BIO8041
PRACTICAL

A Laboratory Project Investigating Antibiotic Production Among Soilborne Streptomycetes

Ahh, the smell of dirt! That's the smell of geosmins, volatile substances given off by streptomycetes—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 streptomycetes can form pigmented spores or exude pigments into the substrate that make for gorgeous plates of pinks, yellows, greens, blues, violets, or greys.

Ecologically, streptomycetes are incredibly important soil inhabitants. Streptomycetes 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 streptomycetes.

The streptomycetes 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 streptomycetes. To underscore the importance of these organisms, the genomes of two streptomycetes, *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 streptomycetes, which they use to inhibit the growth of troublesome fungi.

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

In this lab project, you will examine aspects of antibiotic production of streptomycetes isolated from different soils. As teams, you will identify, purify, and make a spore suspension of four streptomycete isolates from your soil sample. You will discover the antibiotic inhibition and resistance profile for each of these four streptomycetes as you challenge them against each other, against antibiotic discs, against a set of Gram-positive and Gram-negative bacteria and against target-specific reporter strains. You will each be expected to keep a research notebook and produce a report based on your findings.

PROJECT DAY 1: Isolate bacteria from soil

Divide into teams as indicated by your instructor.

Place 1 g of prepared soil in 9 ml of sterile dH_2O and vortex for 1 minute.

Let sample sit at room temperature for 5 minutes.

While you are waiting, decide what serial dilution protocol to use so that you can determine the total number of colony-forming units (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 dilutent and four oatmeal agar plates (N.B. aim for 30-300 CFU as the target number per plate).

You will use micropipettors to plate 100 µl (0.1 ml) aliquots of your dilutions onto your dilution plates. Get instructor approval before you proceed.

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. More information regarding the spread plate technique can be found here: http://bit.ly/2ih8MeQ

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

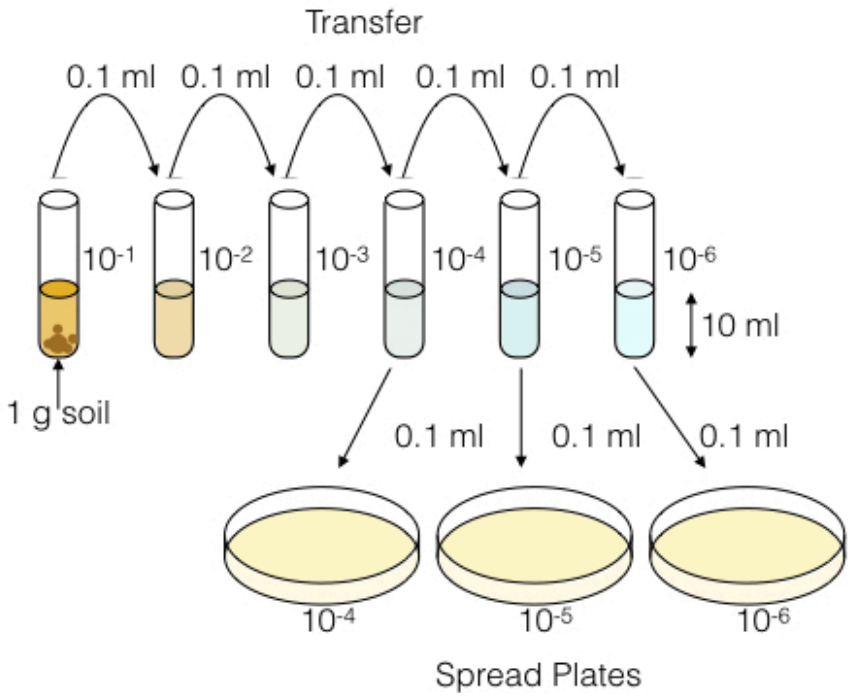

Fig. 1. Outline of serial dilution and spread plating

PROJECT DAY 2: Subculture individual streptomycete isolates

Streptomycete colonies often are small, hard, and friable (easily crumbled). They appear to have a powdery coating and often exhibit a zone of inhibition. They will sometime have liquid droplets on their surface (secondary metabolites - colony farthest right). They will not be gooey or shiny. Streptomycetes can be various colours above the agar (but most often are white or grey) and below the agar (frequently grey or yellow).

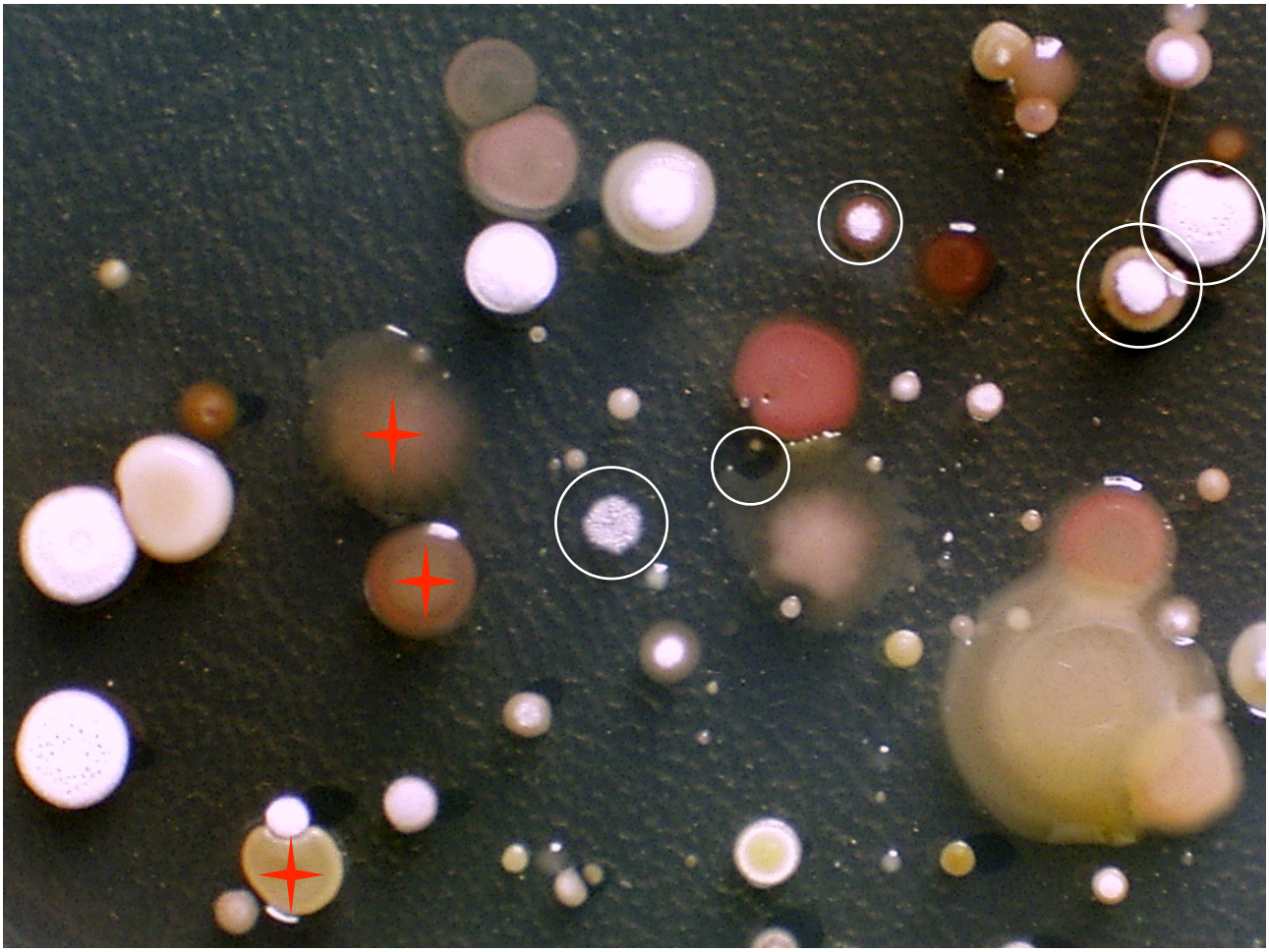

Fig. 2. Typical soil isolation spread plate. Colones with white circles are examples of possible streptomycetes; red crosses indicate non-streptomycete bacteria.

Isolate and purify individual streptomycetes by collecting spores on a sterile toothpick/ or plastic loop and streaking them onto OA. 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 7 days at 28°C. Most contaminating bacteria will grow faster than the streptomycetes so it is possible that some of your plates may be contaminated; pay particular attention to spore collection; be careful. Ask an instructor for guidance. Pure cultures will be made available if you fail to obtain pure cultures.

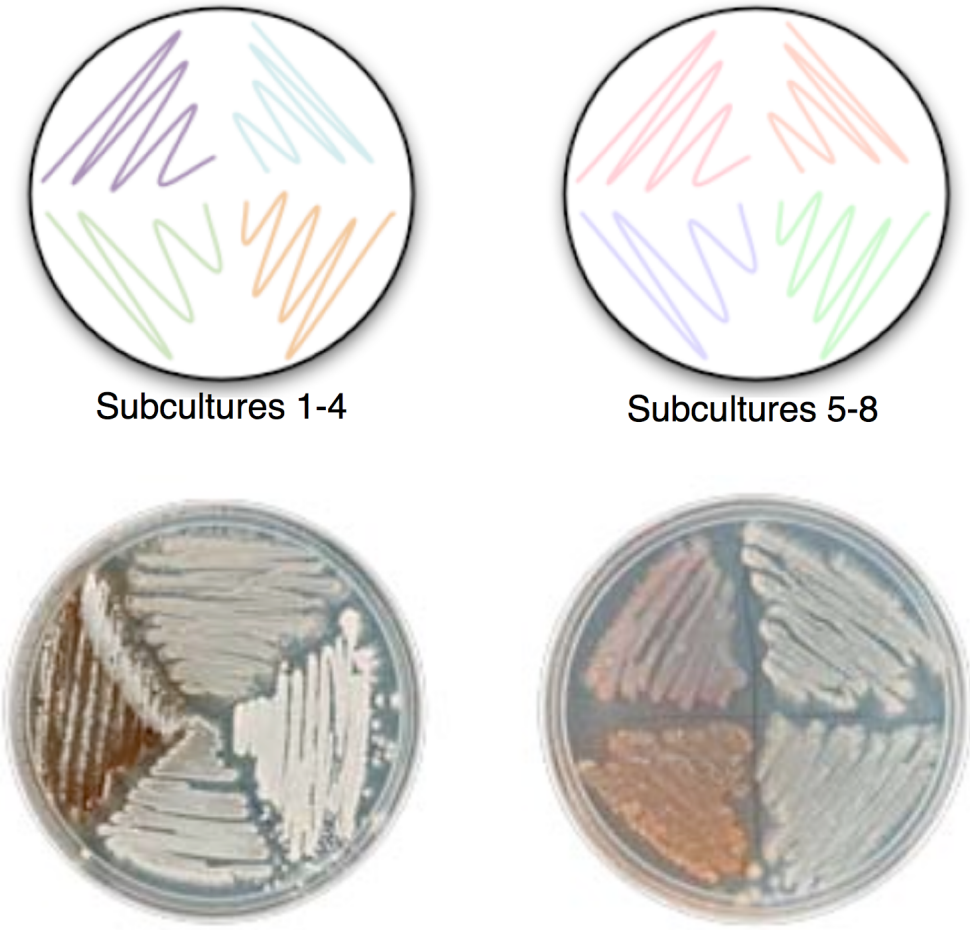

Fig. 3. Typical quadrant subculture.

PROJECT DAY 3: Purify selected streptomycetes subcultures and Gram stain

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 you will; prepare a spore suspension: using a micropipettor,

aliquot 750 μl 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 areas of the plate. Twirl the cotton swab in the microfuge tube to liberate the spores. To guarantee good spore harvest, re-swab 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. Prepare a lawn of each of your four streptomycetes. Get four OA plates and label them streptomycete 1, 2, 3 and 4. Using a micropipettor add 100 µl of

spore suspension 1 to the OA plate labeled streptomycete 1, use a sterile glass bent rod to gentle spread the spores over the entire surface of the plate. Repeat for streptomycetes 2-4. Incubate for 7 days at 28°C.

Fig. 4. Example of spore swab plates after 7 days incubation

Gram 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 neighbours. While the Gram stain is a very commonly used technique in microbiology, it is very often overlooked as a method that requires care and precision, in order to generate a good stain (see examples below) it is necessary to prepare good thin smears and pay particular attention to the timings of the stains and washes.

Place a 10 μl drop of water onto a glass microscope slide. With a pipette tip of wire loop, pick up a small amount of culture from the agar plate and mix it with the water drop. Be careful to take as small an amount as possible, as too much cell material will result in poor quality stains.

Spread the mixture across the surface of the slide to produce a thin smear. Hold the slide with tweezers and heat-fix the smear by rapidly placing in and out of a gently flame - avoid

localised heating, or over-heating. The heating helps retain the cells on surface if the slide, facilitating subsequent rinsing steps.

Perform staining steps over a staining tray - all rinsing steps should be done into a Gram-stain waste bottle. Make sure you are wearing gloves and lab coats

Add 10-20 μl of crystal violet stain to the heat-fixed cells. Let the stain stand for 30-60 seconds.

Pipette excess stain off of the slide and into the waste, rinse off the remaining stain with water from a squeeze bottle - rinse into the waste bottle.

Add 10-20 μl of iodine solution - Let stand for 30 seconds. Pipette off the excess and rinse with water as above.

Add 10-20 μl of ethanol, after 5 seconds rinse off with water - you should see the slide de-colour; there will likely be some remaining spots of purple.

Add 10-20 μl of safranin solution, let stand for 30 seconds and rinse off with water as above.

Using tissue paper to remove excess water from the back and sides of the the slide, place the slide on tissue and allow to air dry.

Examine the slide under a light microscope - view under x 10 and x 40 objectives. When you have a good image under x 40, ask a demonstrator to provide you with lens oil for observation using the x 100 objective - DO NOT get lens oil on the x 10 or x 40 objectives.

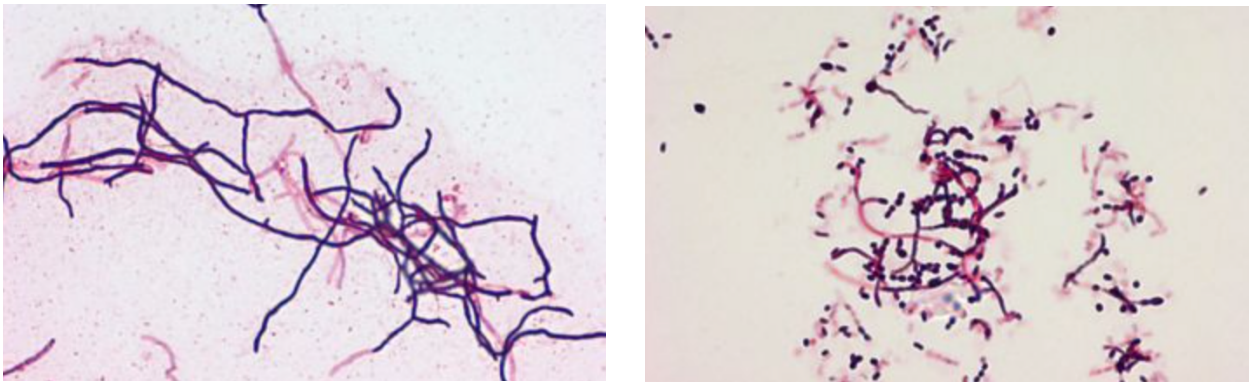

Fig. 5. Representative Gram-stains of two different *Streptomyces* species

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

Take a brass plug borer, flame sterilize it using ethanol (dip in ethanol, touch to side of jar, briefly pass through flame to burn of ethanol - do not hold in the flame) and once it is cool punch at least 10 holes/plugs in your streptomycete plate 1 (you do not need to sterilise between each plug)

Sterilize the cork borer again and punch 10 holes in your streptomyces plate 2. Repeat for plates 3 and 4. Leave the plug in the plate until required, some may get stuck in the borer; push out with tweezers. Each plug contains any antibiotic that the streptomycete produced.

Preparation of antibiotic screening plates:

You will be supplied with cultures of *E. coli*, *Pseudomonas fluorescens* (Gram-negative), *Bacillus subtilis* and *Micrococcus luteus* (Gram-positive); tubes of top agar (held at 47^{o}C) and four Muller-Hinton agar plates.

Label the Muller-Hinton plates with the name of one of the species above.

For each species above, add 100 μl of the culture to the to the top agar. Quickly mix the tube and pour the contents onto a pre-warmed (37°C) Muller-Hinton agar plate. Immediately rock the plate gently so that the top agar solution evenly covers the entire surface. Cool the plates with the lids on until fully solidified (ca. 5 mins).

For each plate add one plug from each of your streptomycetes - place onto the surface of agar such that the mycelium/spore material is on the top of the plug.

Use the template attached to the back of this report to add in the placement of your plugs (place under the plate). Incubate the plates at 30^{o}C overnight.

Mode of action screening plates:

You will be supplied with agar plates containing *Bacillus subtilis* reporter strains. These strains have promoters - from genes known to be upregulated upon exposure to antibiotics - fused to the reporter gene *lac*Z. These strains can be used to determine the mode of action (MoA) of antimicrobial compounds (see the BIO8041 website for further details).

Place one agar plug from each of your four streptomycetes strains on each reporter panel. Place a positive control on each reporter as follows (use the template attached to aid in the placement of your plugs and antimicrobials):

*din*B: Naladixic acid.
*yvg*S: Rifampicin.
*yvq*I: Bacitracin.
*ypu*A: Cefoxitin.
*yja*X: Triclosan.

Incubate plates overnight at 37^{o}C and place in a fridge to aid in colour development.

PROJECT DAY 5: Data collection and analysis

Record your data in the table below and then hand the sheet it to your instructor. The data will be made available to the class for the practical report/questions. Use your initials to give your strains a code (e.g. JS1 for Jem Stach's streptomycete 1)

Strain	**Zone of Inhibition (mm)**				**LacZ activity (blue colour around plug)**				
	E. coli	*P. fluorescens*	*B. subtilis*	*M. luteus*	*dinB*	*yjaX*	*ypuA*	*yvgS*	*yvqI*

Example table.

Strain	**Zone of Inhibition (mm)**				**LacZ activity (blue colour around plug)**				
	E. coli	*P. fluorescens*	*B. subtilis*	*M. luteus*	*dinB*	*yjaX*	*ypuA*	*yvgS*	*yvqI*
JS1	0	0	12	14	+	-	+	-	-
JS2	0	0	13	13	-	-	++	-	-
JS3	1	0	23	25	-	-	-	-	+++
JS4	0	0	0	0	-	-	-	-	-