

A Laboratory Project Investigating Phenotypic Diversity Among Soilborne Streptomycetes

Resource Type: Curriculum: Laboratory

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Authors

Kim Mogen

University of Wisconsin-River Falls

River Falls, Wisconsin

Email: kim.l.mogen@uwrf.edu

Linda Kinkel

University of Minnesota

St. Paul, Minnesota

Email: kinkel@umn.edu

Abstract

In this project the phenotypic diversity (antibiotic resistance, inhibition, pigmentation, and hyphal structure) of antibiotic-producing streptomycetes isolated from varying soils is examined. As a team, students (junior- and senior-level undergraduates) prepare a dilution series and perform a plate count on their soil. As individuals, students identify, purify, and make spore suspensions of the four best antibiotic-producing streptomycete isolates from their soil sample. They challenge their streptomycete isolates against antibiotic-containing discs, gram-positive and gram-negative bacteria, and other streptomycetes isolated from their soil. As a team, students prepare short oral reports, collect data, and present their experimental results.

Activity

Invitation for User Feedback. If you have used the activity and would like to provide feedback, please send an e-mail to MicrobeLibrary@asmusa.org. Feedback can include ideas which complement the activity and new approaches for implementing the activity. Your comments will be added to the activity under a separate section labeled "Feedback." Comments may be edited.

Learning Outcomes.

At the completion of this activity, students will have

1. worked cooperatively in teams to follow an experimental protocol, collect and organize data, analyze data, and communicate results.
2. demonstrated the ability to enumerate the number of total soil bacteria and of streptomycetes in particular, using serial dilution techniques.
3. demonstrated the ability to use aseptic techniques to isolate and purify streptomycetes and harvest streptomycete spores.
4. demonstrated the ability to use micropipettors to spot microbial samples and read zones of inhibition.
5. demonstrated the ability to use brightfield microscopy and staining procedures to view varying aerial hyphae with chains of conidiospores.
6. observed the diversity of streptomycete phenotypes.
7. explored the antibiotic interactions of soilborne streptomycetes.

PROCEDURE

Materials.

- 60°C heating oven
- sterile distilled water blanks (5/team is adequate)
- 28°C incubator
- 12 oatmeal agar plates amended with 100 µg/ml cycloheximide per student
- 2 Mueller-Hinton tryptic soy agar or nutrient agar plates/student
- sterile toothpicks
- sterile 20% glycerol (3 ml per student)
- sterile cotton swabs
- antibiotic discs
- rulers (mm)
- #3 cork borers or glass Pasteur pipettes
- sterile microcentrifuge tubes
- micropipettors and sterile tips (10 to 100 µl volume)
- bent glass rods and ethanol for flaming
- balance and weigh boats for 5 and 1 g weights
- sterile forceps or spatula

- plastic zip-top bags for soil collection

Student Version.

Available as a PDF

[PDF of student version](#)

Instructor Version.

Available as a PDF

[PDF of instructor version](#)

Safety Issues.

Unidentified infectious pathogens may be present in the soil samples, but students are encouraged to avoid collecting soil from sites where fecal contamination (fields spread with manure, feedlots, active pastures) is probable. Also, the plates are incubated at 28°C for several days which may select against human pathogens. However, a few streptomycete species or strains are reported to be human pathogens. Thus, aseptic techniques for biosafety level 2 organisms should be followed throughout the lab project. Students should wear lab coats, goggles, and gloves while handling their dilution bottles and plates. Discarded toothpicks, plates, pipette tips, swabs, and spore suspensions should be autoclaved before disposal. After the first set of subculturing, bacterial or fungal contaminants are rare.

ML Safety Statement regarding Environmental Isolates

The Curriculum Resources Committee recognizes that isolated organisms can be a powerful learning tool as well as a potential biological hazard. We strongly recommend that:

- Environmental enrichment laboratories should only be performed in classes in which students have been trained to work at a BSL2.
- Direct environmental samples (eg. soil, water) which are known to contain infectious organisms should be handled according to the biosafety level of that infectious agent.
- Cultures of enriched microorganisms, derived from environmental samples, should be handled using Biosafety Level 2 precautions.
- Mixed, enriched or pure cultures of microorganisms from environmental samples with a significant probability of containing infectious agents should be manipulated in a biosafety cabinet if available.
- Where possible, media used for the enrichment of environmental isolates should contain an appropriate anti-fungal agent.
- Instructors should be aware if they are teaching in regions with endemic fungi capable of causing systemic infections, and should avoid environmental isolations.

ASSESSMENT AND OUTCOMES

Suggestions for Assessment.

I assign four graded components to this project as described below. These components are due at varying times during the course of the project.

1. At the beginning of the project, a team 10-minute oral report to the class on assigned topic area (soil microbiology and plant rhizosphere, streptomycete antibiotic discovery and current antibiotic diversity, streptomycete genetics, or industrial antibiotic production); at least two team members must report; one page summary with three main references to be turned in to instructor on day of presentation (15 points)
2. Individual lab notebook, containing entries for all lab work and final data analysis (10 points)
3. Individual Gram or simple stain of streptomycete (5 points)
4. A team 5-minute oral report of analyzed inhibition and resistance data (10 points)

Field Testing.

I have done a streptomycete lab project for 4 years. I typically have 45 to 50 students split into two 20 to 25-student labs. Each lab section is divided into four teams of students. The students are a mix of biomedical, biotechnology, food science, and animal science majors. The photographs are examples of student plates taken by the author (KM). Students were asked to evaluate this lab project (as well as others) and typical comments are below. Generally, they greatly enjoyed "seeing" the antibiotic activity of the streptomycetes and wanted to know more about their particular samples. About half of the students voted this project their favorite, over their unknown identification project. "I loved learning about where our antibiotics come from, I don't think I ever really knew before this project."

"It would be neat to get very, very diverse samples instead of using just relatively local-type soil."

"It would be interesting to run more tests on the Streptomyces and figure out individual species of each."

"I would like to know the exact antibiotics that each one was producing as well."

Student Data.

Example data sheet.

	Zone size (mm)				
	Spot, plug, or disc				
Lawns	Streptomycete 1	Streptomycete 2	Streptomycete 3	Streptomycete 4	Antibiotic disc

Streptomycete 1	XXXX	1	2	3	6
Streptomycete 2	1	XXXX	2	4	0
Streptomycete 3	3	0	XXXX	0	7
Streptomycete 4	0	0	5	XXXX	12
Gram-negative 1	4	0	2	0	XXXX
Gram-negative 2	3	0	4	5	XXXX
Gram-positive 1	1	0	0	0	XXXX
Gram-positive 2	0	0	0	0	XXXX

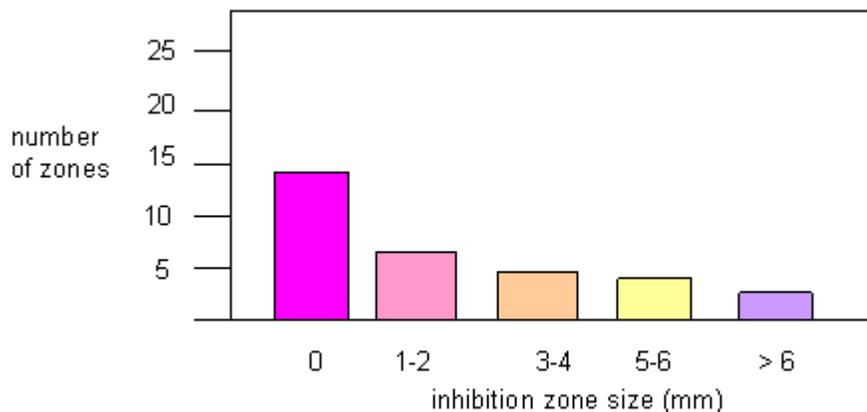
Bacterial and streptomycete densities.

Usually too numerous to count on lowest dilutions and a few colonies on highest dilution, among all the plates students can easily find 8 to 10 streptomycetes.

Number of inhibitions and zone sizes.

The number of inhibitions produced by each streptomycete and the size of the zones each streptomycete produces are highly variable.

Example Bar Graph (question 2)



Inhibitory activities of streptomycetes against other bacteria.

Inhibitory interactions are often highly specific. Individual streptomycetes frequently have very different inhibitory profiles. Gram-positive bacteria are generally more susceptible to inhibition by streptomycetes than gram-negative bacteria, though there are many exceptions.

Antibiotic susceptibility of streptomycetes.

Resistance to antibiotic inhibition is also highly specific, with resistance profiles varying widely among individual streptomycete isolates. Streptomycetes are generally more susceptible to antibiotics produced by other streptomycetes (like tetracycline and erythromycin) than to antibiotics produced by other microbes (like penicillin and polymyxin B).

SUPPLEMENTARY MATERIALS

Possible Modifications.

Ecology studies:

Do streptomycetes from different soils (a plot containing native, undisturbed vegetation versus a plot with insecticides, herbicides, fungicides or antibiotics applied) vary in the antibiotic inhibitory or resistance activities? Is antibiotic activity (a competitive trait) related to streptomycete or total bacterial density in soil? How deep in the soil are streptomycetes normally found? Are they found in aquatic soils? What are the potential advantages or disadvantages for streptomycetes to inhibit or to resist inhibition by organisms from the same or different soils? Why are patterns of interaction so complex?

Phenotype studies:

How quickly can the isolated streptomycetes degrade complex recalcitrant polymers like chitin, lignin, cellulose, pectin, and keratin? How variable in structure are the aerial hyphae with chains of conidiospores?

Grading Rubrics.

Grading rubric for oral presentation topic:

Informative, well-researched	5 pts
Presentation within time, well organized, practiced, interesting	5 pts
Handout complete, well-organized information, within space_____	5 pts
Total points possible	15 pts

Grading rubric for individual lab notebook:

Entries complete, appropriate detail, dates	4 pts
Entries well organized, easy to follow, easy to find information	4 pts
Neat and legible_____	2 pts
Total points possible	10 pts

Grading rubric for individual Gram stain:

Proper density on smear, hyphae and spore pattern visible	3 pts
Proper staining_____	2 pts
Total points possible	5 pts

Grading rubric for oral team data presentation:

Results properly analyzed and interpreted	7 pts
Results presented in organized way, easy to follow, within time_____	3 pts
Total points possible	10 pts

Recipes.**Oatmeal agar amended with cycloheximide**

1. Use a 2 liter flask.
2. To 500 ml of oatmeal extract* add 500 ml of distilled water.
3. Add 15 g of agar.
4. Autoclave and cool slightly.
5. Add 10 ml of cycloheximide solution,** swirl to mix but avoid large bubbles.
6. Makes about 65 to 67 15-ml plates.

***Oatmeal extract**

1. Use a 2 liter flask.
2. For 1 liter mix 20 g of oatmeal (grocery store regular oats) with 1 liter of distilled water.
3. Autoclave for 30 minutes (put flask in tray filled with a couple inches of water).
4. Strain the mixture through cheesecloth.
5. Dispose of the oatmeal chunks.
6. Distribute extract into 500 ml flasks (250 ml of extract each).
7. Reautoclave and store RT until use.

****Cycloheximide solution (100 µg/ml in 70% ethanol)**

1. Autoclave a clean, empty 250 ml flask.
2. Add 74 ml of 95% ethanol and 26 ml of sterile distilled water.
3. Add 1.0 g of cycloheximide powder.
4. Store at 4°C.

All oatmeal agar plates used in this activity should contain cycloheximide.

Difco Mueller-Hinton agar, Difco tryptic soy agar, or Difco nutrient agar can be purchased from Becton, Dickinson and Company, Sparks, MD 21152, USA.

References.

1. **Bentley, S. D., et al.** 2002. Complete sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**:141–147.
2. **Currie, C. R., J. A. Scott, R. C. Sumerbell, and D. Malloch.** 1999. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* **398**:701–704.

3. **Sylvia, D. M., J. J. Fuhrmann, P. G. Hartel, and D. A. Zuberer.** 1998. Principles and applications of soil microbiology, Pearson Prentice-Hall, Upper Saddle River, N.J.
4. **Williams, S. T., M. E. Sharpe, and J. G. Holt (ed.)**. 1989. Bergey's manual of systematic bacteriology, vol. 4, p. 2451-2492. The Williams and Wilkins Company, Baltimore, Md.

PROCEDURE – STUDENT VERSION

A Laboratory Project Investigating Phenotypic Diversity Among Soilborne Streptomyces

Introduction. Ahh, the smell of dirt! That’s the smell of geosmins, volatile substances given off by streptomyces—aerobic, gram-positive, spore-forming, filamentous bacteria found abundantly in most soils of the world. Growing on agar these bacteria look somewhat like fungi. They form filamentous hyphae inside the agar, called substrate hyphae, as well as aerial hyphae. The aerial hyphae eventually form chains of nonmotile spores. This gives the colonies a powdery or chalky look. The hyphae make the colonies tough and leathery, too, instead of soft and moist like most bacterial colonies. While the colonies are often white, the streptomyces can form pigmented spores or exude pigments into the substrate that make for gorgeous plates of pinks, yellows, greens, blues, violets, or grays.

Ecologically, streptomyces are incredibly important soil inhabitants. Streptomyces can degrade a wide range of organic materials, but most especially the difficult, recalcitrant materials like cutin, pectin, and lignin, and turn those materials into soil-conditioning humus. This is beneficial for plants growing in the soil. In turn, plant roots secrete, exude, and leak nutrient-rich materials that may be an important food source for the streptomyces.

The streptomyces have another talent as well. To compete with the thousands of other bacteria, fungi, and protozoa in each gram of soil, they produce the microbial version of weapons of mass destruction—antibiotics like chloramphenicol, streptomycin, neomycin, erythromycin, nystatin, and tetracycline. Over 70% of the antibiotics currently used for human and veterinary health are produced by streptomyces. To underscore the importance of these organisms, the genomes of two streptomyces, *Streptomyces coelicolor* A3(2) and *Streptomyces avermitilis*, have already been completely sequenced. Interestingly, ants figured out the value of having antibiotic producers around at least 50 million years ago. The leaf cutter ants common in North and South America coat their bodies with streptomyces, which they use to inhibit the growth of troublesome fungi.

This raises interesting and intriguing questions about streptomyces in soil. What other organisms do streptomyces inhibit? What may be the advantages or disadvantages of inhibiting some groups and not others? If a streptomyces is a good inhibitor of other organisms, is it also good at resisting antibiotic inhibition? And what might patterns of inhibitory interaction among streptomyces suggest about the ecology of these organisms in soil?

In this major lab project, you will examine aspects of the phenotypic diversity of streptomyces isolated from different soils. As individuals, you will identify, purify, and make a spore suspension of four streptomyces isolates from your soil sample. You will discover the antibiotic inhibition and resistance profile for each of these four streptomyces as you challenge them against each other, against antibiotic discs, and against a set of gram-positive and gram-negative bacteria. You will each be expected to keep a research notebook. As a team, you will give two short oral reports—one on an assigned streptomyces-related topic, and one on your experimental results.

This project is worth 40 points and has four components:

1. A 5 to 8-minute team oral report to class on your topic area* (15 pts); reports are given in lab; at least two team members must report; a one-page summary with three main references is to be turned in to the instructor. Due (date) in lab.

2. Individual lab research notebook (10 pts); this lab research notebook (provided) should contain ALL of your lab and analysis work. Due (date) in lab.
3. Individual Gram stain of your chosen streptomycete isolate (5 pts). Due (date) in lab.
4. A 5-minute team oral data report of experimental results for your soil (10 pts); reports are given in lab, at least two team members must report (different members than first oral report). Due (date) in lab.

*topic areas: microbial community ecology of soil and rhizosphere

antibiotic diversity of streptomycetes

streptomycete genetics and physiology

industrial antibiotic production, “from microbe to medicine”

Your team may collect soil from your own site or use soil supplied by the instructor. To collect your own soil, get a new pint- or quart-sized zip-top storage bag from your instructor. Loosen the soil if necessary. Collect about 1/4 cup of soil. Avoid contaminating the soil by touching it with your hands. If you push the outside bottom of the bag into and through the inside of the bag with your hand you can scoop the soil into the bag without touching it. Seal the bag and get it to your instructor at least 2 days before the laboratory project starts. Soil can be collected from any site (e.g., fields, gardens, woodlands, under turf, near sidewalks, roadside ditches). While unlikely, it is possible that because you are isolating unknown organisms from the soil, you may isolate pathogens. To minimize the collection of any human pathogens, avoid sites where fecal contamination is possible (e.g., fields spread with manure, feedlots, active pastures).

PROJECT DAY 1: Isolate bacteria from soil

Divide into teams as indicated by your instructor.

Assigned research topic = _____

Record soil source = _____

Place 1 g of prepared soil in 9 ml of sterile dH₂O and vortex for 1 minute.

Let sample sit at room temperature for 30 minutes.

While you are waiting, decide what serial dilution protocol to use so that you can determine the total number of CFU per gram of soil and the number of streptomycete CFU per gram of soil.

Expect a typical total CFU per gram of soil to be about 10^6 to 10^7 . Your team will have four or five 9 ml sterile water tubes as diluent and four oatmeal agar plates. You will use micropipettors to plate 100 μ l aliquots of your dilutions onto your dilution plates. Get instructor approval before you proceed.

Plate 100 μ l of your dilutions on oatmeal agar amended with cycloheximide (100 μ g/ml). These plates will be referred to as OA plates. Use a sterile bent glass rod to spread the sample.

Incubate for 5 days at 28°C. Streptomycetes are considered slow-growers compared to the typical bacteria used in microbiology labs.

PROJECT DAY 2: Determine total bacteria and streptomycete population densities and subculture individual streptomycete isolates

Determine population of total bacteria and of streptomycetes using plate count methodology. Streptomycete colonies often are small, hard, and friable. They appear to have a powdery coating and often exhibit a zone of inhibition. They will not be gooey or shiny. Streptomycetes can be various colors above the agar (but most often are white or gray) and below the agar (frequently gray or yellow).



Typical soil dilution plate showing small, white, hard streptomycete colonies.

Dilution plate	Number total CFU	Number streptomycete CFU
10 ⁻¹		
10 ⁻²		
10 ⁻³		
10 ⁻⁴		



Close up of area from soil dilution plate showing white, hard streptomycete colonies powdery, granular appearance of white streptomycetes

Number of total CFU per gram of soil: _____

Number of streptomycetes per gram of soil: _____

What percentage of the soil bacteria are streptomycetes? Is this consistent for all soil sources used in class?

Why might the percentage of streptomycetes be artificially high?

Isolate and purify individual streptomycetes by collecting spores on a sterile toothpick and streaking them onto OA. Handle the toothpick just as you would an inoculating loop. Use a gentle touch as you glide it across the agar when streaking the OA plate. Collect eight isolates total and streak them onto two OA plates. Label these plates “subcultures 1–4” and “subcultures 5–8”. Incubate for 2 days at 28°C. If after 2 days your plates appear to be growing only streptomycetes,

continue incubating them at 28°C. If after 2 days you see any growth that looks like a contaminant, restreak your isolates onto new OA plates using sterile toothpicks. Most contaminating bacteria will grow faster than the streptomycetes so you can usually find a section of the streptomycete colonies that appears clean and streak from that area. Remember that you only need four isolates, so if some are beyond purifying, that's okay.



PROJECT DAY 3: Purify selected streptomycetes subcultures

Among the eight streptomycetes selected above, choose the four “best” isolates for further work. The “best” streptomycete characteristics will be those that are fast growers (have spread the most on your plate or produced the most powdery spores), those that appear pure, and those that show diverse phenotypes (colors). To do this, use a sterile toothpick to streak each isolate as heavily as possible onto half a plate of OA. Use two OA plates, two isolates per plate. Label these plates “spore collection 1–2” and “spore collection 3–4.” Incubate for 5 to 7 days at 28°C.



PROJECT DAY 4: Spore collection, antibiotic inhibition and resistance assays

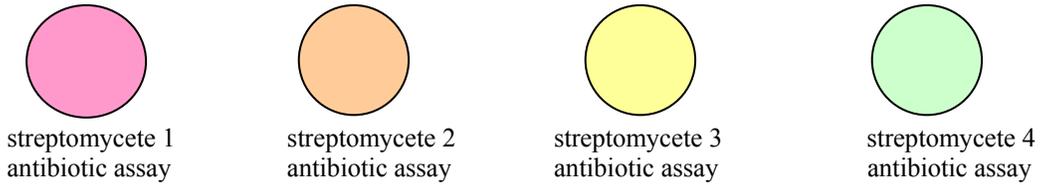
Spore collection:

Using a micropipettor, aliquot 0.75 ml of sterile 20% (v/v) glycerol into four 1.5 ml sterile microfuge tubes. Label the tubes “spore suspension 1, 2, 3, and 4.” Moisten a sterile cotton swab by dipping it into one of the microfuge tubes of sterile 20% (v/v) glycerol. Collect the streptomycete spores by gently rolling the moist sterile swab across the surface of the spore collection plate, one isolate at a time, being careful not to move the swab into the other half of the plate. Twirl the cotton swab in the microfuge tube to liberate the spores. To guarantee good spore harvest, reswab two more times, using the same swab and liberating the spores into the same tube. Repeat the spore collection process for each of the four isolates using one fresh swab for each isolate. Save these swabbed spore collection plates as you will use them again to collect plugs.

Antibiotic assays:

Get four OA plates and label them “streptomycete 1–4 antibiotic assay.” Using a micropipettor, add 100 µl of your spore suspension 1 to the OA plate labeled “streptomycete 1 antibiotic assay,” add 100 µl of your spore suspension 2 to the OA plate labeled “streptomycete 2 antibiotic assay,”

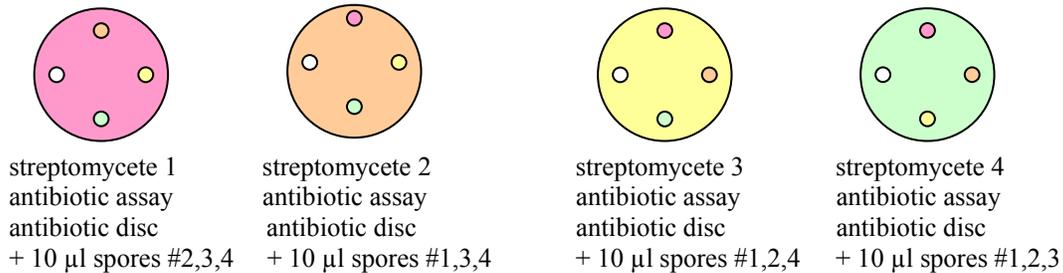
and repeat until you have made all four streptomycete lawns. Use a sterile bent glass rod to spread the spores and make good, solid lawns.



Allow the lawns to dry about 5 minutes before proceeding.

Use a sterile forceps to add one antibiotic disc (your choice of those supplied by your instructor, use the same antibiotic for each plate) to each antibiotic assay plate. Tap the disc down with the forceps to make sure it adheres to the agar.

On each antibiotic assay plate make three 10- μ l spots of the other streptomycete spore suspensions. Use a fresh tip each time.



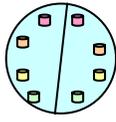
Set these plates aside and at the end of the class period invert them and incubate 7 days at 28°C.

Get two Mueller-Hinton (MH), tryptic soy agar (TSA), or nutrient agar plates. Label one of these plates “gram-negative antibiotic assay” and the other “gram-positive antibiotic assay.” Divide one MH plate in half and use two sterile swabs to make two half-lawns of the two gram-negative bacteria provided. Divide the second MH plate in half and use two sterile swabs to make two half-lawns of the two gram-positive bacteria provided. Label each half-lawn with the name of the swabbed bacteria.

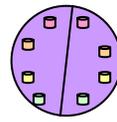


Get a cork borer, flame sterilize it using ethanol, and once it is cool punch four holes in your swabbed spore collection plate 1. Sterilize the cork borer again and punch four holes in your swabbed spore collection plate 2. Repeat for plates 3 and 4. The plugs should stay in the plate. Each plug contains any antibiotic that the streptomycete produced that diffused into the agar.

Use a sterile forceps or spatula to carefully pick up the plugs and place them onto each MH lawn, upside down or right side up (doesn't matter).



gram-negative antibiotic assay
+ plugs from each streptomycete



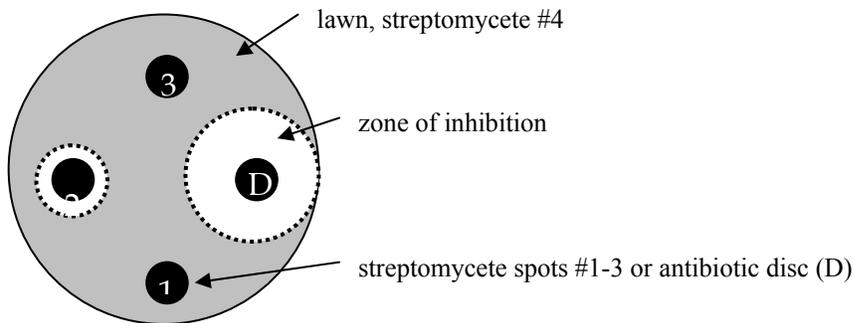
gram-positive antibiotic assay
+ plugs from each streptomycete

Incubate these plates 7 days at 30 °C. Do NOT invert these plates or the plugs may fall off.

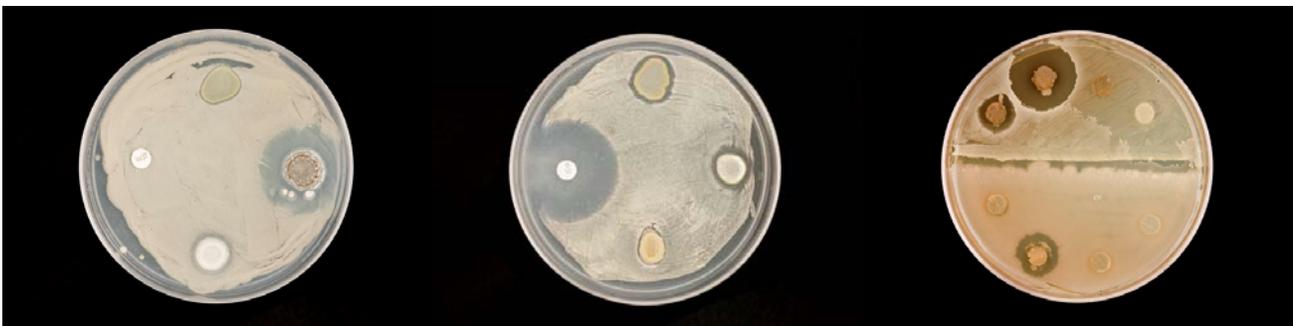
PROJECT DAY 5: Data collection and analysis

Zones of inhibition:

Measure to the nearest mm any zones of inhibition. Measure from the edge of the dotted streptomycete spore isolate or disc or plug to the edge of the zone. Record your data on the data sheet provided.



In the above example, streptomycete #1 (spot) did not inhibit streptomycete #4 (lawn), as no zone of inhibition was produced, and conversely, streptomycete #4 was resistant to whatever antibiotic streptomycete #1 produced. Also, streptomycete #2 did inhibit streptomycete #4, and conversely, streptomycete #4 was susceptible to the antibiotics produced by streptomycete #2.



Plates 1 and 2 contain streptomycete spore suspension lawns on OA plates challenged with the other spore suspensions of the collection and an antibiotic disc, plate 3 contains two lawns of gram-positive bacteria challenged with antibiotic-containing streptomycete plugs on MH agar.

Analysis:

1. How many total CFU/g are in your soil? How many of those are streptomycetes? What differences or similarities in substrate and aerial hyphae did you observe in your streptomycete collection?
2. How many zones of inhibition were produced? How many of each size were produced? Construct a bar graph to show your results.

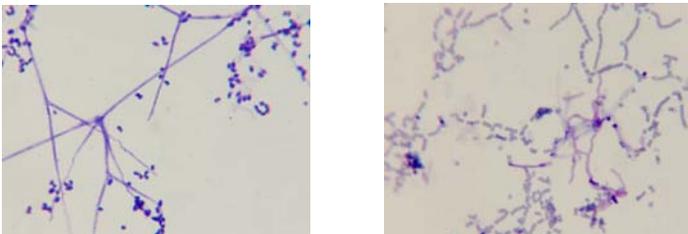
3. How many microbes did each of your streptomycetes inhibit? Construct a bar graph to show your results.
4. How many microbes did each of your streptomycetes resist? Construct a bar graph to show your results.
5. Are your streptomycetes resistant or susceptible to the antibiotic in the disc? Is it an antibiotic naturally produced by a streptomycete?

Answer the following questions after class data has been shared:

6. Do your streptomycetes appear to be strong or weak resistors when compared to others in class? Explain.
7. Do your streptomycetes appear to be strong or weak inhibitors when compared to others in class? Explain.
8. Does any particular soil source seem to produce more effective antibiotic producers? Explain.
9. Why do you think the streptomycetes produce antibiotics? How do you think their antibiotic-producing ability affects the microbial soil community? Explain.

Staining:

From a portion of a plate that contains any streptomycete isolate, use a toothpick to gather aerial filaments and spores and perform a standard Gram stain. Your Gram stain should yield a gram-positive reaction. Note in particular the filamentous nature of the streptomycetes and find the spore pattern of your streptomycete isolate. View the spore pattern of other streptomycetes stained by your neighbors.



Typical stains of streptomycetes

DATA SHEET

	Zone size (mm)				
	spot, plug, or disc				
Lawns	Streptomycete 1	Streptomycete 2	Streptomycete 3	Streptomycete 4	Antibiotic disc
Streptomycete 1	XXXX				
Streptomycete 2		XXXX			
Streptomycete 3			XXXX		
Streptomycete 4				XXXX	
Gram-negative 1					XXXX
Gram-negative 2					XXXX
Gram-positive 1					XXXX
Gram-positive 2					XXXX

PROCEDURE – INSTRUCTOR VERSION

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2. Individual lab research notebook (10 pts); this lab research notebook (provided) should contain ALL of your lab and analysis work. Due (date) in lab.
3. Individual Gram stain of your chosen streptomycete isolate (5 pts). Due (date) in lab.
4. A 5-minute team oral data report of experimental results for your soil (10 pts); reports are given in lab, at least two team members must report (different members than first oral report). Due (date) in lab.

*topic areas: microbial community ecology of soil and rhizosphere

antibiotic diversity of streptomycetes

streptomycete genetics and physiology

industrial antibiotic production, “from microbe to medicine”

Your team may collect soil from your own site or use soil supplied by the instructor. To collect your own soil, get a new pint- or quart-sized zip-top storage bag from your instructor. Loosen the soil if necessary. Collect about 1/4 cup of soil. Avoid contaminating the soil by touching it with your hands. If you push the outside bottom of the bag into and through the inside of the bag with your hand you can scoop the soil into the bag without touching it. Seal the bag and get it to your instructor at least 2 days before the laboratory project starts. Soil can be collected from any site (e.g., fields, gardens, woodlands, under turf, near sidewalks, roadside ditches). While unlikely, it is possible that because you are isolating unknown organisms from the soil, you may isolate pathogens. To minimize the collection of any human pathogens, avoid sites where fecal contamination is possible (e.g., fields spread with manure, feedlots, active pastures).

This ongoing lab project allows students to explore the antibiotic activity of soilborne streptomycetes. It exposes them to slightly different laboratory techniques (using toothpicks instead of loops or needles and spotting bacteria on top of bacterial lawns and collecting bacterial spores) and allows viewing of filamentous bacteria. It builds on aseptic techniques and microscopy skills they already have accomplished. It also exposes them to the idea of microbial ecology, a topic often lacking in a one-semester general microbiology course. If you collect soil for your students rather than having them gather their own soil, you may want to consider the possible modifications listed in the supplementary materials. I group the students into teams based on the number of students per lab bench. My benches hold five students, and I have five benches, thus there are up to five teams of five students per lab section. I have two to four lab sections per semester.

On the first day of the project I give students minimal background information about streptomycetes and then assign them, as teams, topics to research. The topics are: soil and the rhizosphere, antibiotic history and diversity, streptomycete genetics, and industrial antibiotic production. Other topic ideas are ecological competition or niche. The students prepare for and give a 10-minute oral report to the lab class one week later. If students are more familiar with streptomycete ecology, genetics, physiology, and antibiotics, they appreciate and understand the project more, however, they will gain a lot from the project even if they do not do the research and give an oral report.

The day before the project starts, have all soil gathered and ready. Teams that are bringing in their own soil must get it to you ahead of time. Soils can be collected from the root zone of particular plants or from other areas (deciduous woodland, coniferous woodland, pasture, garden, lawn, etc.). Weigh out ~5 g of fresh weight soil and dry overnight in weigh boats under two layers of sterile cheesecloth. The next day (the day the project starts), prior to the start of the lab, weigh out a 1 g subsample of the dried soil. Break apart large clumps of soil by grinding soil in a glass petri plate with a weigh boat or spoon. Cover the soil sample with a sterile cheesecloth and place it in a heating oven at 60°C for 90 minutes. While this step can be omitted, it

helps to reduce the number of competing, spreading bacteria and fungi in the sample and makes it much easier to obtain pure cultures of streptomycetes.

PROJECT DAY 1: Isolate streptomycetes from soil (1.5 hours)

Have all students on the same team use the same source of soil. They will accumulate more data about a specific soil this way. Split them into small groups or individuals to come up with the protocol for how to perform the soil dilution series and let them compare their protocols with others before you verify it. You can have them prepare the soil dilution bottles as one team and split the team back into individuals to prepare the plates for colony counts. This way they will be able to replicate the plate count data and have several plates from which to locate and choose streptomycetes.

Divide into teams as indicated by your instructor. **Assign each team a topic to research.**

Record soil source = _____

Place 1 g of prepared soil in 9 ml of sterile dH₂O and vortex for 1 minute (**need one 9 ml water blank per team**).

Let sample sit at room temp for 30 minutes.

While you are waiting, decide what serial dilution protocol to use so that you can determine the total number of CFU per gram of soil and the number of streptomycete CFU per gram of soil. Expect a typical total CFU per gram of soil to be about 10⁶ to 10⁷. Your team will have four or five 9 ml sterile water tubes as diluent and four plates. You will use micropipettors to plate 100 µl aliquots of your dilutions onto your dilution plates. Get instructor approval before you proceed. (**need four 9 ml water blanks per team, 10⁻² to 10⁻⁵ serial dilutions work well**)

Plate 100 µl of your dilutions on oatmeal agar amended with cycloheximide (100 µg/ml). These plates will be referred to as OA plates. Use a sterile bent glass rod to spread the sample. (**do as individuals, each student needs four OA plates**)

Incubate for 5 days at 28°C. Streptomycetes are considered slow-growers compared to the typical bacteria used in microbiology labs.

PROJECT DAY 2: Determine total bacteria and streptomycete population densities and subculture individual streptomycete isolates (1 hour)

Determine population of total bacteria and of streptomycetes using plate count methodology. Streptomycete colonies often are small, hard, and friable. They appear to have a powdery coating and often exhibit a zone of inhibition. They will not be gooey or shiny. Streptomycetes can be various colors above the agar (but most often are white or gray) and below the agar (frequently gray or yellow). **Another group, the *Streptoverticillium*, may be mistaken for the *Streptomyces* at this stage. They also produce antibacterial compounds and have colored substrate and aerial mycelium. It is okay if the students happen to include them in this study. They will be easier to identify when they are grown out in pure culture. If not today, then the next time they go to subculture the streptomycetes, the students should notice the smell of “soil.” Even without opening their plates this distinctive odor wafts from the incubator.**

Dilution plate	Number total CFU	Number streptomycete CFU
10 ⁻²		
10 ⁻³		
10 ⁻⁴		
10 ⁻⁵		



Typical soil dilution plate showing small, white, hard streptomycete colonies.

Close-up of area from soil dilution plate showing powdery, granular appearance of white streptomycetes.

Number total CFU per gram of soil: usually 10⁶ to 10⁷

Number of streptomycetes per gram of soil: **usually 10 to 100 fold less than above, overall a large percentage of soilborne bacteria are streptomycetes.** Students should discover that the streptomycetes comprise a significant portion of soil microbes, however, in this experiment they need to realize they have selected for them (using OA and the heating step done by the instructor), so the percentage will be artificially high.

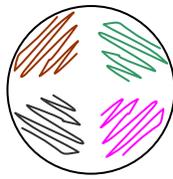
What percentage of the soil bacteria are streptomycetes? Is this consistent for all soil sources used in class?

Why might the percentage of streptomycetes be artificially high?

Isolate and purify individual streptomycetes by collecting spores on a sterile toothpick and streaking them onto OA. Handle the toothpick just as you would an inoculating loop. Use a gentle touch as you glide it across the agar when streaking the OA plate. Collect eight isolates total and streak them onto two OA plates. Label these plates “subcultures 1–4” and “subcultures 5–8”. Incubate for 2 days at 28°C. If after 2 days your plates appear to be growing only streptomycetes, continue incubating them at 28°C. If after 2 days you see any growth that looks like a contaminant, restreak your isolates onto new OA plates using sterile toothpicks. Most contaminating bacteria will grow faster than the streptomycetes so you can usually find a section of the streptomycete colonies that appears clean and streak from that area. Remember that you only need four isolates, so if some are beyond purifying, that’s okay. **Toothpicks are used instead of loops as the cracks within the toothpick pick up considerably more spores. Have the students check their choices with you before they subculture. Four isolates are put on one plate and only two plates are used, all to save costs. If more resources are available, isolates could be streaked onto separate plates or more isolates could be streaked.**



subcultures 1-4



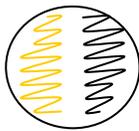
subcultures 5-8



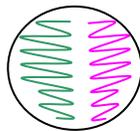
Typical quadrant subcultures

PROJECT DAY 3: Purify selected streptomycetes subcultures (0.5 hour)

Among the eight streptomycetes selected above, choose the four “best” isolates for further work. The “best” streptomycete characteristics will be those that are fast growers (have spread the most on your plate or produced the most powdery spores), those that appear pure, and those that show diverse phenotypes (colors). To do this, use a sterile toothpick to streak each isolate as heavily as possible onto half a plate of OA. Use two OA plates, two isolates per plate. Label these plates “spore collection 1–2” and “spore collection 3–4.” Incubate for 5 to 7 days at 28°C. **After 2 to 3 days of incubation you can usually tell if the plates are contaminated. Restreaking is necessary mostly for isolates contaminated with *Bacillus* spp. The longer incubation is required to get good spore production. The aerial mycelium of *Streptoverticillium* will have a very cottony appearance. The aerial mycelium of *Streptomyces* will look more powdery. Have the students check their choices with you before they make their spore collection plates. Usually only 10% of the students find contaminated plates and need to subculture. Plates need to incubate a minimum of 5 days to produce an abundant spore population, 7 days is better.**



spore collection 1-2



spore collection 3-4

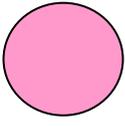
PROJECT DAY 4: Spore collection, antibiotic inhibition and resistance assays (2 hours)

Spore collection:

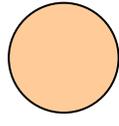
Using a micropipettor, aliquot 0.75 ml of sterile 20% (v/v) glycerol into four 1.5 ml sterile microfuge tubes. Label the tubes “spore suspension 1, 2, 3, and 4.” Moisten a sterile cotton swab by dipping it into one of the microfuge tubes of sterile 20% (v/v) glycerol. Collect the streptomycete spores by gently rolling the moist sterile swab across the surface of the spore collection plate, one isolate at a time, being careful not to move the swab into the other half of the plate. Twirl the cotton swab in the microfuge tube to liberate the spores. To guarantee good spore harvest, reswab two more times, using the same swab and liberating the spores into the same tube. **The tube should darken and become turbid as it fills with spores.** Repeat the spore collection process for each of the four isolates using one fresh swab for each isolate. Save these swabbed spore collection plates as you will use them again to collect plugs. **The swab collection process works well and results in little cross contamination even though the students are isolating from open, mixed plates. These streptomycete spores are much less mobile than those from typical laboratory fungal samples or contaminants.**

Antibiotic assays:

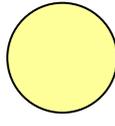
Get four OA plates and label them “streptomycete 1–4 antibiotic assay.” Using a micropipettor, add 100 µl of your spore suspension 1 to the OA plate labeled “streptomycete 1 antibiotic assay,” add 100 µl of your spore suspension 2 to the OA plate labeled “streptomycete 2 antibiotic assay,” and repeat until you have made all four streptomycete lawns. Use a sterile bent glass rod to spread the spores and make good, solid lawns.



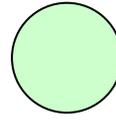
streptomycete 1
antibiotic assay



streptomycete 2
antibiotic assay



streptomycete 3
antibiotic assay

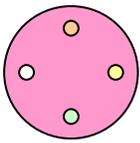


streptomycete 4
antibiotic assay

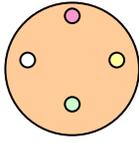
Allow the lawns to dry about 5 minutes before proceeding.

Use a sterile forceps to add one antibiotic disc (your choice of those supplied by your instructor, use the same one for each plate) to each antibiotic assay plate. Tap the disc down with the forceps to make sure it adheres to the agar.

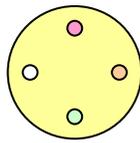
On each antibiotic assay plate make three 10- μ l spots of the other streptomycete spore suspensions. Use a fresh tip for each spot.



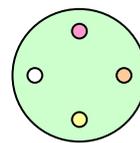
streptomycete 1
antibiotic assay
antibiotic disc
+ 10 μ l spores #2,3,4



streptomycete 2
antibiotic assay
antibiotic disc
+ 10 μ l spores #1,3,4



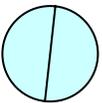
streptomycete
antibiotic assay
antibiotic disc
+ 10 μ l spores #1,2,4



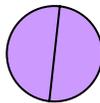
streptomycete 4
antibiotic assay
antibiotic disc
+ 10 μ l spores #1,2,3

Set these plates aside and at the end of the class period invert them and incubate 7 days at 28°C. **Wait to invert the plates or the 10- μ l spots will spread out.**

Get two Mueller-Hinton (MH), tryptic soy agar (TSA), or nutrient agar plates. Label one of these plates “gram-negative antibiotic assay” and the other “gram-positive antibiotic assay.” Divide one MH plate in half and use two sterile swabs to make two half-lawns of the two gram-negative bacteria provided. Divide the second MH plate in half and use two sterile swabs to make two half-lawns of the two gram-positive bacteria provided. Label each half-lawn with the name of the swabbed bacteria. **Gram-negative bacteria that have worked well are *Escherichia coli*, *Pseudomonas* species, and *Serratia marcescens*. Gram-positive bacteria that have worked well are *Bacillus cereus*, *Micrococcus luteus*, and *Staphylococcus epidermidis*. Others without spreading habits may work as well.**



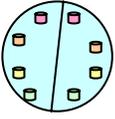
gram-negative antibiotic assay
name of bacteria



gram-positive antibiotic assay
name of bacteria

Get a cork borer, flame sterilize it using ethanol, and once it is cool punch four holes in your swabbed spore collection plate 1. Sterilize the cork borer again and punch four holes in your swabbed spore collection plate 2. Repeat for plates 3 and 4. The plugs should stay in the plate. Each plug contains any antibiotic that the streptomycete produced that diffused into the agar. **If #3 cork borers (6-mm diameter) are not available, the bulb-side of sterile glass pipettes works equally well.**

Use a sterile forceps or spatula to carefully pick up the plugs and place them onto each MH lawn, upside down or right side up (doesn't matter).



gram-negative antibiotic assay
+ plugs from each streptomycete



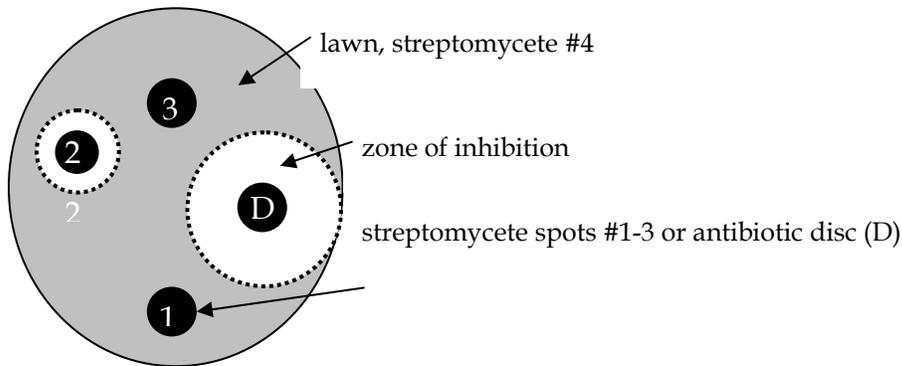
gram-positive antibiotic assay
+ plugs from each streptomycete

Incubate these plates 7 days at 30°C. Do NOT invert these plates or the plugs may fall off.

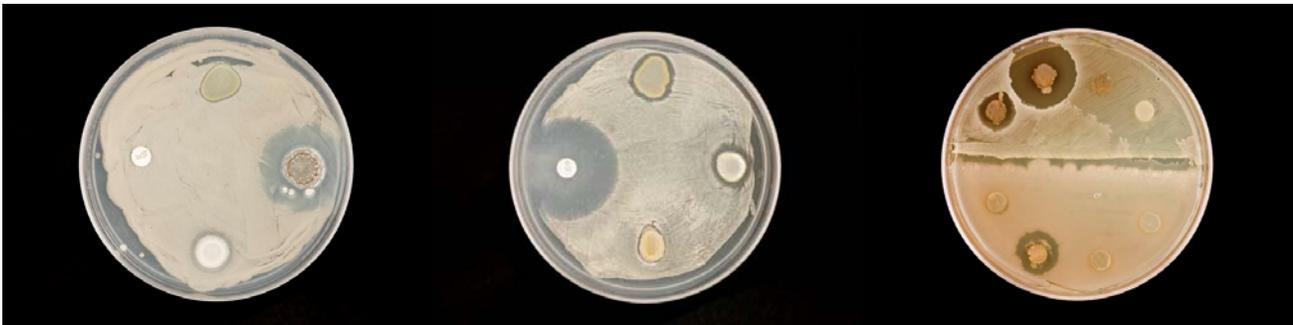
PROJECT DAY 5: Data collection and analysis (2 hours)

Zones of inhibition:

Measure to the nearest mm any zones of inhibition. Measure from the edge of the dotted streptomycete spore isolate or disc or plug to the edge of the zone. Record your data on the data sheet provided. **Occasionally two zones are visible: a distinct clear, kill zone and an opaque inhibit zone. Either have the students ignore any opaque zones and measure only kill zones, or have them measure both. Rarely, a spot of spore suspension yields very little growth. As the students were to select streptomycetes with relatively fast growth, it is most likely due to the lawn streptomycete inhibiting the spot streptomycete rather than the spot streptomycete being of low viability. However, sometimes students select streptomycetes that are not very vigorous growers. If that is the case, each time a lawn or spot is made of that spore suspension the growth would be minimal. Have the student determine what would be the appropriate treatment of that data.**



In the above example, streptomycete #1 (spot) did not inhibit streptomycete #4 (lawn), as no zone of inhibition was produced, and conversely, streptomycete #4 was resistant to whatever antibiotic streptomycete #1 produced. Also, streptomycete #2 did inhibit streptomycete #4, and conversely, streptomycete #4 was susceptible to the antibiotics produced by streptomycete #2.



Plates 1 and 2 contain streptomycete spore suspension lawns on OA plates challenged with the other spore suspensions of the collection and an antibiotic disc, plate 3 contains two lawns of gram-positive bacteria challenged with antibiotic-containing streptomycete plugs on MH agar.

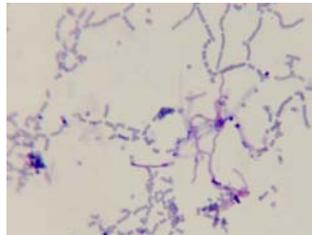
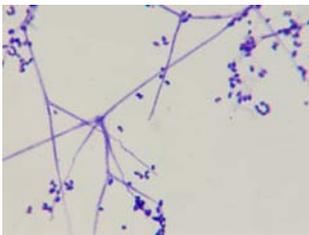
Analysis:

1. How many total CFU/g are in your soil? How many of those are streptomycetes? What differences or similarities in substrate and aerial hyphae did you observe in your streptomycete collection?
2. How many zones of inhibition were produced? How many of each size were produced? Construct a bar graph to show your results. (**Y axis = number of zones, X axis = inhibition zone size**)
3. How many microbes did each of your streptomycetes inhibit? Construct a bar graph to show your results. (**Y axis = number of microbes inhibited, X axis = streptomycete #1, #2, #3, or #4**)
4. How many microbes did each of your streptomycetes resist? Construct a bar graph to show your results. (**Y axis = number of streptomycetes resisted, X axis = streptomycete #1, #2, #3, or #4**)
5. Are your streptomycetes resistant or susceptible to the antibiotic in the disc? Is it an antibiotic naturally produced by a streptomycete?

Answer the following questions after class data has been shared:

6. Do your streptomycetes appear to be strong or weak resistors when compared to others in class? Explain.
7. Do your streptomycetes appear to be strong or weak inhibitors when compared to others in class? Explain.
8. Does any particular soil source seem to produce more effective antibiotic producers? Explain.
9. Why do you think the streptomycetes produce antibiotics? How do you think their antibiotic-producing ability affects the microbial soil community? Explain.

Staining: From a portion of a plate that contains any streptomycete isolate, use a toothpick to gather aerial filaments and spores and perform a standard Gram stain (**or simple stain to just check morphology**). Your Gram stain should yield a gram-positive reaction (even though plates are > 24 hours old). Note in particular the filamentous nature of the streptomycetes and find the spore pattern of your streptomycete isolate. View the spore pattern of other streptomycetes stained by your neighbors. **The biggest problem the students have in staining their bacteria is that they make too thick a smear—make sure they spread out their sample. *Streptomyces* spp. have straight, looped, or spiraled aerial hyphae bearing chains of many spores. The spores are smooth or ornamented with spines, warts, or hairs. *Streptoverticillium* spp. have straight aerial hyphae bearing branches in whorls from which the spores arise. Using low magnification it is described as resembling barbed wire.**



Typical stains of streptomycetes

Background:

Students will be expected to be able to use pipettes and micropipettors and be fairly proficient in aseptic techniques. Students will be expected to be able to do a Gram stain or simple stain and use a microscope by the last day of the project. Students will be expected to understand how to set up a serial dilution series and calculate viable cell

counts using the standard plate counting method. If this project is started about one-third to one-half of the way through the semester, then the students should have developed these skills to the necessary level. Data will be collected and analyzed from zones of inhibition. It is helpful, but not necessary, to have done an antibiotic disc diffusion test prior to reading the plates. It is helpful, but not necessary, to have discussed the main types of antibiotics and their modes of action before the zones are measured.