photolyase.
   e. Share your data with the other teams in your section. Are either of your isolates conspicuously resistant in comparison to other groups? If so, why do you think that is?

2. For heat shock and freeze-thaw experiments:
   a. For each replicate of each isolate, calculate survival as the percentage of CFU/mL in the treatment culture vs. the non-heat shocked control. Calculate the mean and 95% confidence interval of this value.
   b. Plot percent survival for both you isolates as a single bar graph with error bars.
   c. Use t-tests to determine if your isolates are different than each other.
   d. Share your data with the other teams in your section. Are your isolates conspicuously more resistant than other isolates? If so, why do you think this might be?

LAB WORKSHEET #5a
"Anxiety"

In these experiments, you investigated the response of your organisms to two of four different stress conditions. Use your data to answer the following questions.

1. For the two tests you chose to perform, provide the required graphs and statistical tests described in the "Analysis" section.

2. Are either of your isolates significantly more resistant to these stresses than the other? Compare the error bars -- do they overlap? Is the same isolate more resistant to both? Is there a correlation between stress resistance in these experiments and your isolates' antibiotic and metal resistance profiles (Worksheet 5)?

3. Based on the stress tolerance and antimicrobial profile of your organism, **refine the hypothesis** you stated in Worksheet 4. If either of your isolates is strongly resistant and/or strongly inhibitory, how does this give them an advantage? If they are generally susceptible to damage, why do you think they can get away with this? What does this say about their ecology?

4. Write a "Methods" section for these experiments (both the Antimicrobials and Stress Tolerance experiments).

CHAPTER 11
MICROBIAL ~~COMBAT~~ COMPETITION

Like all organisms, microbes have evolved for billions of years, adapting to the many physical and chemical environments offered by planet Earth. In fact, microbes evolve much more rapidly than larger organisms because of their short generation times and vast population sizes. For this reason, it's a good bet that the most abundant organisms in an environment -- i.e., the ones you're most likely to isolate -- are very well adapted to that environment. However, if they are coexisting, it's also a good bet that they are differentiated somehow based on their metabolic requirements. In other words, coexisting organisms generally occupy different **niches**. Traditionally, ecological theory predicts that only one species can occupy a single niche in a single place at a time, an idea called **the competitive exclusion principle**.

In this experiment, we'll learn how to measure the fitness of two organisms using direct head-to-head competitions, which will give us insights into what niches different organisms occupy. To do this, your team and another team will choose isolates to "fight" in different environments like microscopic gladiators.

By now you've done quite a few tests on your two environmental isolates. You know your isolates' fundamental niches, and you know how resistant they are to a variety of stresses and whether or not they You've probably seen some tests that suggest scenarios where one isolate grows better than the other in a certain environment. You may have also noticed that other teams' isolates also have different metabolic abilities and environmental preferences; some are similar to yours, others are quite different. In other words, the organisms have different niches, and we can hypothesize that, if forced to share a niche, one will competitively exclude the other.

In this exercise, you and one other team will pick a pair of isolates to wage war against each other in two different arenas of your choosing.

MICROBIAL COMPETITION RULES!

1) *You must choose isolates that produce colonies that can be clearly distinguished from each other on agar media. There are two ways to make this happen:*
    a. *Pick isolates that make very different colonies (color, shape, or both) on a common medium, or*
    b. *Pick isolates that each have a particular type of medium where only one of them will grow at all.*

2) *You will pick two environments (or "arenas") for the battle to take place. Each team gets to pick one environment. You should pick your environment such that you expect your warrior to "win the battle". Some ideas:*
    a. *Different temperature, pH, or salinity*
    b. *Nutrient broth supplemented with different carbohydrates*
    c. *Anaerobic vs. aerobic growth*
    d. *Solid vs. liquid media*
    e. *Environments exposed or not exposed to some sort of stress, like heat shock or UV*

3) *Both arenas have to support the growth of each organism*

4) *Choose your isolates carefully -- it's okay to play dirty tricks, like picking antibiotic producers.*

Competition Day -1:

DIRECTIONS

1. Decide what arenas you want to perform your competitions in, figure out what media and resources you will need and how much, and tell your TA.
2. Write down your predictions about which isolates will prevail in each arena.

Competition Day 0:

DIRECTIONS

1. Label 2 tubes (or plates) of each competition medium.
2. Inoculate each competing isolate BY ITSELF into one tube or plate.
3. If isolates are to be grown in broth, inoculate with a single isolated colony.
4. If isolates are to be grown on plates, streak for a confluent lawn.
5. Incubate the organisms under the competition conditions. They will stay there until you begin the competition. Note that this step is to acclimate the cells to the competition conditions, so that they start out "on a level playing field" metabolically.

Competition Day 1:

YOUR TEAM WILL NEED

- *3 tubes/plates of each competition medium -- these are the **arenas***
- *18 quantification plates (probably R2A or BHI, but can vary)*
- *12 9.9 mL saline dilution blanks, plus 2 extra for each competition done on solid media*
- *Extra sterile saline*
- *Acclimated unknown cultures*
- *Sterile Eppendorf tubes*

DIRECTIONS

1. Label 3 arenas "Cond1-1" through "Cond1-3" meaning, for instance, "Condition 1, replicate 1". Label the other 3 arenas "Cond2-1" through "Cond2-3". Also include your teams' names on your tubes/plates.
2. Label your dilution blanks "C1-1" through "C2-3", #1 and #2. In other words, 2 dilution blanks per arena.
3. Label quantification plates "C1-1" through "C2-3" #1, #2, and #3. In other words 3 plates per arena. Also write "Day 1" on each plate.
4. FOR COMPETITIONS DONE IN LIQUID MEDIUM ARENAS:
    a. Vortex your acclimated isolates cultures to mix.
    b. Add approximately 10^6 CFU/mL of each isolate to each arena tube.
    c. Vortex arena tubes. Pipet 100 μL from each arena tube into the appropriate #1 dilution blank.
    d. Place tubes in appropriate incubation conditions.
5. FOR COMPETITIONS DONE ON SOLID ARENAS:
    a. Pipet 1 mL of sterile saline from one dilution blank onto each acclimated unknown plate.
    b. Using a cell spreader, carefully resuspend the bacterial growth from the unknown plate into the saline. Tilt the plate so that the saline collects at the bottom, and "wash" the agar surface to get as much growth as possible into the saline.
    c. Pipet as much of the saline as you can off of the plate and into an eppendorf tube.
    d. Label 2 dilution blanks with your organisms' names.
    e. Pipet 50 μL of resuspended cells into the appropriate dilution blank and vortex. Use OD to dilute to approximately 10^7 CFU/mL
    f. Pipet 50 μL of each competitor into the center of each arena plate.
    g. For most competitions, you will spread the competitors across the entire surface of the agar using a flamed spreader.

    h. Rarely, you may wish to leave the cells in the center of the plate (e.g., a motility race). In this case, leave the plates lid-side up until the 100 μL of saline soaks into the agar.
    i. place in appropriate incubation conditions.
    j. Pipet 50 μL from each unknown dilution tube into the appropriate #1 dilution blank.
6. Vortex dilution blanks and pipet 100 μL from each #1 blank into each #2 blank.
7. Vortex #2 blanks.
8. Spread plate 5 μL from #2 blanks onto appropriate #1 plates.
9. Spread plate 50 μL from #2 blanks onto appropriate #2 plates.
10. Spread plate 5 μL from #1 blanks onto appropriate #3 plates.
11. Incubate plates at 30º C.
12. Important: place dilution blanks (and eppendorf tubes) in the refrigerator until the next class!

Competition Day 2:

YOUR TEAM WILL NEED

- *18 9.9 mL saline dilution blanks*
- *18 quantification agar plates*
- *Sterile Eppendorf tubes*

DIRECTIONS

1. First, check your Day 1 plates. There should be at least one plate with between 20-500 colonies and with both of your competitors represented.
2. If none of your plates have a countable number of colonies, go back to your saved dilution blanks and plate more or less as necessary to achieve countable plates. MAKE SURE TO KEEP UP WITH YOUR DILUTION FACTORS!
3. For today's plating, label dilution blanks "C1-1" through "C2-3", #1 through #3. In other words 3 dilution blanks per competition tube.
4. Label quantification plates "C1-1" through "C2-3" #1, #2, and #3. In other words 3 plates per arena. Also write "Day 2" on each plate.
5. FOR COMPETITIONS DONE IN LIQUID ARENAS:
    a. Vortex arena tubes.
    b. Pipet 100 μL from each arena tube into the appropriate #1 dilution blank.

6. FOR COMPETITIONS DONE ON SOLID ARENAS:
    a. Pipet 1 mL of sterile saline from one dilution blank onto each competition plate.
    b. Using a cell spreader, carefully resuspend the bacterial growth from the arena plate into the saline. Tilt the plate so that the saline collects at the bottom, and "wash" the agar surface to get as much growth as possible into the saline.
    c. Pipet as much of the saline as you can off of the plate and into a sterile Eppendorf tube.
    d. Pipet 100 μL from the Eppendorf tube into the appropriately labeled dilution blank #1.
7. Vortex dilution blanks and pipet 100 μL from each #1 blank into each #2 blank.
8. Vortex #2 blanks and pipet 100 μL from each #2 blank into each #3 blank.
9. Spread plate 5 μL from #3 blanks onto appropriate #1 plates.
10. Spread plate 50 μL from #3 blanks onto appropriate #2 plates.
11. Spread plate 5 μL from #2 blanks onto appropriate #3 plates.
12. Incubate plates at 30º C.
13. Important: place dilution blanks (and eppendorf tubes) in the refrigerator until the next class!
14. Count your Day 0 plates. Use red and black sharpies to mark the different competitors.

Competition Day 3:

DIRECTIONS

1. Count your Day 1 plates. Use red and black sharpies to mark the different competitors.
2. If none of your Day 1 plates have countable numbers of colonies (20-500), go back to saved dilution blanks and plate more or less to achieve a countable number of colonies.

LAB WORKSHEET #5b
"Combat"

In these experiments, you pitted one of your isolates against another team's isolate. Use the data you collected during this gladiatorial combat to answer the following questions.

1. Describe the arenas that you used. What did you predict the outcomes of your competition experiments would be? Why?

2. For each time point, calculate CFU/mL using the dilution factor and your colony count. Do this for each competitor separately. Then, for each competition, calculate the **Malthusian parameter** of each competitor in each replicate:

$$m = \ln \frac{N_t}{N_0}$$

3. Express the fitness of each competitor as either the ratio or the difference of the Malthusian parameters (do this separately for each replicate). Both are legitimate methods for calculating fitness; only the latter is possible if either of the organisms decreased in abundance over the course of the experiment.

4. Compute the mean, standard deviation, and 95% confidence intervals of fitness measurements for each set of replicates. Plot these as a bar graph with error bars showing the 95% confidence interval.

5. Use an **unpaired t-test** (see Appendix 3) to determine if the difference between the means is statistically significant.

6. Are the results you observed here consistent with your predictions from before the competitions were performed? If so, can you think of reasons why your predictions might have been wrong?

7. Based on the competition results and antimicrobial profile of your organism, **refine the hypothesis** you stated in Worksheet 2 – what allows these organisms to coexist when they are under constant competition? How might their interactions be different in the soil environment?

8. Write a "Methods" section for these experiments (both the Antimicrobials and Competition experiments).

CHAPTER 12
HYPOTHESIS TESTING

You have now spent weeks getting to know your bacterial isolates. You've learned their names, what they like to eat, how to make them comfortable, and what they are afraid of. You've even tried to make them look pretty. It's like you're best friends! Now it's time to sit down and have a heart-to-heart discussion with them -- they have all sorts of stuff they'd like to tell you.

Here, you will design and execute new experiments to learn something previously unknown about your microbes and how they make a living together in their natural environment. Use what you've learned in lab as well as what you've learned in lecture (and anywhere else) to figure out some aspect of your isolates' biology that 1) you know enough about to ask meaningful questions about it but 2) raises some kind of question that your team finds interesting. Make sure you pick something that you are *interested* in -- you will need to write about it, and it's a lot easier to write about something if you're interested in it.

Some guidelines:

1. Start by pulling all of your information together into one place -- environmental tolerances, carbon substrates and terminal electron acceptors utilized, stress resistance phenotypes, whatever you've learned based on the identification of your organism. Use your completed worksheets as a resource.
2. Now look at the photos of your agar art. Pay close attention to how your isolates interact with each other and with other species. If anybody else used your isolates, look at their art too. Make a list of strange, unexpected, or perplexing things they did in the art. Add to this list any other "weird" observations you've made about how your isolates act -- times they didn't do what they were supposed to do, various ways that they are finicky to grow, things that affected their appearance or color, etc.
3. Look at these two lists -- the "objective science" list and the "art and other weird stuff" list and start thinking about it. Brainstorm out loud. Do you think any of the things in one list are related to things in the other? Write down a few possible ideas.
4. Now look at your hypotheses and start thinking of ways they could be tested using experiments. Settle on one that is both interesting and "doable" using the resources in the lab.

Once you've settled on a hypothesis, it's time to design your experiments. Here's a checklist to get you started:

1. Make sure that every measurement you make is **replicated**. Have in mind what statistics you will use to test your hypothesis before you collect your data. (Appendix 7 has a useful flow chart for deciding what statistics are appropriate for different kinds of hypotheses.)
2. Make sure you include **controls**. When you are thinking about what controls to use, try to imagine what criticisms a person might raise about your experimental design, and craft controls to counter those concerns.
3. Plan to spend several weeks working on your experiments. Plan at least one follow-up experiment based on the results of your first experiment. Also be prepared to repeat experiments that don't work the first time.

Your final paper will be written in style of a professional research paper, written as a team effort. First, your team should work together to analyze the data and produce figures, tables, and an abstract. At that point, you should decide how to divide the labor of writing the final paper. A good solution is to have different team members write the first draft of each section, and then get together to edit the finished document. Using Google Docs is a good way for everyone to work on the final draft together at the same time.

Try to get as much done as possible before the second-to-last lab period -- we will peer review each other's work at that point and try to sharpen up the final papers.

You will also make a poster and present it on the last day of lab. These posters are all eligible to be presented at university undergraduate research expos, and we would love to help you make that happen if you’re interested!

Last, your data from these experiments might be used by subsequent students as the basis for their own experiments. We ultimately hope to be able to turn some of your discoveries into publishable papers, and if that happens you will have the opportunity to participate in the manuscript preparation process, and to be a co-author when the paper is finally published.

So have fun, and do some science!

APPENDICES

Appendix 1: Calculating Dilution Factor

CALCULATING THE DENSITY OF A CULTURE

The viable count plating method works by diluting a culture until plates can be spread with only about ~100 cells. If we know how much the culture was diluted prior to plating, we can back-calculate to figure out how dense the original culture was. The easiest way to do this is with a **dilution factor**.

To calculate a dilution factor for a simple dilution, divide the volume of the original solution by the total volume of the diluted solution. For instance, if you put 100 μL of a culture into 9.9 mL of sterile saline, the dilution factor would be $\frac{0.1 \text{ mL}}{10 \text{ mL}} = 0.01$. If you perform multiple sequential dilution steps, you simply multiply each dilution factor to get a final overall dilution factor. So, if you diluted the culture above into a *second* 9.9 mL sterile saline blank, giving a second dilution factor of 0.01, the overall dilution factor would be 0.01 x 0.01 = 0.0001 or 1×10^{-4}. If you were to then plate 50 μL of this second dilution, you simply multiply by the volume plated: 0.01 x 0.01 x 0.05 mL = 5×10^{-6} mL. Note that the final value isn't a dilution, so you don't divide by the final volume like in the previous steps. Also notice that the final dilution factor for a plating is expressed in milliliters, whereas the dilution factor for transfer to liquid media doesn't have a unit. One way of thinking about this, is that plating 50 μL after two 100-fold dilutions is the same thing as plating 5×10^{-6} mL of the original culture (which of course would be impossible).

Once you have the total dilution factor, you can calculate **CFU/mL** (cell density) given a colony count by dividing the colony count by the dilution factor. Let's say we do the above dilution scheme and then count 215 colonies. We would calculate CFU/mL like this:

$$215 \text{ colonies} \div 5 \times 10^{-6} \text{ mL} = 4.3 \times 10^{7} \text{ colonies(CFU)/mL}$$

Practice it:

You dilute a culture twice by placing 20 μL into 9.98 mL of sterile saline, and then you plate 75 μL onto agar. A day later, you count 97 colonies. What was the cell density in the original culture?

Solution:

Dilution factor = 0.02 mL/10 mL * 0.02 mL/10 mL * 0.075 mL = 3×10^{-7} mL

97 CFU / 3×10^{-7} mL = 3.23×10^{8} CFU/mL

Note: what is a "CFU"? A CFU is a "colony forming unit". We usually assume that each colony originated as a single bacterial cell. However, it is possible that multi-cell structures can initiate colony formation; in fact, in some bacterial groups such as the *Streptomyces* this is the norm. Thus, we use "CFU per mL" instead of "cells per mL" to more accurately reflect what we are measuring.

Important note: Whenever we do spread-plates, we do multiple dilutions trying to hit the "sweet spot" where there are enough colonies to count to be a representative sample, but not so many we can't tell one from another. **You only need to count one plate** from each dilution series -- ideally one with between about 50 and 300 colonies.

Appendix 2. Calculating Confidence Intervals

CONFIDENCE INTERVALS AND THE *t* DISTRIBUTION

Why do scientists collect scientific data? It's because we want to test hypotheses of different kinds. For instance, in the experiments in Chapter 6, we were interested in testing the hypothesis:

H1: Temperature influences bacterial growth rates.

We made a lot of growth rate measurements for a lot of different isolates at several temperatures. However, the measurements weren't all the same, even when we measured the same exact thing multiple times (i.e. we replicated the measurements). How do we know if two sets of measurements reveal actual differences between samples, and not just "random noise" caused by measurement error?

To do this, we use statistics. All of the methods we're going to talk about here rely on three values. First, there's the **mean** of a set of measurements, which is just what we normally think of as the "average". Second, there's the **variance**, which is a measure of how much the individual measurements differ from the mean. Third is the **sample size**, usually symbolized by *n*. The bigger the variance, the bigger the "cloud" of points surrounding the real value and the larger the sample size needs to be if we want to be sure differences between the means of two samples are real. Our measure of variance is the **standard deviation**, σ, which is obtained with the formula:

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \mu)^2}{n-1}}$$

where n is the sample size, x_i is the i^{th} measured value, and μ is the mean of all the measured values. Of course the easier way to calculate mean and standard deviation is using a spreadsheet. In Microsoft Excel, you can compute the mean and standard deviation with the following formulas:

Mean: **=average(***selected values***)**

Standard deviation: **=stdev(***selected values***)**

(**`stuff in this font`** is code that can be typed or pasted right into Excel; *stuff in italics indicates information you have to provide, or cells you have to select*)

You can think of these values as representing the ***confidence*** you should have in the predictive power of the dataset. For instance, if variance/standard deviation is low, then any given measurement is likely to be pretty close to the "true value" of whatever you're trying to measure. If variance is high, on the other hand, many measurements are likely to be very far off from the desired real value. However, as long as the measurements are equally likely to be high as low, then if you take LOTS of measurements, you can EVENTUALLY develop a good idea of the real value. Thus, **the higher the variance, the more measurements you need** to make a good prediction about the real value of something you're trying to study.

Once we know the mean and the standard deviation of a dataset, we can calculate a **95% confidence interval** for the data. We can't be sure that the mean we measured is the "real" mean of the data, but based on the variance of our replicate measurements we can give a range of values that the real mean is 95% likely to be within. This calculation assumes that the "error" of our measurements is random, but the SIZE of the error is "normally distributed", meaning values closer to the real mean are more likely than values farther away. Without going into too much mathy stuff, the distribution of error probabilities is described by something called a ***t* distribution**, and gets smaller when we have more measurements or when the measurements have smaller variances. The formula for calculating the 95% confidence interval *CI* is:

$$CI = \frac{\sigma \, t_{0.05,n-1}}{\sqrt{n}}$$

where $t_{0.05,n\text{-}1}$ is the value of the *t* distribution for *n*-1 "degrees of freedom" with 95% confidence (1-0.05). You can look that value up in a table or you can just use Excel:

`=tinv(.05,`*n*-1**`)`**

Thus the Excel formula for the 95% CI is:

`=stdev(`*selected values***`)*tinv(.05,`**n-1**`)/sqrt(`***n***`)`**

When you make a graph with measured values in it, the 95% CI describes the **error bars** that should go on the graph. If you have two samples, and neither sample's 95% CI overlaps the other sample's mean, then you can say the two samples are **significantly different** at a confidence level *P* of

0.05. This latter point forms the basis of the ***t*-test** which we'll cover in the next Appendix.

For visual instructions on how to produce such a graph, see this video:

https://www.youtube.com/watch?v=xCYO1u7G6vQ&list=PLqTuWB3uliCzKB5aEaTePYq2ZL7E7tShu&index=4

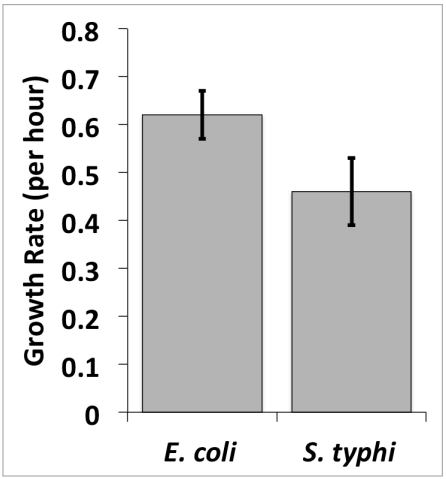


Figure A1. A bar graph with error bars representing the 95% confidence intervals of the two mean growth rates depicted. Neither error bar overlaps the mean of the other bar, so these two organisms have *significantly different* growth rates.

Practice:

You want to know whether two different species of motile bacteria have significantly different swimming speeds. After inoculating both onto 5 replicate plates each of "swim agar" and incubating them for 2 days, you measure how far growth has expanded from the point of inoculation. You get the following data:

Organism 1 (5 replicate plates): 5 mm, 7 mm, 6 mm, 8 mm, 5 mm

Organism 2 (5 replicate plates): 9 mm, 6 mm, 8 mm, 4 mm, 12 mm

Calculate the mean and 95% confidence interval of each organism, then plot them as bar graphs with error bars. Is one significantly faster than the other?

Solution: Organism 1: 6.2 +/- 1.6 mm; Organism 2: 7.8 +/- 3.8 mm. The two are NOT significantly different; Organism 2's lower error bar overlaps Organism 1's mean.

Appendix 3. The *t*-TEST

Calculating confidence intervals is very useful for visually representing the variance of a data set in a graph, but sometimes we want a more precise measurement of how confident we are that two sample means are different. One of the simplest ways to achieve this is with a *t*-test.

The *t*-test was developed in the early 20th century by the Guinness Brewery in Dublin, Ireland, in order to compare different batches of barley used in the brewing of their famous Irish stout. Afterward, it became a widely used tool for biologists. The *t*-test is based on the *t* distribution described in Appendix 2, which basically modifies the normal distribution to account for smaller sample sizes. When *n* gets very large, the *t* distribution is the same as the normal distribution, but when *n* gets very small, the distribution gets wider. What this means is that we can't be as confident about a sample with only a few measurements as we can about a sample with thousands of measurements.

A t-test typically compares a **control** sample to an **experimental** sample and asks if the mean of some measured value is different between the two samples. In each test, we first calculate a **test statistic** called ***t*** that we then compare to a table of values to determine how likely it is that the means of the two samples are actually different. There are three common varieties of the *t*-test, each described in turn below: the one-sample *t*-test, the unpaired *t*-test, and the paired *t*-test.

A. THE ONE SAMPLE T-TEST

In a ***one-sample*** *t*-test, a dataset is compared to a known standard value. For instance, in Chapter 9 we measured antibiotic resistance by measuring the diameter of the zone of inhibition around an antibiotic-containing disc, and each antibiotic had a target zone diameter that indicated clinical levels of resistance. If we have a group of *n* measurements *x* of zones of inhibition (with mean $\bar{x}$ and standard deviation σ) that we want to compare against a test value *V*, we can calculate the test statistic *t* with the formula:

$$t = \frac{\sqrt{n}(\bar{x} - V)}{\sigma}$$

Here is the Excel formula:

=tdist(sqrt(*n***)*(average(***measured values***)-***V***)/stdev(***measured values***),***n***-1,2)**

which gives a ***p* value**, or the probability (from 0 to 1) that the measured values are actually different than the test value *V*. **By convention, *p* <**

0.05 (i.e., 95% confidence) is the cutoff for saying that the measurements are "significantly" different.

Practice: You read in a paper that *E. coli* has a growth rate of 0.45 per hour in LB media at 37 C. When you measure a set of cultures in your lab, you collect the following growth rates from replicate cultures:

0.53 per hour, 0.61 per hour, 0.46 per hour, 0.51 per hour, 0.48 per hour

Does your strain of *E. coli* significantly different from the published value?

Solution: The *V* test value is the published rate of 0.45 per hour. The average of your cultures is 0.52 per hour, which is higher – in fact all of your cultures measured higher than the published value. However, there is also quite high variability between the measurements, so when you plug the values into the equation above, the *p* value is only 0.06 – close, but not statistically significant. However, it is quite close, so you would be advised to measure growth rates in more replicate cultures to increase your **statistical power** to detect a difference between the two.

B. THE UNPAIRED T-TEST

In an ***unpaired*** t-test, we are comparing two groups of measurements and asking if their means are significantly different. We assume that the values in each group being compared are all repeated measurements of a single "real" value. These replicates are not paired in any meaningful way, so all we have to know is the mean and standard deviation of each group in order to calculate *t*. For samples 1 and 2 with means $\bar{x}$, standard deviations σ, and sample sizes *n*:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{{\sigma_1}^2}{n_1} + \frac{{\sigma_2}^2}{n_2}}}$$

Here is the way to do it in Excel:

=t.test(*Group 1 values***,***Group 2 values***,2,3)**

Practice: Let's consider the test data from Appendix 2. Remember, based on our graph of the 95% confidence intervals, we assumed that the two samples were NOT significantly different. Here, we can plug them into the t.test function to make sure. When we do that, we get a *p* value of 0.32, confirming our initial conclusion.

C. THE PAIRED T-TEST

A *paired* t-test, on the other hand, compares individuals measured once each under two different conditions, essentially using each individual as their own control. The paired design is more powerful because it eliminates the effect of variability between individuals. For example, if you measured CFU/mL in 8 cultures of different species of bacteria before and after exposure to UV, you could use a paired *t*-test to see if the treatment had a significant effect on the organisms even if the starting CFU/mL was very different for the different species.

The paired *t*-test is a special case of the one-sample *t*-test because it reduces each pair to a single value -- the *difference* between the means -- and compares those differences to a test value. Usually, this test value is 0 -- the hypothesis that there is no difference between the two samples.

Calculating *t* for a paired *t*-test requires two steps. First, compute the difference X_D for each of *n* pairs. Note that in some cases it may make more sense to calculate the *absolute* difference (i.e., disallowing negative values). Then calculate the mean $\bar{X}_D$ and standard deviation σ_D of these differences, and (assuming the test value is 0) use this formula to calculate *t*:

$$t = \frac{\bar{X}_D \sqrt{n}}{\sigma_D}$$

The *p*-value for a paired *t*-test can also be easily calculated in Excel with the formula:

=t.test(*Group 1 values***,***Group 2 values***,2,1)**

Make sure that the values are in order such that value 1 in Group 1 is paired with value 2 in Group 2, and so forth.

Practice: You have isolated 6 different kinds of bacteria and measured their growth rates at pH 7 and at pH 4. You hypothesize that each will grow faster at pH 7, but the problem is that they have very different overall growth rates. Is the *difference* large enough to be significant, despite the major differences between species in overall growth rates? You collect the following data:

pH	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
7	.45	.12	.88	.39	.61	.22
4	.23	.03	.45	.24	.36	.10

Again, make *sure* that the values are entered into Excel in this order!

Solution: The *differences* for each isolate are as follows: 0.22, 0.09, 0.43, 0.15, 0.25, 0.12. The average difference is thus 0.21. Using the Excel t.test function, we obtain a *p* value of 0.009, strongly supporting the hypothesis that the organisms grow faster at pH 7.

REPORTING AND INTERPRETING *T*-TEST RESULTS

Note that when reporting the results of a *t*-test in a manuscript, you should always indicate the **sample size and *p* value** of the test, as well as whether you used a one-sample, unpaired, or paired *t*-test. For example, for the bar graph in Figure A1, you might say:

***Escherichia coli* grew significantly faster than *Salmonella typhi* (unpaired *t*-test, n = 6, *p* = 0.003).**

This result would be interpreted as: "There is 99.7% chance that *E. coli* actually grows faster than *S. typhi*, and a 0.3% chance that the apparent difference is just a result of measurement or other experimental errors."

Practice: Express the results from the previous three practice exercises in a way appropriate for a paper.

Solution:

1. Our E. coli strains had a higher mean growth rate than the published value (0.52 vs. 0.45), but the result was not significantly different (one-sample t-test, n=5, p=0.06).
2. Organism 1 and Organism 2 moved 6.2 +/- 1.6 mm and 7.8 +/- 3.8 mm away from the point of inoculation, respectively. These movement rates were not significantly different. (unpaired t-test, n=5, p=0.32)
3. Despite widely varied growth rates, the organisms grew significantly slower at pH 4 than at pH 7 (paired t-test, n=6, p=0.009).

Appendix 4. Correlation Analysis

Sometimes we want to know if the value of one parameter is related to another one. For instance, we might like to know if cultures that have higher growth rates at 30º C tend to also have higher growth rates at 37º C. We can do this using a **correlation test**. This test gives us a value *R* that is 0 if the two parameters are completely unrelated, 1 if both parameters increase or decrease together, and -1 if an increase in one parameter is matched by a decrease in the other.

R isn't hard to calculate, but we're just going to cheat and use Excel to do it here. If we have two datasets *x* and *y* that have paired values, we can use Excel to calculate the correlation coefficient:

=correl(*x,y***)**

The correlation coefficient needs to be above a certain level before we can say we've discovered a significant correlation. We'll test that level using the *t* distribution (Appendix 2):

$$t = R\sqrt{\frac{n-2}{1-R^2}}$$

You can then use this *t* statistic to determine a *P* value for the correlation as described above, as described in Appendix 3. Here's the complete formula for Excel:

=tdist(abs(correl(*x,y***)*sqrt((***n***-2)/(1- correl(***x,y***)^2))),***n***-1,2)**

Again, $P < 0.05$ is generally considered statistically significant.

Practice: You measure the growth rates of 5 organisms at 30 C and again at 37C. The 30 C rates for organisms 1-5 were 0.3, 0.35, 0.25, 0.4, and 0.5. The 37 C rates were 0.5, 0.4, 0.5, 0.2, and 0.55. What is the correlation coefficient for these measurements, and is it statistically significant?

Solution: The correlation coefficient is -0.074, which perhaps unexpectedly shows a weak *negative* correlation – as one growth rate increases, the other (slightly) decreases. A look at the data shows that this is probably because of organism 4, which unlike the other samples grows slower at 37 C (by 50%). The correlation is therefore not significant (p = 0.9).

Appendix 5. LINEAR REGRESSION.

Many scientific arguments revolve around the hypothesis that some phenomenon influences some other phenomenon. "Effects of X on Y" is a common title trope for scientific papers. A t-test comparing a treatment and control can inform us as to whether an effect exists, but it has trouble telling us how *big* of an effect there is. Indeed, statistics like t-tests can be very misleading when sample sizes get very big, because they can show significant differences even when "effect sizes" are infinitesimally small (a big problem in social science and medical research).

Linear regression, on the other hand, shows the size of an effect very clearly. In a linear regression experiment, we have some ***independent variable*** that we hypothesize to have an effect on a ***dependent variable***. We then experimentally alter the amount of the independent variable and measure the response in the dependent variable. For instance, in the experiments in Chapter 9, ***UV exposure time*** was the independent variable, and ***CFU/mL*** was the dependent variable. Our hypothesis was that CFU/mL would decrease at a constant rate under exposure to UV.

Mathematically, linear regression starts with a plot of independent variable data (x-axis) vs. dependent variable data (y-axis) and looks for a straight line that is the "best fit" for the real data. This line minimizes the value of the ***residuals***, or the distances between the actual data points and the nearest point on the line (Fig A2). This line is defined by a ***slope*** and an ***intercept*** (e.g. y=mx+b), although for most purposes the slope is all we care about. A line is "statistically significant" if its slope is different than 0; the size of the 95% confidence interval of the slope is a function of the size of the residuals, or how well the line fits the actual data.

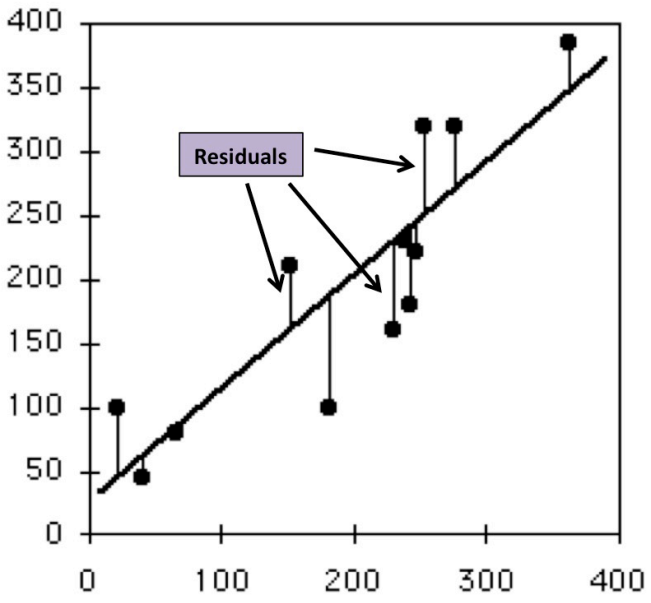


Figure A2. A linear regression.

In order to calculate a linear regression in Excel, the best choice is to use the function **`linest`** (Fig. A3). This formula is input in a somewhat different manner from most Excel formulas, because it covers multiple cells. In order to do a **`linest`**, select a 2x3 (width vs. height) block of cells and type

=linest(*cells containing dependent variable values***, *** cells containing independent variable values***, 1, 1)***

then hit **command+shift+enter** on a Mac or **ctrl+shift+enter** on a PC. Note that the number of dependent and independent variable values must be the same, and must be in the same order (i.e., value 1 for dependent variable matches value 1 for independent). This will result in a 2x3 output that contains the following values:

Slope (m)	Y-intercept (b)
Standard error of the slope	Standard error of the intercept
r^2 value (from 0 - 1)	Standard error of the y-estimate

These values are interpreted as follows:

Slope: The slope of a regression means the same thing as it does in the familiar equation for a straight line: it's "rise over run", or the amount that the dependent variable changes for every unit change of the independent variable. This is usually the most important value from a regression analysis.

Y-intercept: This is the other familiar parameter from the straight-line equation, or where the best-fit line crosses the Y-axis. It is not usually important analytically, but it is critical if you want to calculate what dependent variable value you would expect for a given value of the independent variable -- e.g., if you are trying to develop a ***standard curve***.

Standard errors of the slope, intercept, and y-estimate: These values are derived from the size of the residuals (i.e., how well the line actually fits the data) and the number of data points used to generate the line. To calculate the **95% confidence interval** of the slope and intercept, we need to know how many ***degrees of freedom*** we have, which in the case of

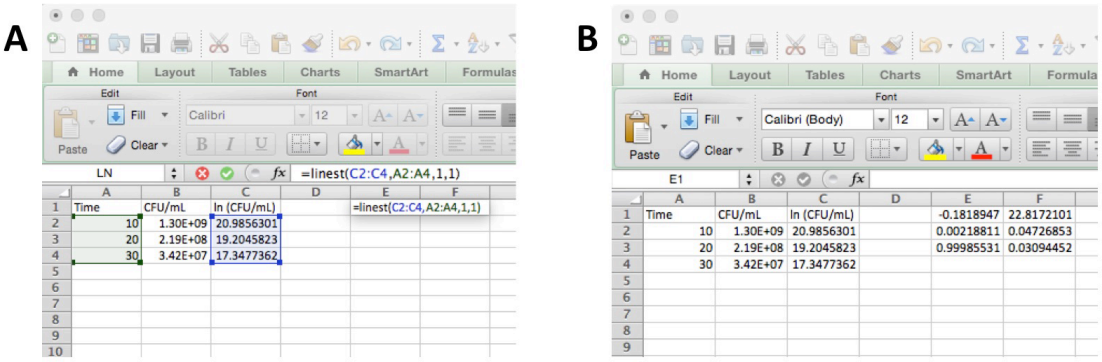


Figure A3. Screenshots of the "linest" function in Excel.

linear regression is the number of dependent variable measurements (***n***) we have minus 2. The Excel formula for the confidence interval is thus:

= *standard error* *** tinv(0.05,***n***-2)**

To determine if the slope is significant, perform a **one sample *t*-test** (Appendix 3) using the value of the slope as the "mean" and 0 as the test value. Here is the Excel code to get a p-value:

=tdist(*slope***/***standard error***,***n***-1,2)**

r^2 value: The r^2 value is a measure of how well the line fits the data. If there is a perfect match (no residuals) then $r^2 = 1$. The lower the r^2 the worse the fit. If you are producing a standard curve, you should be very worried if your r^2 value is less than 0.9.

In some cases you might be interested to see if two slopes are significantly different from each other. For example, are your two isolates killed at different rates by exposure to UV radiation? This is easily calculated as a *t*-test. For two slopes m_1 and m_2 with standard errors s_1 and s_2, calculate *t* as:

$$t = \frac{|m_1 - m_2|}{\sqrt{{s_1}^2 + {s_2}^2}}$$

Here is the Excel formula to get a p-value:

=tdist(abs(*slope1***-***slope2***)/sqrt(***s1***^2+***s2***^2),***n***-1,2)**

When reporting the results of a linear regression, always give the sample size and r^2 value of the regression. Usually you will also give the value of the slope (make sure to include proper units!) as well as its *p*-value in comparison with a test value of 0. For instance:

Viability of Isolate 1 decreased during UV exposure (linear regression of CFU/mL vs. time of exposure, n = 18, r^2 = 0.86, m = -0.24 per second, p = 0.004).

In order to use your regression results to predict a value (as in the standard curve generated in Chapter 6), simple multiply the predictor variable (e.g., the optical density measurement) by the slope, and add the intercept. If error bars are required for this estimate, use the "standard error of the y-prediction" from the linest output.

Practice: You measure the zone of inhibition of one of your isolates across a range of concentrations of the antibiotic streptomycin and collect the following data (note that there are replicate measurements for each concentration):

[Streptomycin] (ug/mL)	Zone of Inhibition (mm)
1	1.1
1	1.3
5	2.8
5	3.2
10	4.4
10	3.8
20	6.2
20	4.1

How strongly does increasing streptomycin affect inhibition?

Solution: By running a linear regression with ZOI as the y-value and [Streptomycin] as the x-value, we calculate a slope of 0.19 mm per ug/mL of streptomycin, indicating that the ZOI increases with dose (as expected). The 95% confidence interval of the slope is 0.14 however, which is nearly as high as the slope itself! Indeed, the r^2 value is only 0.78, and when we calculate a p-value of the comparison of the slope to a value of 0 (no relationship between x and y), we get a p value of 0.23, indicating the slope isn't statistically significant. A graph of the data shows the likely reason is the widespread different between the 20 ug/mL measurements – despite the fact there is a clear effect, the data is too noisy for statistical significance. The solution? Do more replicates!

Appendix 6. THE CHI-SQUARED TEST.

Linear regression and t-tests are appropriate for *continuous data*, or data where the measured values aren't limited to integers. However, sometimes your data is *discrete*, where it can only take certain values. The most common reason you can have this sort of data is because you are counting something, often "successes" and "failures" or yes/no kinds of questions. If you are interested to know if the number of "yes" answers in one group is significantly different from that in another group, you can test this using ***Pearson's chi-squared test*** on a **contingency table**. To make a contingency table, each row should be an experimental condition, and each column should be a possible result. The cells contain the number of observations for each condition that were of the column's result. The chi-square test statistic χ^2 is calculated with this formula:

$$\chi^2 = \sum_{i=1}^{N} \frac{(s_i - E_i)^2}{E_i}$$

where *N* is the number of cells in the table, s_i is the count in the *i*th cell, and E_i is the *expected* count for the cell (equal to the number of total observations in the row times the expected likelihood for the column's result). If we take a simple coin-flipping example, we might ask if a coin is "fair", i.e., it comes up heads or tails with equal probability. Let's say we flip it 10 times and count 9 successes. We can make a very simple contingency table:

Heads	Tails
9	1

The total number of flips is 10 and the *expected probability* is 0.5 for both heads and tails, so E_i is 0.5 * 10 = 5 for both cells. We can then calculate chi-squared for the two cells:

Heads	Tails
$(9-5)^2/5 = 3.2$	$(1-5)^2/5 = 3.2$

Sum these up and you get $\chi^2 = 6.4$. We can get a p-value with this excel code:

=chisq.dist.rt(χ^2,1)

If we type in this code, we see that the p-value is 0.011. This is the probability of getting 9 heads on a fair coin -- about 1%. Because this is

less than 0.05, we can reject the idea that this is a fair coin -- the actual number of successes is significantly higher than we would expect from a fair coin.

In practice, we usually don't have an expected probability of a success, like we do in a coin toss. In this case we have to use a **contingency table**. Here's an example: in Experiment 9B you used EcoPlates to see how many different carbon sources your isolates could use. Let's say one isolate used 6 of the 31 possible sources and the other used 18. To do our chi-squared test, we make a table:

	Used	Not Used
Isolate 1	6	25
Isolate 2	18	13

To calculate our test statistic, we use the equation above *for each cell in the table,* using the null hypothesis that the probability of a "yes" is the total number of "yes" divided by the total number of observations, or (6+18)/(31+31) = 0.39. Thus, the probability of a "no" is 1-0.39=0.61. We then add up the chi-squared statistics in each cell. So, we can make a second table:

	Used	Not Used
Isolate 1	3	1.9
Isolate 2	3	1.9

The sum of the cells is 9.79. Plugging into Excel we derive a p value of 0.002 – so these isolates do in fact use significantly different proportions of substrates. (Note that the fact that the values are identical down each column is caused by the fact that the sample sizes are exactly the same, 31.)

Practice: You notice that your organism sometimes forms yellow colonies, and sometimes forms red colonies. You think this might be affected by the incubation temperature, with the red pigment being a molecule that protects against high temperature stress. You incubate spread plates at 30 C and at 42 C and count the number of red colonies and yellow colonies on each plate:

Temperature	Red Colonies	Yellow Colonies	Total Colonies
30 C	12	31	43
42 C	55	62	117

Is this organism significantly more likely to be red at 42 C?

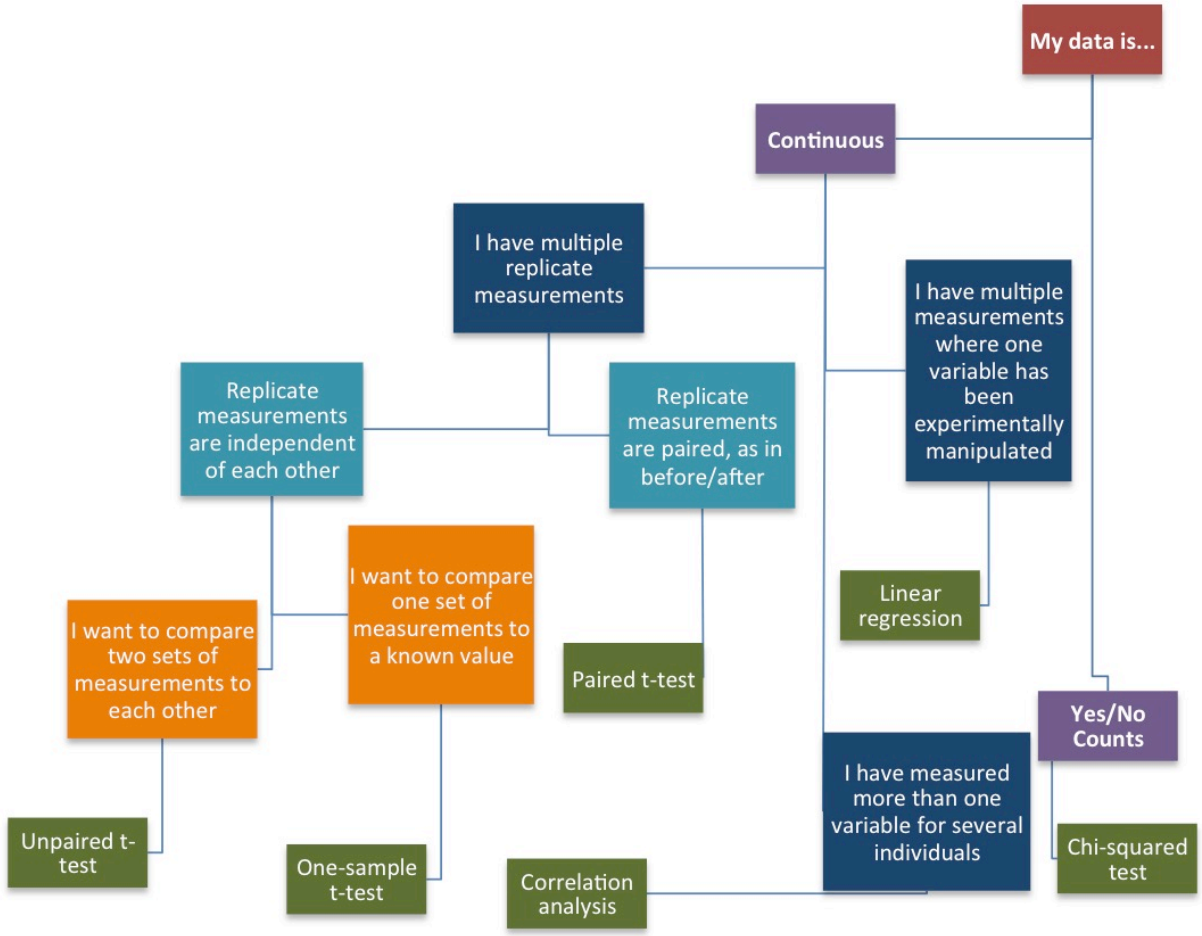
Solution: The "red" and "yellow" columns above are a contingency table. The overall probability of being red is (12+55)/(43+117)=0.42, and the probability of being yellow is 1-0.42 = 0.58. Using the chi-squared formula on each cell of the contingency table, we get:

Temperature	Red	Yellow
30C	2	1.44
42C	0.74	0.53

The sum of the cells is 4.71, which yields a *p* value of 0.03. The two samples do in fact have significantly different proportions of red colonies.

Appendix 7. CHOOSING THE RIGHT STATISTICAL TEST.

Here is a flow chart to help you decide which statistical test is right for the data you are planning to collect. Note that only the tests described in this manual are considered; there may be better tests available, but these are sufficient for our purposes.



Appendix 8. MEDIA RECIPES

Sterile Saline

Dissolve 8.5 g of NaCl in 1 L of deionized water
Autoclave

R2A Broth

Dissolve in 1L deionized water:
0.5 g yeast extract
0.5 g proteose peptone
0.5 g casamino acids
0.5 g dextrose/glucose
0.5 g soluble starch
0.3 g sodium pyruvate
0.3 g dipotassium phosphate
0.05 g $MgSO_4$ * 7 H_2O
Autoclave

R2A agar, Standard

Make 1 L R2A broth as above
add 15 g agar prior to autoclaving

BHI Broth/Agar

Use commercial (BD) BHI broth or agar powders according to manufacturer's instructions.

R2A/BHI Swim Agar

Make 1 L R2A or BHI broth
Add 3 g of agar prior to autoclaving

Low-carbon R2A Swim Agar

Dissolve in 1L deionized water:

0.1 g	yeast extract
0.1 g	proteose peptone
0.1 g	casamino acids
0.1 g	dextrose/glucose
0.1 g	soluble starch
0.06 g	sodium pyruvate
0.3 g	dipotassium phosphate
0.05 g	$MgSO_4$ * 7 H_2O
3 g	agar

Autoclave

pH-adjusted R2A/BHI agar

For pH 5, 7, and 9, make R2A/BHI agar as above, but adjust pH prior to autoclaving using 1N HCl or NaOH

For pH 3, make R2A/BHI and add 2.2 mL concentrated HCl ***after*** autoclaving

NaCl-adjusted R2A/BHI agar

Make 1L of R2A/BHI as above.

Add NaCl before autoclaving:

0.5% NaCl	5 g
5% NaCl	50 g
10% NaCl	100 g
15% NaCl	150 g

Soil/Water Extract

Add 200 g of unfertilized garden soil to 1 L deionized water in a 2L Erlenmeyer flask

Autoclave

Pass through a coffee filter into a storage bottle

Autoclave again

Soil Broth/Agar

Dissolve in 950 mL deionized water:

7 g $K_2HPO_4 * 3\ H_2O$

2 g KH_2PO_4

1 g Ammonium sulfate

0.5 g Sodium citrate dihydrate

Autoclave, and while still hot aseptically add 50 mL soil/water extract

For plates, add 15 g agar prior to autoclaving

Heavy Metal Solutions

For the antimicrobial experiment, prepare 200 uM $Pb(NO_3)_2$, 12.5 mM $MnCl_2$, and 250 uM $ZnCl_2$. Filter sterilize solutions prior to use.

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