

Antimicrobial Olympics: Isolation and Characterization of Antibiotic-Producing Microorganisms

Resource Type: Curriculum: Laboratory

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Authors

Mark Gallo
Niagara University
Lewiston, New York
USA
Email: mgallo@niagara.edu

Abstract

Students work individually to selectively enrich, isolate, purify, and characterize microorganisms from the soil belonging to two of the groups (the actinomycetes and the fungi) well known for their ability to produce compounds belonging to the world of secondary metabolites. The secondary metabolites encompass any materials that are not part of primary metabolism and we know many of these products such as pigments, odors, and most importantly antibiotics. The students evaluate isolates for their ability to produce antibiotics as part of a group project. Zones of inhibition for the various isolates are measured against a panel of test organisms. This laboratory is an excellent starting point for discussions of antibiotic discovery and use, microbial ecology, diversity, and antibiotic resistance. Group discussion and a formal report are used to assess the project.

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.

INTRODUCTION

Time Required.

The laboratory work will require several hours spread out over parts of four lab periods. A soil sample must also be collected prior to the first lab period.

Pedagogical Function.

This activity was designed to teach about selective enrichment, secondary metabolism, and diversity of soil microorganisms. Additionally, students will gain experience in experimental design, data analysis, and scientific writing. Successful students will also improve time management, teamwork, and technical lab skills.

Students will gain experience in the care and maintenance of an organism with uncharacterized properties. They are required to use keen observational skills to: describe their isolate, define its macro- and microscopic appearance, recognize its unique metabolic profile, and be able to discriminate it from other organisms. They will also be responsible for subculturing and preserving their isolate, as they are the principle investigator for the organism.

Background.

Before beginning the project, all students should have demonstrated proficiency in laboratory safety. Basic techniques such as aseptic handling of organisms, bacterial isolation procedures, and general use of differential and selective media should be mastered.

The key objective of this laboratory exercise is for students to gain an appreciation for the biological warfare that is being waged by organisms in the environment. Their experience should lead to a better recognition that microbes interact with each other in the soil and that these interactions encompass a number of types of relationships—some beneficial, some neutral, and some damaging or lethal. These observations have been noted in the past by such famous microbiologists as Alexander Fleming, Ernst Chain, Howard Florey, Selman Waksman, Rene Dobus, and Giuseppe Brotzu. The pharmaceutical industry has relied on straightforward observations, such as inhibition of growth, as the discovery path for many antibacterial, antifungal, antihelminthic, antiviral, and anticancer agents.

Each student needs to consider the importance of secondary metabolites for the producing organism in its natural habitat. Why is there such diversity in the products, both in terms of their potency and their biochemical origin? What is the gain for the producing organism? Does the production of a secondary metabolite incur a cost to the producing organism? If secondary metabolites are so good, how do other organisms compete against producers? How do the producers protect themselves from the activity of the compounds? Would one expect organisms from varied environments to produce different types of antibiotics? How is production regulated, and would a different antibiotic profile be detected if the cells were grown in the presence of different competitors?

PROCEDURE

Materials.

Materials normally available in a microbiology teaching laboratory are required. These include:

- Bunsen burners
- Inoculating loops
- Ethanol
- Pasteur pipettes
- Test tubes
- Microscopes
- Staining reagents
- Incubators
- Millimeter rulers

One large glass baking dish per indicator organism

Disposable sealable plastic bags (e.g., Ziploc)

Bacteriological media, as specified below

Useful resources include a reference copy of Bergey's Manual and information on fungi.

Instructor Version.*Week one*

1. Site identification and collection of samples.

Students are asked to collect a soil sample in a sealable plastic bag for food storage (e.g., Ziploc bag). Students are instructed on a way to acquire the sample by inverting the bag and grabbing soil, then pulling soil into the bag and sealing it.

2. Inoculation of media.

A small amount of the soil (pea-sized scoop) is placed in a 15-ml conical-bottom tube containing 5 ml of sterile, distilled water. The sample tube is shaken to dislodge the organisms from the soil particles. Several agar plates containing various media are each inoculated from this sample using a sterile inoculating loop. Sabouraud glucose agar works well for selective enrichment of fungi; glycerol arginine agar and starch casein nitrate agar work well for the actinomycetes. Students also inoculate a nutrient agar and a minimal agar plate. Although some of the organisms in these two groups grow quite slowly, incubation of the plates at room temperature (25°C) allows for sufficient growth by one week.

Week two

3. Observation and enumeration.

Students count colonies on the plates and note diversity based on colony morphology. The fungi typically have a fuzzy or fluffy appearance. The actinomycetes may appear fluffy, fuzzy, leathery, or powdery. In some instances, one can detect zones of inhibition around certain colonies. Many fungi and actinomycetes are also pigmented. Colonies of these two groups of organisms are friable or pliable, never sticky or slimy.

Students perform wet mounts on prospective positive colonies and restreak organisms that appear filamentous on similar agar. See Fig. 1 for examples of student isolates and their patterns of growth.

FIG. 1.*Week three*

4. Antibiotic production (Antimicrobial Olympics).

Students insert the wide bore end of a sterile Pasteur pipette or 1,000-microliter sterile disposable micropipette tip into the agar and slowly rotate it until a plug of agar is cut cleanly from a region of the plate containing sufficient growth of their actinomycete or fungal isolate. Often the plug remains in its original location on the agar plate; occasionally it remains in the pipette. In either case, the plug is carefully removed with sterile forceps (produced by dipping the end of the forceps in 95% ethanol and passing through a flame) and placed upright on the surface of a large glass baking dish containing a suitable agar medium that has been seeded with an indicator organism of interest (*Escherichia coli*, *Pseudomonas fluorescens*, *Staphylococcus epidermidis*, and *Saccharomyces cerevisiae* have all been successfully used.). It is important to use a medium that supports the growth of the indicator organism. The author recommends looking at a manual for suggested media or consulting [Media for Isolation and Enumeration](http://archive.microbelibrary.org/edzine/curriculum/incubator/incubator3a.htm) (<http://archive.microbelibrary.org/edzine/curriculum/incubator/incubator3a.htm>) for more information. It is also recommended that the instructor place known antibiotic disks on the baking dish for comparison purposes.

To prevent condensation problems on the baking dish, it is recommended to autoclave the baking dish and the agar separately and only after the agar and the baking dish have cooled should the agar be poured into the dish. The glass dish is covered with foil and incubated upright (do not invert). The dish is observed one and three days after inoculation with the indicator organism and the test samples. (It can be stored in a refrigerator until the next class period after growth of the indicator organism has occurred.)

Week four

5. Analysis of antibiotic production.

Students measure zones of inhibition using a clear plastic ruler. Comparisons are made among the isolates and "winners" are chosen based upon their ability to inhibit each indicator organism. See Fig. 2 for an example of a completed contest.

FIG. 2.

Students note the number of indicator organisms that their isolate was able to inhibit and compare their results to the spectrum of activity of known antibiotics. Students are asked to suggest the mode of action of their antibiotic based on this profile.

Safety Issues.

Actinomycetes and molds are both spore producers, therefore there is the possibility of contamination with these agents. It is very important that students be extremely careful with plates containing fungi, lest they release them. It is important to dispose of the plates according to your institution's biosafety protocols.

It is advisable to talk to the students prior to sample collection about the potential hazards of microorganisms. Instructors would be well-advised to steer students away from soil sources contaminated with human wastes or likely to contain human pathogens. As these organisms are spore formers, there is also the very real potential of intentional or inadvertent release. Some individuals have allergies to mold spores. Mycoses are a possibility. Lab coats, eye protection, and appropriate footwear are highly recommended, in addition to all standard safety measures when working with flames and glass. The glass baking dishes are autoclaved after the conclusion of the experiment to destroy the indicator and test organisms. It should be stressed that microorganisms are able to produce some very potent compounds and that there is a very real possibility that working with unknown isolates could turn up a potentially dangerous microbe.

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.

Instructors should plan time for discussion of likely habitats prior to the first lab period. As both groups of organisms are very prevalent in most terrestrial environments, it is usually not a problem to acquire them.

Students should recognize the macroscopic and microscopic properties of these groups of organisms. It is important that they recognize that these two groups of organisms look very similar macroscopically (aerial mycelia) and microscopically (filamentous), act very similarly (antibiotic producers), and share a similar life cycle (spore formers). However they should also recognize the differences between these two groups of organisms, the primary one being that they belong to two different domains (kingdoms) as the actinomycetes are eubacteria (prokaryotes) and the fungi are eukaryotes.

After the zones of inhibition are measured it is important to begin discussions on the properties of antibiotics. Antibiotics are biochemically selective; they inhibit some function of a cell. There are certain shared properties of cells (e.g., a membrane), therefore agents that target such an element will have a broad spectrum of activity. Conversely, all organisms also have unique properties, and agents that affect a specific target in a certain organism will have a limited range.

Each student completes a formal research report in the format of a scientific paper. Assessment is based on (i) experimental design, (ii) ability to follow format guidelines, (iii) quality of data evaluation, and (iv) quality of discussion of rationale for differences observed in antibiotic production patterns. Questions addressed in the discussion should include: "What are the properties of these two groups of organisms that are utilized during the initial enrichment?", "What are the distinguishing properties of these two groups of organisms that allow for relatively straightforward identification?", "Why are there other organisms on the initial enrichment plates, what must be the properties of these organisms?", and "How significant are these two groups in the soil, in terms of their numbers and diversity?"

Problems and Caveats.

Laboratory strategies may include:

- Comparison of specific antibiotic batteries (e.g., cell wall synthesis inhibitors and protein synthesis inhibitors) to evaluate the biological effects of structural analogs or altered targets. This will focus on antibiotic mechanisms.
- Correlation between environmental source of isolates and antibiotic sensitivity patterns. This will focus on selective pressures, diversity, and evolutionary theory.
- Comparison of bacterial isolates to evaluate multidrug resistance. This will focus on genetics, gene transfer, and gene frequency between populations.

SUPPLEMENTARY MATERIALS

Possible Modifications.

The exercise may be extended in any of the following ways:

- Modification of selection conditions—does it influence efficiency and diversity of recovery?
- Medium-dependent production of antibiotic—can one optimize production?
- Comparison of potency of antibiotic versus known concentrations of antibiotics—what is the level of production of the compound?
- Extraction and partial purification of antibiotic—is there one, or are there many active compounds responsible for the activity?
- Properties of antibiotic stability—do temperature, light, and oxygen affect stability?
- Characterization of antibiotic producer—is it related to known antibiotic producers?

Recipes for Media.

For media recipes, see the Difco Manual (Difco Laboratories, Detroit, Mich.) or [Media for Isolation and Enumeration](http://archive.microbelibrary.org/edzine/curriculum/incubator/incubator3a.htm) (<http://archive.microbelibrary.org/edzine/curriculum/incubator/incubator3a.htm>).

Figure 1.
Representative *Actinomycetes*
Pure Cultures

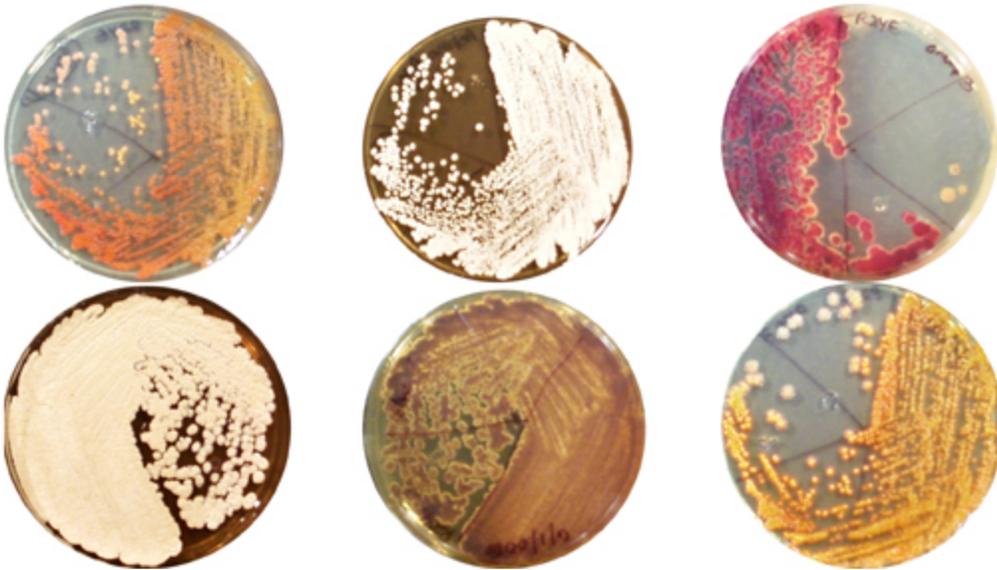
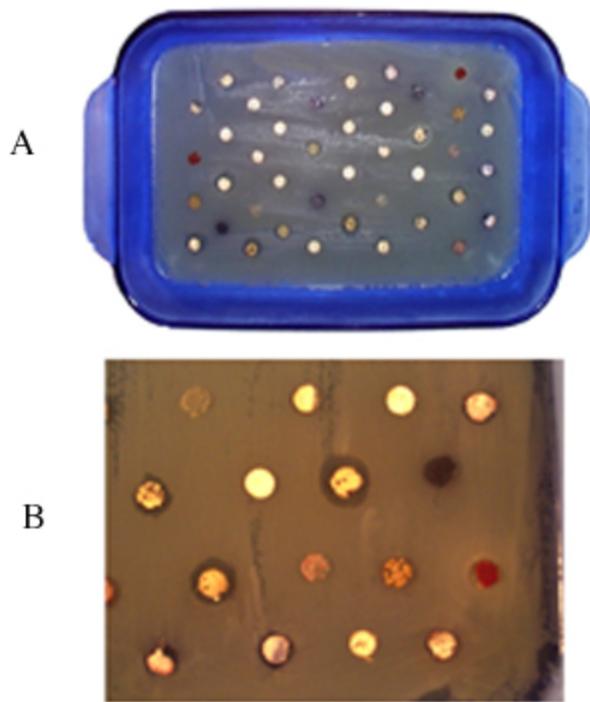


Figure 2.
"Anti-Microbial Olympics"



A, baking dish with plugs on lawn of *E. coli*;
B, close-up of dish showing zones of inhibition.

Media for Isolation and Enumeration

Note: Media marked with an * indicate they are readily available commercially.

Non-selective or general purpose media

1. ***PCA – Plate Count Agar** (a.k.a. Standard Methods agar). A medium used for the routine enumeration of bacteria in food.
2. ***TSA – T-soy agar** (a.k.a. Tryptic Soy Agar or Trypticase Soy Agar). A widely used medium for the cultivation of many heterotrophic bacteria.
3. ***R2A – R2 Agar**. A low nutrient medium devised for the recovery of bacteria from treated potable water. Energy sources range from pyruvate to starch.
4. ***NA – Nutrient Agar**. A classic medium for growth of bacteria. Beef extract provides a complex mixture of minerals, vitamins and some carbohydrates.

Differential Media

1. ***King's Medium B** (a.k.a. Pseudomonas F or Flo agar). A differential medium for the detection and isolation of fluorescent *Pseudomonas spp.* Enhances the elaboration of fluorescent pigments. View colonies under UV light to observe fluorescence.
2. ***Sheep Blood Agar** (5% sheep blood in T-soy agar). A differential medium for the detection of hemolytic bacteria. A standard medium for isolating bacteria from clinical specimens.

Selective Media

1. ***Eosin Methylene Blue (EMB) Agar**. A differential plating medium for detection and isolation of gram-negative coliforms. Eosin inhibits the growth of gram-positive bacteria. Differentiates lactose-fermenters from lactose-nonfermenters. Fecal coliforms, such as *E. coli* are blue-black with a greenish metallic sheen; *Enterobacter* forms large pink, mucoid colonies; lactose nonfermenters such as *Salmonella* and *Shigella* are translucent and amber or colorless; *Acinetobacter spp.* turns bright blue.
2. ***Mannitol Salt Agar**. Selective and differential medium for isolation and differentiation of *Staphylococcus*. Contains mannitol, 7.5% sodium chloride and phenol red indicator. The NaCl inhibits organisms other than staphylococci. A yellow color indicates fermentation of mannitol.
3. ***PEA Agar**. Phenol ethanol Agar is a selective medium for isolating staphylococci and streptococci in the presence of gram-negative organisms such as *Proteus spp.* or *Escherichia coli*.
4. ***Sabouraud Glucose (Dextrose) Agar**. This medium is for the isolation and cultivation of fungi (yeasts and molds). The selective property of this medium is the relatively low pH (about 5.6). To make the medium more selective for fungi, 0.05 g/l chloramphenicol can be added. The Emmon's formulation of this medium is buffered to a pH of 7; it will not be as inhibitory to bacteria.
5. **Actinomycete Isolation Agar**.

There are several approaches to selection of actinomycete type organisms. Two examples are given below.

Glycerol Arginine Agar. This medium is somewhat selective for actinomycetes (particularly streptomycetes). It is suggested that cycloheximide (50 µg/ml) and nystatin (50 µg/ml) be added to prevent growth of fungi. The basis for selectivity appears to be the high carbon/nitrogen ratio,

which favors actinomycetes over the unicellular bacteria. Per liter of distilled water: glycerol 12.5 g, arginine 1.0 g, NaCl 1.0 g, K₂HPO₄ 1.0 g, MgSO₄·7H₂O 0.5 g, Fe₂(SO₄)₃·6H₂O 0.01 g, CuSO₄·5H₂O 0.001 g, ZnSO₄·7H₂O 0.001 g, MnSO₄·H₂O 0.001 g, agar 15 g.

Starch Casein Nitrate Agar. This medium is somewhat selective for actinomycetes. It is suggested that cycloheximide (50 µg/ml) and nystatin (50 µg/ml) be added to prevent growth of fungi. Selectivity is partially based on use of complex substrates as well as the high carbon/nitrogen ratio. Per liter of distilled water: starch 10.0 g, casein 0.3 g, KNO₃ 2.0 g, NaCl 2.0 g, K₂HPO₄ 2.0 g, MgSO₄·7H₂O 0.05 g, CaCO₃ 0.02 g, FeSO₄·7H₂O 0.01 g, agar 18.0 g.

Note: Actinomycetes often are slow growing. Incubation should continue for at least one to two weeks. Also, since these media are not 100% selective, actinomycete colonies will have to be identified by colonial morphology or by microscopic examination.

6. **Medium BG-11 for Cyanobacteria.** NaNO₃ 1.5 g, K₂HPO₄ 0.04 g, MgSO₄·7H₂O 0.075, CaCl₂·2H₂O 0.036 g, citric acid 0.006 g, ferric ammonium citrate 0.006 g, EDTA (disodium salt) 0.001 g, Na₂CO₃ 0.02 g, trace metal mix A5 (see below) 1.0 ml, agar (if needed) 10.0 g, distilled water 1.0 L. The pH should be 7.1 after sterilization.

Trace metal mix A5: H₃BO₃ 2.86 g, MnCl₂·4H₂O 1.81 g, ZnSO₄·7H₂O 0.222 g, Na₂MoO₄·2H₂O 0.39 g, CuSO₄·5H₂O 0.079 g, Co(NO₃)₂·6H₂O 49.4mg, distilled water 1.0 L.
Light is the energy source for cyanobacteria.

Other media suggestions

1. To inhibit fungi.

To inhibit fungal growth, cycloheximide can be added to a medium at a concentration of 50 mg/ml. This is especially important for soils that may contain pathogenic fungi such as species of *Histoplasma* or *Coccidioides*.

2. Variations of T-soy agar.

Used to select for organisms able to grow with fewer nutrients or in the presence of more sodium chloride.

0.1x TSA – Tryptone 1.5 g, Soytone 0.5 g, NaCl 0.5 g, agar 15 g per liter. [Note that T-soy broth has glucose and dipotassium phosphate and different amounts of tryptone and soytone per liter than T-soy agar.]

TSA + 5%NaCl – Tryptone 15 g, Soytone 5 g, NaCl 50 g, agar 15 g per liter.

3. Variations on minimal agar.

Minimal agar is minimal with regard to organic compounds. Bacteria with the capacity to synthesize all organic compounds from a limited organic carbon source can grow on this medium. Bacteria that require specific growth factors will not grow by themselves but may form satellite colonies around other colonies that secrete such nutrients.

Minimal agar – Dextrose 1 g, dipotassium phosphate 7 g, monopotassium phosphate 2g, sodium citrate 0.5 g, magnesium sulfate 0.1 g, ammonium sulfate 1 g, agar 15 g per liter.

Minimal agar + 0.1% benzoate – To minimal agar ingredients add 1 g sodium benzoate per liter.

Minimal agar medium + 0.1% selected fatty acid salt – To minimal agar ingredients add 1 g fatty acid salt (e.g. sodium palmitate).

Nitrogen-free minimal agar medium + 0.1% selected fatty acid salt – Minimal agar ingredients minus the ammonium sulfate (substitute sodium or potassium sulfate) plus 1 g of a selected fatty acid salt. This medium would require the bacteria to grow by fixing nitrogen from the air; a carbohydrate such as mannitol could be substituted for the fatty acid salt.

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