From Microbes to Medicines Laboratory

In this laboratory, you will get to explore the bacteria that live in your own backyard. You will gather soil, isolate the bacteria that live in your soil, and determine whether your bacteria can kill your lab partner's bacteria! If you are lucky, you might even discover bacteria that make novel antibiotics!

What do you think of when you hear the word bacteria? Maybe you think of sickness or infections? While it is true that many bacteria can wreak havoc on the human body, not all bacteria are bad! In fact, over half of our clinically used antibiotics actually come from bacteria that live in the soil. You might be asking why bacteria would make antibiotics. That is an excellent question! The major hypothesis for this is that bacteria make antibiotics to protect themselves from other competing bacteria in the soil. Essentially, bacteria are the original creators of chemical warfare! Microorganisms (bacteria and fungi) are fantastic chemists capable of making very complicated molecules often much faster than we humans can. Below is shown one such molecule, penicillin (**Figure 1A**). Penicillin was originally discovered by a happy accident. Dr. Alexander Fleming, a scientist at St. Mary's Hospital in London, was growing *Staphylococcus aureus* (the cause of MRSA infections) on a petri dish. He went on vacation and came back to find that his petri dish was contaminated with another microorganism, the fungus *Penicillium*. Most people would have thrown away the contaminated plate. However, Fleming noticed that *Penicillium* appeared to be preventing the growth of *S. aureus*. Specifically, there was a "zone of inhibition" or a region where the bacteria did not grow (**Figure 1B**). *Penicillium* was later found to produce an antibiotic that we now call penicillin. It took many years and a team of many other scientists to isolate penicillin and develop ways to produce it on a useful scale. Their hard work combined with Fleming's discovery ultimately allowed for the production of one of the most important antibiotics. This discovery went on to be of extreme importance during World War II (**Figure 1C**). Interestingly, Fleming credits his upbringing on a small farm in Scotland and his childhood experiences exploring the outside world for his curiosity and attention to detail that ultimately led to his discovery of penicillin (**Figure 1D**).

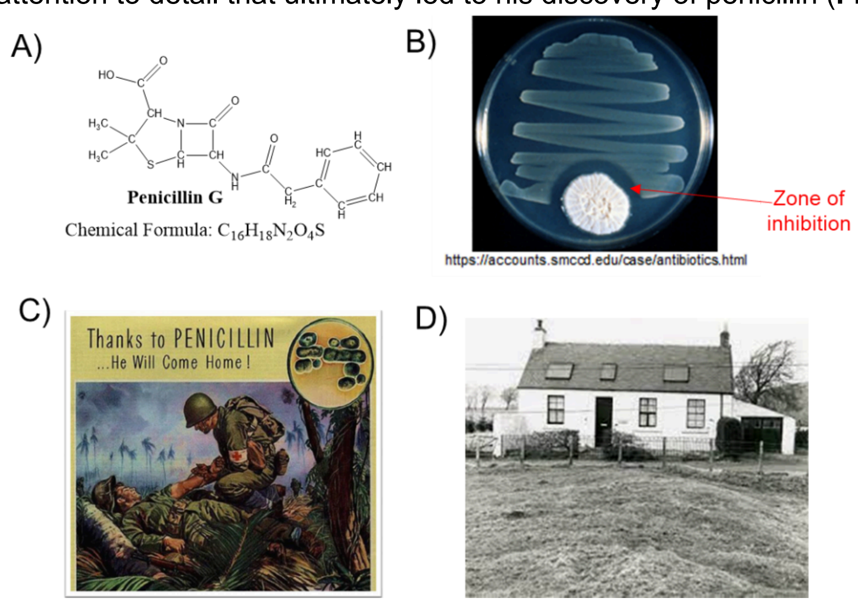
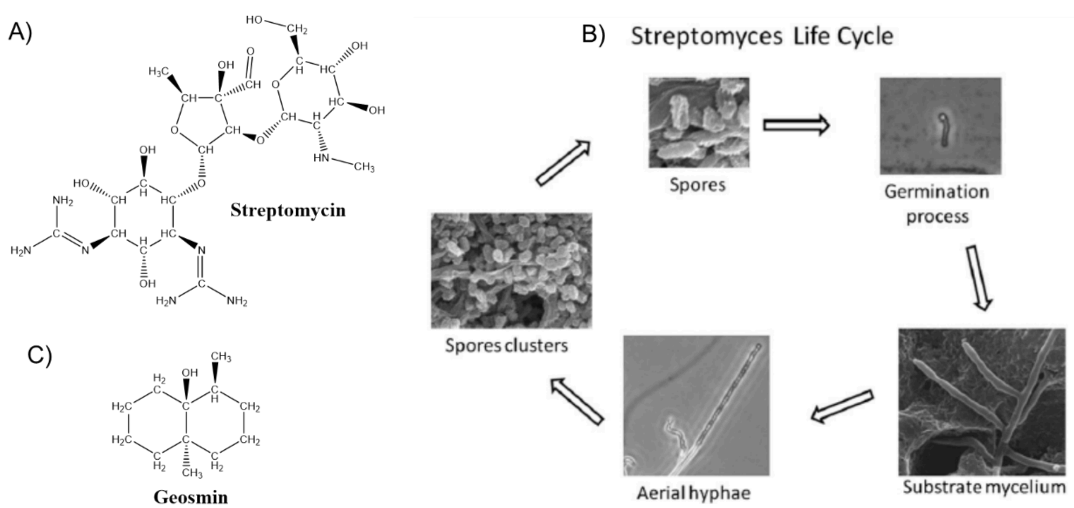


Figure 1. A) Structure of the antibiotic penicillin. B) Petri dish with two different microorganisms. One (the white circle) is producing a compound that inhibits the growth of the other, as evidenced by the zone of inhibition. C) Poster from World War II. D) The schoolhouse that Alexander Fleming attended until he was 13 years old.

A few years after the discovery of penicillin, streptomycin (an antibiotic used in the treatment of tuberculosis, **Figure 2A**) was discovered from the soil-dwelling bacteria *Streptomyces griseus*. *Streptomyces* are Gram-positive bacteria that are ubiquitous in nature, growing in soil in practically every environment on earth. They grow as vegetative branching hyphae (or slender threadlike structures, **Figure 2B**) that can differentiate into spores when conditions do not promote growth. Spores can survive very long periods of time (up to 70 years!) and very intense conditions including high and low temperatures, drought, and flood. Very few human or plant diseases are caused by *Streptomyces*. One of the very few examples of a *Streptomyces* causing illness is potato scab, which is caused by *S. scabies.* On the other hand, *Streptomyces* have been extremely useful for human health and the agricultural industry! Bacteria from the genus *Streptomyces* are one of the most prolific producers of bioactive molecules. Many FDA-approved drugs including antibiotics, anticancer agents, and immunosuppressants originate from *Streptomyces*. Additionally, *Streptomyces* produce insecticides and fungicides that have been invaluable in agriculture. They also produce many other interesting, if less useful, compounds. For example, the smell of dirt just after it rains. This smell is actually due to the molecule geosmin (Greek for “earth” and “smell”, **Figure 2C**).

Figure 2. A) Structure of the antibiotic streptomycin. B) Life cycle of *Streptomyces*. This figure is reproduced from (Anunciação de Jesus Sousa, 2016) C) Structure of geosmin, the molecule responsible for the smell of dirt after it rains.



Pre-Lab Questions

1. It is hypothesized that microorganisms make antibiotics as chemical warfare. What do you think they are fighting over? Why do you think they make these molecules?

2. Do you know of anyone who has ever taken amoxicillin (*aka* “the pink stuff”)? Do you know what it is commonly used to treat? Explore the CDC website (https://www.cdc.gov/antibiotic-use/common-illnesses.html) for common illnesses by ctrl+F for amoxicillin to find out more!

3. How do the structures of amoxicillin and penicillin G differ?
    a. Look up the structure of amoxicillin and penicillin and draw them below. Note that https://pubchem.ncbi.nlm.nih.gov/ is a great place to look up chemical structures!
    b. Circle the portions that differ from penicillin G.
    c. How many sp^3 hybridized carbons are there in amoxicillin?
    d. How many sp^2 hybridized carbons are there in amoxicillin?
    e. How many sp hybridized carbons are there in amoxicillin?

4. Researchers often use mass spectrometry (a technique that measures mass to charge ratio of molecules) to determine whether a molecule is present in a sample. With mass spectrometry, one detects the exact mass of the molecule. The chemical formula for penicillin is $C_{16}H_{18}N_2O_4S$.
    a. Calculate the exact mass of penicillin
    b. Calculate the molecular weight of penicillin G.
    c. Are these numbers the same or different? Why?

5. How do penicillin (and amoxicillin) kill bacteria? That is, what is their “mode of action”? See https://courses.lumenlearning.com/microbiology/chapter/mechanisms-of-antibacterial-drugs/ for a great discussion of how antibiotics can kill bacteria without harming human cells.

6. Norman Heatley, an early researcher on the isolation of penicillin, discovered that it was more easily purified by adjusting the pH.
    a. Define pH.
    b. Give an example of a compound with a low pH. Is it acidic or basic?
    c. Give an example of a compound with a high pH. Is it acidic or basic?
    d. Which proton is most acidic on penicillin? Why?

7. Who is “Moldy Mary”? What did she discover that helped the penicillin movement? You can learn more about Moldy Mary by reading this article; https://www.washingtonpost.com/history/2020/07/11/penicillin-coronavirus-florey-wwii-infection/

8. In Alexander Fleming's acceptance speech for the Nobel Prize, he warns that "There is the danger that the ignorant man may easily underdose himself [with antibiotics] and by exposing his microbes to non-lethal quantities of the drug make them resistant". Unfortunately, Fleming was correct and antibiotic resistance is quite commonplace.
    a. How do bacteria become resistant to antibiotics? Explore [How Antibiotic Resistance Happens | Antibiotic/Antimicrobial Resistance | CDC]() to learn more about this!
    b. What can you do to help slow antibiotic resistance development?
    c. Find a recent news story about antibiotic resistance. Briefly describe it below. Remember to provide a reference for the article!

9. *Streptomyces* require carbon and nitrogen sources to grow. You will be using *Streptomyces* isolation media to isolate bacteria. This media consists of casein (protein from milk) and starch along with other components.
    a. Both proteins and starches are polymers. Define polymers.
    b. What are the monomers that make up protein?
    c. What are the monomers that make up starch?
    d. Which monomers might serve as a source of nitrogen?
    e. Which monomers might serve as a source of carbon?
    f. In addition to casein and starch, we also add cycloheximide and nalidixic acid. What are these molecules? Why do you think we added them?
    g. What other factors might affect the ability of the bacteria to grow?

10. Sterile equipment is necessary when working with bacteria because contamination of equipment by microorganisms is very prevalent. List some ways you can maintain a sterile environment when performing this laboratory.

11. *Streptomyces*, like many other bacteria, reproduce by binary fission (*i.e.* one cell divides into 2 daughter cells). However, *Streptomyces* generally grow much slower than other bacteria. Their doubling time is 4 hours compared to *E. coli*, which has a doubling time of approximately 20 min.
    a. How long will it take to get over 1000 cells of *E. coli* if we start from one cell?
    b. How long will it take to get over 1000 cells of *Streptomyces* if we start from one cell?
    c. Hint: Fill out the below table to help you find your answer.

# cell divisions	**# bacteria**	**Time for *E. coli* (hours)**	**Time for *Streptomyces* (hours)**
1	1	0	0
2	2	1/3	4
3	4	2/3	8
4	8	1	12
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			

12. Oftentimes bacteria cultures are very concentrated, which makes it not feasible to directly count how many bacteria cells are in a culture. However, it is necessary to know how many bacteria are present in order to control your experiments. To determine the number of bacteria, we typically use a process called serial dilution. In this process, the scientist will make multiple 10-fold dilutions and plate each onto a plate. The scientist will then allow the bacteria to grow until they are small dots (colonies). The scientist can then use the number of colonies and the dilution factor to determine the number of bacteria in the original sample. Use the figure below to answer the following questions about my recent experiment

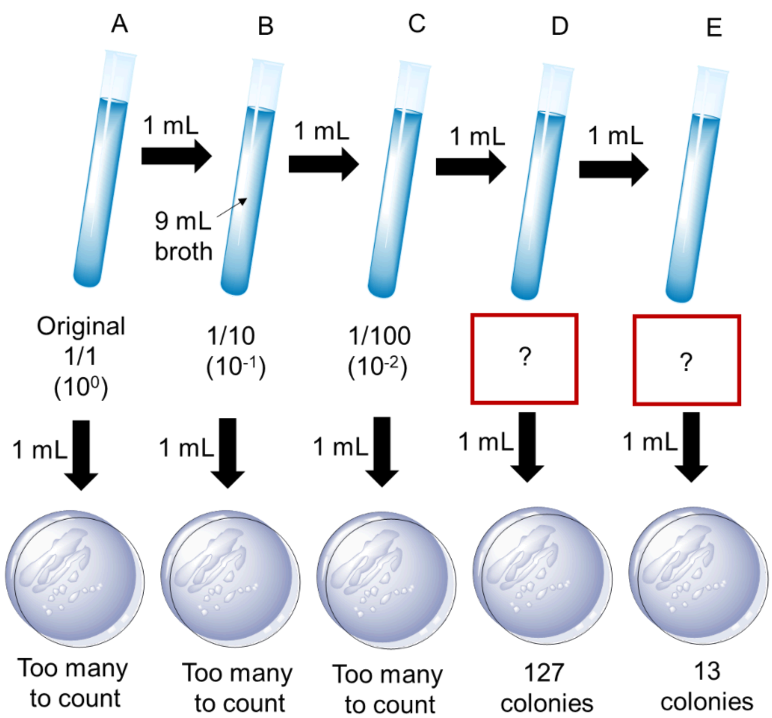
    a. What is the dilution for sample D?

    b. Based on the number of colonies in sample D, how many cells per milliliter were in the original sample?

    c. What is the dilution for sample E?

    d. Based on the number of colonies in sample E, how many cells per milliliter were in the original sample?

    e. Are your answers for b and d the same or different? Explain.



Materials

"Lab in a Box" Kit (*i.e.* things we will provide via mail):

- Plastic sandwich bags and spoons (1 per student)
- 15 mL plastic conical tube (3 per student)
- Small sieve
- Petri dishes with agar and cycloheximide added (3.5 per student)
- Q-tips
- Gloves (medium)
- Toothpicks
- pH strips

Required materials (*i.e.* not provided)

- Phone app for recording location/temperature
- Camera (or phone) for taking picture of site and plates
- Graduated cylinder capable of measuring 10 mL
- Pasteur pipettes with bulbs
- Bottled water
- Scale capable of measuring 0.25 g
- Hot water bath or hot plate with large beaker of water capable of maintaining between 50-80 °C for 30 min
- Sharpies
- Fridge to store agar plates

Safety Precautions

- Always make sure to wash your hands before and after you handle soil or your bacteria! While the majority of bacteria from soil are safe, there are some that can cause disease. Washing your hands with soap and water will protect you from them.
- Be careful when using a hot water bath or hot-plate. It is possible to burn yourself. Additionally, if accidentally left unattended, they can be a fire risk.
- Make sure to wear gloves whenever you handle your petri dishes. They contain the antifungal agent cycloheximide, which is toxic to humans. In addition to wearing gloves, always wash your hands after handling the petri dishes.

Procedure

Part 1. Collect Soil. (At least 3 days)

1. Choose a site to collect your soil. This can be from your backyard, a local park, or any other public land. Take a picture of your location. Also, determine the latitude, longitude, and elevation of the location. There are many free apps that can be used to do this such as Coordinates. Finally, record the temperature and time of day that you gathered the soil. Record this information in Question #1 of the Data Collection Sheet.
2. Fill a small plastic bag ~1/3 full of soil using a spoon and seal the bag tightly. Try to avoid rocks, sticks, plants, bugs, and other small debris. Then, put the first bag inside a second bag to ensure that you do not lose your soil.
3. Dry your soil at room temperature in a dark place in a glass loosely covered with aluminum foil until time to complete Part 2 (at least 3 days but up to a month is ok). It should be very dry after this step.

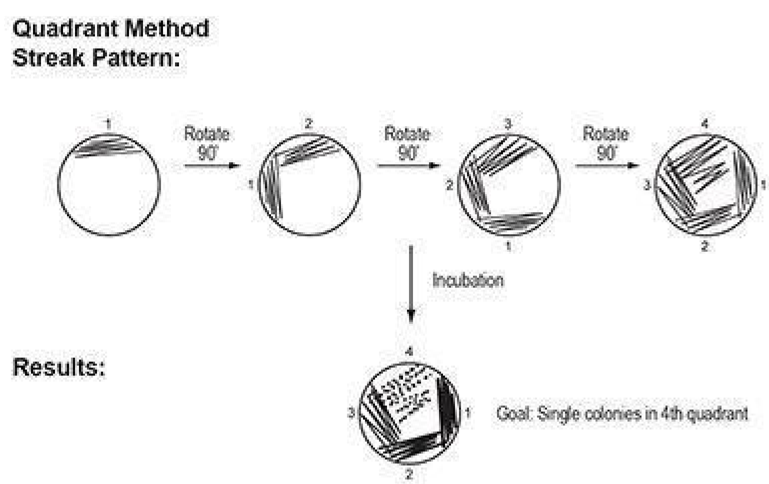
Part 2. Plate soil for bacteria isolation. (~1 week)

1. Put your soil through the sieve provided to remove any rocks or twigs
2. Measure out 0.25 g of soil and place in a 15 mL conical tube.
3. Add 10 mL of water and mix thoroughly. This is your dilution #1.
4. Use the pH strips to determine the pH of the water (dip the strip into the water and compare to the key). Record this information in Question #2 of the Data Collection Sheet.
5. Place your conical tube containing the soil and water in a 50°C water bath for 30 min (Note: If you do not have a water bath, you can instead use a large beaker filled with water heated on a hot plate. It is NOT necessary to be exactly 50°C. As long as you are above 45°C and below 80°C, it should work!). This step will kill many of the microorganisms that cannot form spores.
6. Shake closed tube to resuspend the soil and use a Pasteur pipette to transfer 1 mL to a new conical tube containing 9 mL of water (Total of 10 mL) and mix thoroughly. This is your dilution #2. (serial dilutions!)
7. Add 5 drops of well mixed dilution #2 to one of your labeled agar plates and spread using a clean Q-tip. Be careful when handling the agar plates because they contain cycloheximide. Make sure you wear gloves. Cycloheximide is a ribosome inhibitor that will prevent the growth of fungus (but it is also toxic to humans!). In this lab, we are isolating bacteria (not fungi) that might make interesting natural products!
8. Shake closed tube for dilution #2 and add 1 mL to a new conical tube containing 9 mL of water and mix thoroughly. This is your dilution #3.

9. Add 5 drops of well-mixed dilution #3 to your second labeled agar plate and spread using a clean Q-tip.
10. Put your plates (dilution #2 and #3) in a warm dark place and allow to grow for at least 1 week (up to 3 weeks is acceptable).

Part 3: Isolate bacteria (~1 week)

1. After 1 week, you should see growth (small dots called bacterial colonies). Count the number of colonies on each of your plates (if over 200, just state over 200). Record this information in Question #3 of the Data Collection Sheet.
2. Pick a colony of interest to spread onto a new plate. When picking a colony, try to pick ones that look “furry” not “slimy”. The “fur” is actually spores and indicates that you likely have a *Streptomyces* bacteria. Describe the colony in Question #4 of the Data Collection Sheet.
3. Take a toothpick and use it to “pick” your colony (*i.e.* gently scrape the colony you are interested it without touching any other colonies). Then streak the colony onto a new plate using the pattern shown below (Figure from Sanders, 2012). Allow to grow at room temperature for another week



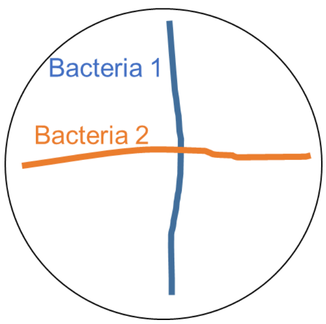
4. After growth, describe your bacteria in Question #5 of the Data Collection Sheet. What color is it (from both the front and the back of the plate—believe it or not this used to be how people determined what type of bacteria it was before genome sequencing!). Does it look like it is forming spores (i.e. is it furry?). What shape are the colonies? What does it smell like? Note: when testing for smell, slightly open the petri dish and waft towards yourself. DO NOT take a big sniff directly from the plate (Remember, while unlikely it is possible that these bacteria could be harmful. Wafting is much safer!)

Part 4: Bacterial Competition! (~1 week)

1. Many bacteria make antibiotics as "chemical warfare"—*i.e.* to kill off their competitors. Now it is your chance to determine whether you have an antibiotic producing bacteria. Pair up with another student whose bacteria you think looks interesting. Describe your partner's bacteria in Question #6 of the Data Collection Sheet.

2. Use a toothpick to pick a colony from your plate of bacteria and streak it as a single line onto a new plate.

3. Allow your partner to do the same but streak it perpendicular to the first streak. The streaks should cross each other (see below).



4. Allow your bacterial co-cultures to grow one week and observe the pattern of growth. Describe the growth in Question #7 on the Data Collection Sheet and take a picture of your plate. Do you think either of your bacteria are making antibiotics? Why or why not?

5. Present your results to your class. The class will then vote on the best 2 bacteria to send back to the Parkinson lab

6. The Parkinson lab will then further evaluate the bacteria (phylogeny and antibiotic activity) and post to a website for you to check)--This will take ~1 month after we receive the strains.

Data Collection

1. Describe the location where you obtained your soil.
    a. Latitude?

    b. Longitude?

    c. Elevation?

    d. What was the weather like? Temperature?

    e. Further description. What was nearby? Was it near plants or buildings? Is the location in a sunny or shady location?

2. What was the pH of your soil?

3. How many colonies were on your plates?

    a. On the dilution #2 plate?

    b. On the dilution #3 plate?

4. Describe the colony that you choose to restreak. What shape was it? Was it flat or growing up out of the agar like a mini-volcano? What color was the colony? Was it a different color from the front and back of the plate? The more details the better!

5. Describe your bacteria after being restreaked. Does it appear the same as previously? Are all of the colonies consistent? Or do you have more than one type of colony on your plate?

6. Describe your partner's bacteria (see question #4 for things to include in the description).

7. Describe the interaction between you and your partner's bacteria. Are there any differences in **morphology** (*i.e.* the outward appearance) of your bacteria when it is close to your partner's bacteria versus when it was grown alone?

Post-Lab Questions

1. Soils can vary dramatically in texture. There are three main types of soil: Sandy, loamy, and clay. Check out https://www.soils4teachers.org/soil-basics and https://www.soils4teachers.org/files/s4t/soils-overview-for-teachers-2020.pdf for more details.
    a. Describe each of these types of soil.
    b. Based on your observations of your soil, which do you think best describes the soil that you obtained?

2. Most bacteria grow best a neutral pH (~7). However, certain bacteria (*e.g.* the *lactobacillus* in yogurt) grow well at lower pH (~4).
    a. Based on this, does the pH of your soil make sense? Is it a desirable pH for growth of most bacteria? If no, how did your bacteria survive (hint: what growth state were your bacteria most likely in when you isolated them from the soil?)
    b. Why do you think most bacterial grow best at neutral pH? Why is it hard for many bacteria to grow at low pH? (*Hint*: Proton motive force)
    c. *Lactobacillus* produce lactate during fermentation. This helps them to generate a low pH around themselves. Why might they want to do this? (*Hint:* Similar reason as to why *Streptomyces* make natural products)

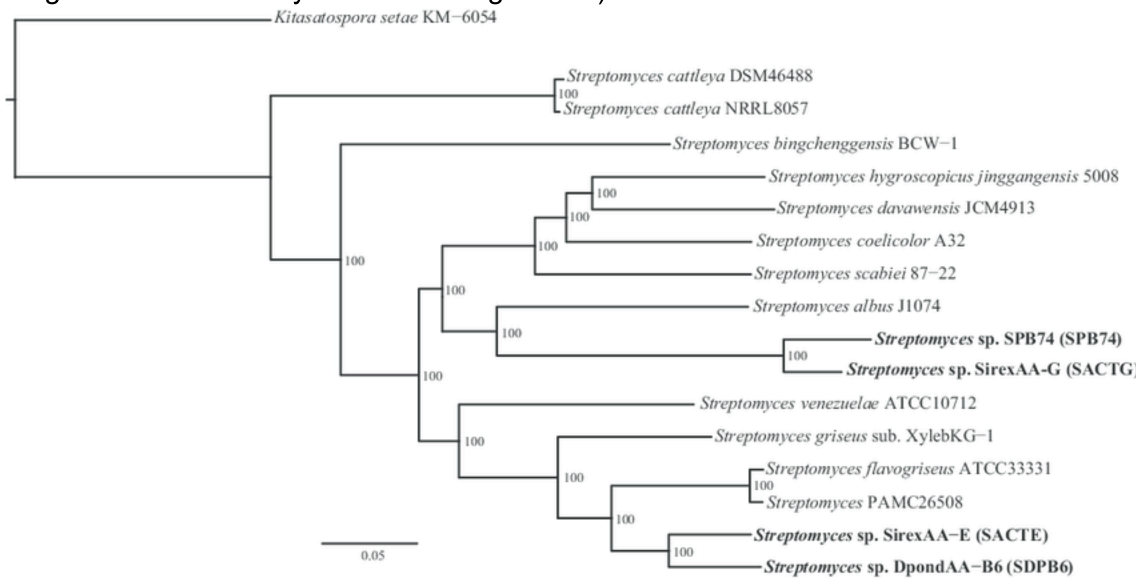
3. Based on the number of colonies that you found on your plates, calculate the number of bacteria per milliliter that were in your original dilution. Compare your values to that of another student. Are they similar? What do you think affects the number of bacteria that can survive in a particular soil?

4. One of the goals of this laboratory is to obtain a "pure culture" (*i.e.* a single type of bacteria). Did you obtain a "pure culture"? Why do you think you did (or did not)? If you did not, what could you do to get a pure culture?

5. Based on the result of your co-culture experiment (where you plated your bacteria with your partner's bacteria), do you think your bacteria produced an antibiotic? Do you think your partner's bacteria produced an antibiotic? Why or why not?

6. After isolation of bacteria, the next step is to determine what type of bacteria they are.
    a. Prior to DNA sequencing, scientists determined phylogeny (*i.e.* the evolutionary history of organisms) by morphology (*i.e.* physical characteristics such as shape and color). What are the advantages and disadvantages to this technique? A hint to consider: Are you closely related to everyone with the same hair or eye color? Do all of your close relatives have the same hair or eye color?
    b. With the advent of DNA sequencing, we now use the sequence of particular genes to determine how closely related bacteria are. What gene(s) are commonly used for this purpose?

c. After sequencing DNA, we generate a phylogenetic tree (or cladogram). These are similar to family trees but are based on the similarity of sequences. Below is given a phylogenetic tree of *Streptomyces* (Figure from Book, 2014). Which are more closely related, *S. coelicolor A32* and *S. albus* J1074 or *S. coelicolor* A32 and *S. scabiei* 87-22 (Hint: Measure the horizontal branch length; the shorter the length the more closely related the organisms).



7. After determining the type of bacteria you have isolated, the next step is to determine what bioactive molecule the bacteria is making. The first thing that is typically done is to compare the mass spectra of the molecule to the known molecules. To do this, we often consult an online database called The Natural Products Atlas (https://www.npatlas.org/). Below are some of the bioactive molecules that we have previously observed. Determine whether they are known molecules (*i.e.* they are in the Natural Products Atlas) or if they are novel. If they are known, describe their activities and what microorganism they come from.
   a. Exact Mass: 560.3534 Chemical Formula: $C_{25}H_{48}N_6O_8$
   b. Exact Mass: 1619.710 Chemical Formula: $C_{72}H_{101}N_{17}O_{26}$
   c. Exact Mass: 731.4608 Chemical Formula: $C_{41}H_{65}NO_{10}$

8. Find another molecule produced by a *Streptomyces* strain that humans now use (can be for medicine, agriculture, or other). Describe its function below.

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