Exploring Microbial Diversity through a Microbe Collection

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Abstract

The diversity of microorganisms inhabiting commonplace environments is phenomenal, yet students are not often given the opportunity to explore this diversity in traditional labs. Having students build a microbe collection enables them to appreciate diversity by isolating taxa from their surroundings. At the beginning of the semester students receive a diversity workbook, which provides background and methods (e.g., construction of Winogradsky column, bait traps, selective (enrichment) media). Working independently or in small groups, students collect 20 microorganisms during the semester. Through their collection, students gain confidence and expertise in isolations, microscopy and other techniques, and an appreciation of microbial diversity.

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

Learning Objectives.
At the completion of this activity, students will have gained an understanding of enrichment cultures and an appreciation for the microbial diversity in their environment. They will also have gained basic microbiological laboratory skills such as microscopy, staining, isolation, streak plating, aseptic technique, and baiting traps.

Background.
Standard laboratory safety and proper disposal of laboratory cultures should be implemented. Basic microbiological techniques such as aseptic technique and streak plating are also useful.

PROCEDURE

Materials.

The majority of materials required for this project are common chemicals and media used in most microbiology labs. Students are expected to gather some of their own materials, such as plastic soda bottles to be used for a Winogradsky column, film canisters and a nylon stocking for the bait traps, and a medium-large glass container and magnet to isolate

Magnetospirillum magnetotacticum. Hard-boiled eggs (for the Winogradsky column) and tuna fish (water packed, not oil) have generally been acquired in small amounts from a campus dining hall. Moth balls, used to enrich for oil degraders, may be purchased from a general store or pharmacy (e.g., K-Mart, Target, and large grocery stores).

Student Version.
Students are given a copy of the diversity workbook (Appendix 1).
Appendix 1. Diversity Workbook

Instructor Version.
Students are given the diversity workbook during the first week of the semester with an explanation of expectations. Students may work individually or in small groups of two to three students. Each student is expected to demonstrate that they have successfully isolated or enriched for each of the organisms regardless of whether or not the work was done as a group.

Grading of the project is based on a sheet in the front of the workbook. This page lists the microorganisms or types of microorganisms each student must collect, along with the "value" of each organism (based on difficulty to obtain). A score key is posted at the bottom of the page indicating the number of points needed to earn an A, B, C, etc. As students obtain their isolates, the professor signs and dates the sheet. At the end of the semester, the sheets are collected and scores are recorded.

This exercise lends itself in an excellent way to development of a scientific notebook. Digital pictures taken along the way lend themselves well to short presentations at the end of course.

As a class, two to three methods and/or species are done together during an initial lab session. This enables students to have a starting point, reduces the feeling that this is a huge endeavor, and clarifies expectations. Depending on locale and when this course is taught during the school year, students may be encouraged to bring soil or water samples back from spring break travels or trips home. I have found this to be extremely useful for obtaining marshy mud for the Winogradsky column. Also note that it is important to encourage students to work on the collection before the last few weeks of the semester, as several species or methods (e.g., Winogradsky column) require several weeks to become established. I have also permitted trading of some cultures (i.e., the transfer of an inoculum from one student plate to the next), as long as each student can reculture the organism to demonstrate it is what they say it is.

NOTES ABOUT ENRICHMENTS

Winogradsky column.
In general this is a very easy enrichment to establish. For greatest success, we have found that using marsh mud is preferable. Also try to ensure that students pack the column tightly and set it in a window with good lighting. After 2 to 3 weeks, distinctly colored zones should be present. In addition to further isolating organisms like *Chromatium, Chlorobium, Desulfovibrio,* and *Thiobacillus,* students may be encouraged to view *Rhodospirillium*. *Rhodospirillium* is a facultative aerobe with purple coloring found near the top of the column. It is fun to view under the microscope with magnification as low as 400x and the diaphragm squeezed down for simulated phase contrast. Students should be able to view large spiral cells that move rapidly.

Soil amoebas.
For best success with this technique, have students use only a small amount of moist soil. The ring of *Enterobacter aerogens* should be laid in a manner so that it does NOT come in direct contact with the soil. When checking samples, make sure students are actually looking at amoebas, not fungal mycelia or nematodes.

Extremophiles, oil degraders, and cellullose, agar, and starch digesters.
Any growth under the identified conditions (with a positive test for loss of indicated product, such as starch) would indicate a positive sample.

Chromatium and *Chlorobium*.
In a Winogradsky column, these microorganisms should be relatively easy for students to obtain and identify. An interesting exercise is to have a student set up a similar enrichment that is maintained in the dark, where they should not observe the presence of these photosynthetic microorganisms.

Desulfovibrio and *Desulfotomaculum*.
Using *Desulfovibrio* medium, tubes incubated at room temperature for 10 to 14 days that result in the formation of black-colored colonies indicates the presence of the microorganism of interest.

Photobacterium.
Bring the culture plates into a dark room. Be sure to take a few minutes for your eyes to adjust to the dim light (the wait is worth it!). The presence of bioluminescence indicates a positive isolation.

Azotobacter.
Included in the methods are controls for isolation of *Azotobacter*. The presence of clear or opaque raised colonies on the soil surface is a positive confirmation.

Serratia.
The presence of a red enteric bacterium on nutrient agar indicates isolation of *Serratia*. Several rapid test kits exist, if you would like further confirmation.

Streptomycetes.
Young colonies of *Streptomycetes* on plates are small with a powdery white appearance. As the colonies age, they often darken in color and release a pigment into the media. Due to their mycelial growth, students should find that the actual transfer of these colonies with a metal loop is difficult, as they often have a leathery appearance and are embedded within the agar matrix.

Thiobacillus.
To confirm the presence of this organism, students may do additional enrichments in a basal salt medium with thiosulfate. Presence of growth and a decrease in pH should be a positive test.

Safety Issues.

Basic laboratory safety and care should be taken any time students are isolating microorganisms from the environment.

Note: most enrichment cultures are incubated at approximately 28^{O}C or cooler, which helps reduce the risk of isolating pathogenic microorganisms. However, the following guidelines should be incorporated when working with environmental samples and/or cultures derived from environmental sources.

- Direct environmental samples (e.g., 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 (BSL-2) precautions.
- Mixed cultures of microorganisms from environmental samples with a significant probability of containing infectious agents should be manipulated in a biosafety cabinet.
- Enriched or pure cultures of BSL-2 microorganisms from environmental samples should be handled within a biosafety cabinet.
- Where possible, media used for the enrichment of environmental isolates should contain an antifungal agent.
- Cultures containing sporulating fungal or mold colonies should be decontaminated without opening the cultures. If manipulation of the cultures is necessary, they should only be opened in a chemical flow hood.

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.
Grading has been based on the value of the collection. A sheet in the front of the workbook identifies the organism or collection method and the number of points each is worth. As organisms are collected or methods completed, the instructor signs and dates the sheet accordingly. At the end of the semester, students hand in the score sheet.

Additional assessment of the collection may be done through class discussions, exams, and presentations.

Field Testing.
This workbook has been used in a general microbiology course (upper division) of 18 to 20 undergraduate students for the past 3 years. The first time it was used, I kept a log of each organism collected for all of the students. This was difficult at best; students often have something under the microscope and want you to “check them off” for it immediately. Students were also constantly checking with me to determine how many more things they needed to isolate or collect. The grading sheet in the front of the workbook was used in years 2 and 3. The sheet made the students responsible for their own collections and minimized the instructor's need for record keeping.

Two other aspects are noteworthy. First, it is important to emphasize the role these organisms play in the environment (both in lab and lecture). If students receive no guidance while working on these collections, I have found they simply view it as a checklist with little regard to the actual organisms. Secondly, sharing or trading of cultures increases collaborative efforts within the class. However, this should be done in a somewhat limited fashion. It is important for students to do the majority of the enrichment work, so that they acquire the lab skills and gain an appreciation of the organisms in their surroundings.

Student feedback has been extremely positive in relationship to what they have learned about diversity. Early in the semester many students complain about the microscope work, however as they become more adept with the microscope, complaints rapidly diminish.

Student Data.
With the exception of *Magnetospirillum magnetotacticum* and *Photobacterium* spp., most students have been able to regularly isolate all of the other organisms. In doing so, students have also isolated some other unique organisms, which I have given them credit for if they can identify either what they is or their role in the environment.

SUPPLEMENTARY MATERIALS

Possible Modifications.
Further evaluation of the student's understanding of the microorganisms may be brought into lecture or laboratory exams. Students may also be required to simple stain, Gram stain, or use phase contrast to determine whether they have a mixed culture of starch degraders (or any other group) or a single isolate. Isolates from the collection may also be used during the semester with other labs. I have had students use these isolates in both our "Unknowns" lab where they use biochemical tests to identify an organism, as well as in our "Growth" lab where they determine growth rates of their microbe under different environmental conditions.

Additionally, the instructor may wish to use a subset of enrichments rather than the exercise in its entirety. This modification could be done in several ways, the simplest where the instructor creates a new grading scale based on a smaller number of enrichments. One could also modify the exercise by using a "theme" approach. For example, students may be directed to enrich and isolate just the organisms they may find associated with a Winogradsky column or from a particular habitat (e.g., terrestrial habitat would include cellulose degraders, starch digester, nitrogen fixers, *Streptomyces* spp., *Rhizobium* spp., and *Azotobacter* spp.). Other modifications include the possibility of performing secondary enrichments, time-lapse photography (e.g., a picture a day) which may then be linked and run, or merely having students give presentations on a particular enrichment.

Note: other groups of organisms may be added to this workbook based on your locale and sampling environments.

Appendices and Answer Keys.
Appendix 1. Diversity Workbook
Appendix 2. Recipes

Microbial Diversity Workbook

Creating a Microbe Collection

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COLLECTION CHECKLIST

Organism or technique (pts)	**Date completed**	**Approved**
Winogradsky column (5)		
Baiting trap (5)		
Soil amoebas (5)		
Thermophile (5)		
Acidophile (5)		
Halophile (5)		
Oil degrader (5)		
Cellulose digester (5)		
Agar digester (5)		
Nitrogen fixer (5)		
Chromatium (10)		
Chlorobium (10)		
Desulfovibrio (5)		
Desulfotomaculum (5)		
Magnetospirillum magnetotacticum (15)		
Myxobacteria (5)		
Photobacterium (and *Lucibacterium*) (15)		
Azotobacter (5)		
Rhizobium (5)		
Serratia species (10)		
Streptomycetes (5)		
Thiobacillus (5)		
Starch degrader (5)		
Total points		

150–130 points A+; 129–110 pts. A; 109–85 pts. B; 84–75 pts. C; 74–55 pts. D;
<55 don't even ask…

INTRODUCTION

Microorganisms interact with the environment in many ways, and no single series of laboratory explorations can cover them all. This workbook is designed to give individuals the opportunity to gain an initial understanding about microbial diversity from their immediate surroundings.

While it's useful to look at natural populations of microorganisms in field samples, we often need large numbers of a specific kind of microbe so we can study its characteristics in the laboratory. To obtain large numbers of a given type of organism, the first step is usually to devise an enrichment culture. That is, we take a field sample of soil, water, or other material likely to have the kind of organisms we want, and then culture the sample under conditions that will favor the desired organism over other kinds. For example:

1. If we are interested in microbes that use light energy (photosynthetic microbes), then we culture the sample under a suitable light source.
2. If we are interested in microbes that can grow at high temperatures (thermophilic organisms), then we culture the sample at a high temperature.
3. If we are interested in microbes that can use a particular nutrient source, such as cellulose, then we add a source of cellulose to the culture.

Essentially, there are two steps in any enrichment.
A. The sample. We start with a sample that is likely to contain the desired microbe.
B. Culture. We culture the sample, manipulating the culture media and conditions in a way that will favor the desired microbe.

If the enrichment is successful, it is often followed by attempts to isolate the organism and grow it in pure culture.

ENRICHMENT METHODS

1. The Winogradsky Column

Introduction.

This classic enrichment technique was devised by the early soil microbiologist Winogradsky. The method selects for organisms that:

1. Use light as an energy source (photosynthetic).
2. Grow in a range of oxygen conditions (from strict anaerobes to obligate aerobes).
3 Use sulfur-containing molecules (such as H_2S, which is a gas that smells like rotten eggs) as electron donors in photosynthesis.

Method.

1. The mud. First, collect about half a bucket of mud from a swamp or marsh. A salt marsh at low tide is usually a good source, soft, smelly mud is best. Take your sample from the surface layer, where there is enough light for photosynthesis.

2. The container. Any tallish, transparent glass or plastic cylinder will work. A good container can be obtained by simply cutting off the top, narrow part of a 1-liter plastic soda bottle. In the lab, glass or plastic chromatography columns or graduated cylinders can also be used.

Preparation.

1. After rinsing the container, place some bits of paper towel and the yolk of a hard-boiled egg in the bottom. These will provide nutrients and, in particular, a source of reduced sulfur (i.e., hydrogen sulfide).

2. Pour in the mud to a level just below the top of the container. Push the mud down until all air bubbles are gone. Pour a bit of water (~2 cm) into the top and cover the open end to prevent the mud from drying out.

3. Put the cylinder in a place where it won't be disturbed and illuminate it from one side by using an ordinary incandescent lamp or simply placing it in a windowsill. (Note: fluorescent light does not work very well.)

Results.

After a few days to a week or so, colored areas will start to appear on the illuminated side. These are colonies of anaerobic and microaerophilic photosynthetic sulfur-using bacteria. Over a longer period, yellowish deposits of elemental sulfur may accumulate.

Implications of the Winogradsky column.

The microbes that appear so colorfully in this enrichment method are quite interesting and may resemble microbial communities present in the early period of life on earth. The atmosphere of the early earth was quite different from the one we have today. For one thing, it had no free oxygen. However, a number of other gases, such as methane and hydrogen sulfide, were present. Hydrogen sulfide is chemically similar to water. Water has the chemical formula H_2O, and hydrogen sulfide has the chemical formula H_2S. As

you can see, the only difference is that water has an oxygen atom in the place where hydrogen sulfide has a sulfur atom.

Modern plants use the energy of the sun to manufacture glucose from water and carbon dioxide, in the process giving off oxygen derived from the water. This is the origin of oxygen in our modern atmosphere. More specifically, the oxygen comes from the water molecule, which the plant cells take apart using the energy of the sun.

In the early days on earth, the first microbes to use the sun's energy did not give off oxygen. Some of them used hydrogen sulfide and carbon dioxide to make simple compounds, in the process giving off pure sulfur. The great sulfur deposits in places such as Louisiana come from the ancient activities of these primitive bacteria.

These bacteria still exist today and are found in sediments and stagnant waters where there is: (i) essentially no oxygen, (ii) some light, and (iii) a source of hydrogen sulfide (usually a breakdown product of the activities of other microorganisms).

In setting up the Winogradsky column you have recreated some of the conditions of the early earth and thereby enriched for very interesting, early types of bacteria. More information can found in Rogan et al. (5).

2. Baiting Traps for Microbes

Introduction.

In this method we enrich for certain protists, ciliates and flagellates, which feed on bacteria and decomposing matter.

Procedure.

Materials.

Start with a film canister (the black cylindrical plastic container that rolls of film come in, any film developing shop will be glad to give you some).

In addition, you will need a small amount of material to serve as a screen, a bit of fine silk or nylon from a discarded stocking or panty hose will work. You can also use a nylon material called Nytex, which is used for making plankton nets.

Making the trap.

Using a sharp knife or razor, cut a small square "window" in the side of the canister. Then glue a square of the silk or nylon material to the edges of the window, forming a screen. Any nonwater-soluble glue should work (e.g., crazy glue, nail polish).

Using the trap.

Remove the lid and put a bit of canned tuna fish (water based, not oil based) in the canister. Put the lid back on and submerge the canister in an aquatic habitat where there is a lot of organic matter and protozoa should be numerous. Make sure to let any bubbles of air in the canister escape. Since the canister floats, you may want to tie a weight to it or simply put a stone inside to weigh it down.

After a period of a day or more, retrieve the canister. With luck, it will contain a dense population of protists, which have been attracted by the tuna and have multiplied inside the trap, feeding on bacteria and perhaps directly on the decomposing tuna fish.

The purpose of the screen is to screen out larger organisms, such as small fish and various invertebrates that would also be attracted by the tuna fish.

You might like to try some variations: other containers, other baits. For example, an early pioneer, Pringsheim, used cheese to attract and enrich for flagellates that could live on small-chain fatty acids ("acetate flagellates," such as *Euglena* and *Chlamydomonas* and their relatives). You might have other ideas. Tuna fish generally works well.

3. Enriching for Soil Amoebas

Introduction.

The soil is full of bacteria, and it is also full of things that eat bacteria. In this method, we enrich for one component of these bacteria, the small soil amoebas.

Materials.

1. Petri dishes containing water agar with no added nutrients.
2. A culture of a suitable food bacterium, such as *Enterobacter aerogenes*.
3. A bit of soil.

Method.

Place a small amount of soil mixed with water on the agar gel surface in the center of the petri dish. Using a bacteriological loop or a dropper, carefully make a ring of food bacteria around the bit of soil. Between the soil and the surrounding bacterial ring there should be bare agar surface, at no point should the soil touch the ring.

Results.

The small amoebas in the bit of soil will crawl away from the soil over the agar surface. When they reach the ring of food bacteria they will start to consume them and will multiply. Using the microscope you will be able to see them clearly after a few days (low power, 100x total magnification).

Since soil is a complex habitat with a rich and diverse biological component, you will often see other things besides soil amoebas coming out in these plates, such as round worms (nematodes) and fungi.

4. Enrichments for Extremophiles

Introduction.

What we consider an extreme condition may be normal for certain organisms. Here are some possible enrichments:

Thermophiles. Inoculate some nutrient broth with a bit of soil or other source (e.g., water from the hot water tap or pasteurized milk), then incubate at a high temperature. Organisms that will grow at 55^{o}C or above are called thermophiles.

If the broth becomes cloudy and smelly from bacterial growth, you may wish to try to isolate a species by making a dilution series. Add 1 ml of the primary enrichment to 9 ml of sterile broth, mix by shaking gently. Then add 1 ml of the first dilution to 9 ml of sterile broth, mix, and so on for several dilutions. With luck, you may obtain a pure or nearly pure culture of the most abundant species in a high dilution.

Acidophiles. Adjust the pH of the nutrient broth to pH 3 and inoculate with soil. After growth appears, you may try isolation by serial dilution, as above. In addition to soil, you might try homemade vinegar, pickles, or sauerkraut.

Halophiles. NaCl is added to nutrient broth to give a final concentration of 8% (8 grams of NaCl per 100 ml of nutrient broth) or more. In addition to soil, seawater or saline inland waters would be good sources, and you might try pickles, salted meats, or sauerkraut.

5. Oil Degraders

Introduction.

Hydrocarbons are produced naturally in marshes and other wetlands by microorganisms. In addition, oil spilled from tankers and pipelines and runoff from highways and streets cause oil contamination of soil and water. Of particular concern are polyaromatic compounds, and especially the chlorinated ones (e.g., polychlorinated biphenyls or PCBs), as many of these are potentially hazardous to humans. Naturally occurring microbes exist that will break these compounds down, and some of these are now used for bioremediation of oil spills.

Method.

A suitable inoculum is any soil or natural water, particularly from a marsh or a site frequently impacted by oil spills (e.g., soil around a gas station).

Here are suggestions for two kinds of basal media that can be used:

1. Standard nutrient broth medium to which hydrocarbon is added.
2. Natural water with added nitrogen, phosphorus compounds, iron, and other trace elements. A convenient source is commercially available plant food, such as Miracle-Gro.

To either of the basal media, add a hydrocarbon. Powdered mothballs (either naphthalene or dichlorobenzene) are a convenient source of aromatic hydrocarbons and form a good enrichment. Similarly, waste oil, which supports growth on an aliphatic compound, works well too. (Note: due to the nearly insoluble nature of naphthalene in water, the relative amount of it to water will not matter. However, one should be aware that by using nutrient broth and naphthalene immediate growth may be obtained in culture from the carbon available in the nutrient media. Therefore, it will take some time to permit establishment of naphthalene degraders.)

Results.

After a week or more, the medium will become cloudy from bacterial growth and the hydrocarbon will start to disappear. A subsequent transfer of the established culture into fresh inoculum at this time may be helpful. Once the oil degrader is established in culture, detection of the microorganism may be confirmed through the elimination of the hydrocarbon, as well as microbial growth. One can also confirm the presence of oil degrader respiration, as one would heterotrophic organisms, by calculating the change in Biological Oxygen Demand or oxygen consumption.

6. Cellulose Digesters

Introduction.

Cellulose, the most common natural polymer, is produced in enormous quantities by plants and then degraded by fungi and bacteria. Animals such as cows and termites have symbiotic microbes in their intestines that can digest this refractory polysaccharide.

Method.

To enrich for cellulose degraders, first find a plausible inoculum source, such as rotting wood, decaying leaves, cow cud, or termite gut. Place a small amount of this inoculum on carboxymethyl cellulose (CMC) agar plates. After a week or more, there should be a significant amount of growth on these plates. Pick a small area of growth and restreak this onto another CMC agar plate. Incubate these plates for approximately a week.

To determine whether cellulose degradation has occurred, stain the plates with Congo red. This is done by pouring a solution of Congo red onto the plates and letting them sit for 10 minutes. After 10 minutes, pour this solution into a waste beaker (to be autoclaved or sterilized using bleach solution). Then destain by pouring a solution of 1 N NaCl onto the plates. Let this sit for 10 minutes and then pour the solution into the waste beaker. The Congo red binds to cellulose. Therefore, degradation of the cellulose should be evident based on "cleared" rings.

7. Agar Digesters

Introduction.

Agar is widely used as an agent to solidify media for microbiological studies. This polysaccharide is produced by red algae and is ultimately degraded by fungi and bacteria.

Method.

To enrich for agar degraders, first find a plausible inoculum source, such as salt marsh mud or soil. Place a small amount of this inoculum on water agar plates. After a week or more, there should be a significant amount of growth on these plates. Check the plates for areas of indentation in the media. This may indicate bacteria are present that are capable of degrading the agar. Pick a small area of growth from one of these indentations and restreak onto another water agar plate. Incubate these plates for approximately a week or two. Deep "pits" in the plate indicate that you have indeed isolated an agar digester.

8. Starch Digesters

Introduction.

Starch is a polymer of α-1,4-linked glucose that is naturally produced by plants (i.e., leaves and seeds). Many fungi and bacteria produce amylase, which is used to digest this polysaccharide. A common bacteria that you may isolate which can hydrolyze starch is the *Bacillus* spp.

Method.

To enrich for starch degraders, first find a plausible inoculum source, such as rotting wood or leaves. Place a small amount of this inoculum on starch agar plates. After a week or more, there should be a significant amount of growth on these plates. Pick a small area of growth and restreak this onto another starch agar plate. Incubate these plates for approximately a week.

To determine whether starch degradation has occurred, stain the plates with iodine-potassium iodide. This is done by pouring a solution of Gram's iodine onto the plates to flood them and letting them sit for 1 minute. After 5 minutes, pour this solution into a waste beaker. Degradation of the starch should be evident based on "cleared" rings. Note: isolation of *Bacillus* is common. Their presence can be established by transferring several likely colonies (i.e., white, waxy texture) to separate plates. After a short period of incubation, confirmation of a positive Gram test and positive spore stain will verify the presence of *Bacillus*.

9. Nitrogen Fixers (4)

Introduction.

Diatomic nitrogen is the most abundant element in the atmosphere, but few organisms can use this resource directly. Nitrogen is often the limiting nutrient in natural ecosystems, as well as in agriculture. Thus, the nitrogen fixers, bacteria that can convert atmospheric nitrogen to biologically available forms, can have great ecological significance. Winogradsky was the first to isolate nitrogen-fixing bacteria from soil. In the Species Isolation section of this workbook (exercises 6 and 7), you will look specifically at two genera: *Azotobacter*, a free-living genus, and *Rhizobium*, a symbiont in the roots of legumes.

Method.

The basic idea of this enrichment is to create an environment containing everything except nitrogen, so that organisms that can use atmospheric nitrogen will be favored. The method described in the isolation exercise for *Azotobacter* is inspired by the classic approach developed by Martinus Beijerinck and involves incubating a paste made of soil and calcium carbonate, with added mannitol as both the carbon and the energy source, and dibasic phosphate as both the buffer and the phosphate source. As you establish an enrichment culture for these microorganisms, it is important to note that nitrogen fixers prefer a slightly alkaline pH. Both *Rhizobium* and *Azotobacter* species are among the relatively few bacteria that can metabolize mannitol, which is produced by plants.

SPECIES ISOLATION

(Note: number after species or group heading indicates reference.)

1. *Chromatium* and *Chlorobium*—photosynthetic bacteria

Introduction.

While most people think of our forests and terrestrial plants as the primary source of photosynthesis, microorganisms contribute more than 65% to the world's total biomass of primary producers. Photosynthetic bacteria are phylogenetically diverse. Two distinct groups are the purple phototrophic bacteria and the green sulfur bacteria. You may isolate these microorganisms from pond muds or a Winogradsky column.

Isolation.

Try to isolate these microorganisms from pond muds or a Winogradsky column. Think about oxygen! What methods are available for anaerobic culture? A simple, readily available method for growing anaerobes using seed germination, a Pyrex dish, and a sheet of glass can be found in Rogan et al. (5).

2. *Desulfovibrio* and *Desulfotomaculum*—sulfate reducers (2)

Introduction.

These organisms live by utilizing anaerobic respiration in which sulfate is used as a terminal electron acceptor. Most organisms that we are familiar with use oxygen as their terminal electron acceptor. These bacteria reduce the sulfate (SO_{4}^{-2}) to sulfide in the form of hydrogen sulfide (H_2S). One way to determine the presence of sulfide (hydrogen sulfide, a substance toxic to most living things) is to see if a black color persists when iron (Fe) is present. Reduced iron, in the form of FeS, is black.

Isolation.

Sulfate-reducing bacteria live in habitats such as freshwater and marine areas that include "ooze" mud and water-saturated soils, although they may also be present in some foods.

1. Dilute mud from a Winogradsky column or soil serially to 10^{-6} using 1 ml of solution to 100 ml of 0.9% sterile saline.

2. Melt tubes of *Desulfovibrio* medium at 100°C (loosen caps); this drives oxygen and all other gases from the medium. Allow to cool to 50°C.

3. Add 1.0 ml of diluted inoculate to each tube in turn.

4. Add 1.0 ml of 0.5% sterile ferrous ammonium sulfate to each tube. DO NOT SHAKE; rather, roll tubes between palms of hands.

5. Incubate at room temperature (~25°C) for 10 to 14 days; check for formation of black-colored colonies on agar.

3. *Magnetospirillum magnetotacticum*—magnetotactic bacteria (1)

Introduction.

These interesting microorganisms contain an internal magnet. The magnet is made of the inorganic materials magnetite (Fe_3O_4) and greigite (Fe_3S_4) that are collected into small structures called magnetosomes. Although the exact function of these internal magnets is not known, some people suggest they make the microorganism better adapted to locating

areas of air and oxygen (microaerophilic zones) in mud (i.e., to help it locate which end is up).

Isolation.
1. Collect fresh water and mud and put in 1L jars with screw-top lids; adjust mixture so that mud takes up ~1 to 2 cm on the bottom and the water from the same collection site makes up 75% of jar volume.

2. Tape or rubber band a magnet to the side of the jar at about midheight of the liquid.

3. Wrap jar with foil and incubate at room temperature for 2 to 3 weeks.

4. Collect bacteria in turbid area next to magnet with Pasteur pipette and observe under phase-contrast microscope. Use a magnet to test for magnetotaxis.

4. Myxobacteria (4)

Introduction.
These microorganisms have the most complex behavior amongst prokaryotes. They have gliding motility, produce multicellular fruiting bodies, and exhibit intercellular communication. Amazingly, these bacteria also produce carotenoid pigments in the presence of light, which appears to be linked to the timing of their fruiting bodies.

Isolation.
1. Prepare petri dishes containing water agar (1.5% agar in double distilled water with no added nutrients).

2. Spread a heavy suspension of virtually any bacterium onto the plate.

3. Place a small amount of soil, decaying bark, rabbit dung, or other natural material in the center of the plate.

4. After several days to a week, examine plates under a dissecting microscope. Look for swarms or fruiting bodies. “Tracks” or lines indicate the presence of the gliding bacteria and were formed as the bacteria moved away from the center of the plate and consumed all the food in their path.

5. *Photobacterium* (and *Lucibacterium*)—a bioluminescent bacteria (1, 2)

Introduction.
These naturally-occurring bacteria can be isolated from marine fish. You should be able to isolate these bacteria from fish purchased at the market as long as they have not been extensively washed. Amazingly, fish or other seafood that have been around too long and have started to spoil will sometimes glow in the dark! A saltwater fish from the local pet store should also work. These bacteria are gram negative, polar flagellated, and bioluminescent (i.e., produce luciferase) when in the presence of reduced flavin mononucleotide ($FMNH_2$) and O_2.

Isolation.
1. Obtain a fresh marine fish or squid and allow it to soak undisturbed in 3% NaCl for several days in the dark.

2. In the dark, examine the fish for luminescent colonies. As colonies may be small and easily overlooked, wait 2 to 3 minutes for your eyes to adjust to the dark and then carefully search the fish surface for luminescent colonies.

3. With a sterile toothpick, pick bacteria and place them on glycerol-peptone agar plates.

4. Incubate the plates at room temperature in the dark.

5. To determine whether you have been successful, observe your plates in the dark. Are the colonies luminescent?

6. Azotobacter—free-living nitrogen fixers

Introduction.

Azotobacter are found in many soils and water systems, and even on the surface of leaves. These organisms are aerobic, heterotrophic nitrogen fixers. An interesting puzzle is the fact that the nitrogen enzyme itself is sensitive to oxygen. How does the cell maintain an anaerobic intracellular environment for the enzyme?

Isolation.

1. Make a thick paste of soil and water, enriched with mannitol (0.75 g/50 g of soil), calcium carbonate (0.1 g/50 g of soil), and 5 ml of 3% dibasic sodium phosphate (Na_2HPO_4) solution. Mix well.

2. Put the paste into small plastic petri dishes. Smooth the surface of each plate with a wet glass slide. The top should be made as smooth as possible; paste should be wet enough to make it glisten after smoothing, but not so wet as to make it watery.

3. For the control, repeat steps 1 and 2 using untreated soil as the base for the paste.

4. Place soil plates in a moist chamber. Incubate at room temperature until colonies form, usually within a few days to a week. *Azotobacter* will make clear or opaque raised colonies on the soil surface.

5. Starting with the colonies on the soil paste, you can now try to purify the culture by streaking on a minimal medium containing mannitol, but with no added source of nitrogen.

7. *Rhizobium* from legume roots—nitrogen fixers

Introduction.

Nitrogen fixation via plant-bacterial interactions is of considerable agricultural importance. This symbiotic relationship provides the bacterium with nutrients, water, and a protected environment, while the plant receives nutrients, vitamins, protection from fungal infection, and most importantly nitrogen.

Isolation.

The roots of most of the leguminous plants, such as peas, beans (soybean), clover, and alfalfa bear numerous nodules or tumor-like growths, which contain the infecting *Rhizobium*. In order to demonstrate that the root nodules contain *Rhizobium*:

1. Select a nodule from the root structure of the legume and crush it in a drop of water between two slides.

2. From this preparation, transfer a loopful to a clean slide, make a smear, and stain it with crystal violet.

3. Examine under a microscope at 1,000x to look for irregularly shaped cells. These are "bacterioid" shapes and appear in the preparation from the nodules, but not in cultures from artificial media where they grow as rods.

8. *Serratia* species—colorful enteric bacteria (4)

Introduction.

Serratia is a relatively common bacteria that can be found in many environments, including soil, water, insect guts, and sometimes the human intestine. These bacteria produce prodigiosins which are red in color. Prodigiosins are a secondary metabolite and possess a unique chemical composition, which has led to much research interest in these compounds. Furthermore, *Serratia* falls within the group of butanediol fermenters along with *Enterobacter* and *Klebsiella*.

Isolation.

The likely places to find *Serratia* are water, soil, insect guts, and sometimes even human intestines.

1. Plate inoculum onto nutrient agar plates containing 50 μg/ml cyclohexamide. Incubate the plates at 30°C for 2 to 3 days.

2. Isolate any red colonies.

3. Confirm identification of the bacteria. This may be done in consultation with a standard microbiology text or *Bergey's Manual of Systematic Bacteriology* (3).

9. Streptomycetes—filamentous bacteria

Introduction.

There are over 500 species of this type of bacteria. Many produce antibiotics you are intimately familiar with such as erythromycin and streptomycin. They also may produce spores and a variety of pigments and have a fairly complex morphology. Many are found in the soil under your feet! These bacteria are also recognized for their ability to produce the compound geosmin, which has a musty forest odor.

Isolation.

1. Suspend soil in sterile water.

2. Spread plate the soil-water solution onto selective media (i.e., actinomycete isolation agar plates, starch agar plates, or others).

3. Incubate the plates at 25°C for 5 to 10 days.

4. Look for "characteristic" colonies (4; picture p. 418–419)

10. *Thiobacillus*—sulfur and sulfide oxidizers (2)

Introduction.

These bacteria are aerobic and use sulfide or elemental sulfur as their energy source; this produces sulfate. They are found in many common habitats and alter their surroundings

by making conditions more acidic (i.e., sulfate production can lower pH to nearly 1 in some environments), thus making it difficult for competing bacteria to live.

Isolation.

There are many good sources of inoculum from which to isolate *Thiobacillus*; coal is a particularly good source, as well as stagnant ponds or marshes. If you have made a Winogradsky column, it is likely that you already have enriched for colonies of *Thiobacillus*. Where in the column should they be located? How can you identify them?

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Appendix 2. Media and Supplies

Note: Many of the media below may be bought through Difco supplies

I.3. Enriching for Soil Amoebas

- Water agar plates with no added nutrients (15 g of agar, 1 L of deionized water)
- A culture of a suitable food bacterium, such as *Enterobacter aerogenes*

I.4. Enrichments for Extremophiles

- **Thermophiles**. Nutrient broth tubes
- **Acidophiles**. Nutrient broth tubes, adjusted to pH 3
- **Halophiles.** 8% NaCl nutrient broth tubes (8 g of NaCl, 100 ml of nutrient broth)

I.5. Oil Degraders

- Basal media that can be used: (i) standard nutrient broth medium and (ii) natural water with added nitrogen and phosphorus compounds and complexed iron and other trace elements; a convenient source is commercially available plant food, such as Miracle-Gro. To either of the basal media, add hydrocarbon; powdered mothballs (either naphthalene or dichlorobenzene) are suitable.

I.6. Cellulose Digesters

- Carboyxmethyl cellulose agar plates (2.5 g of carboxymethylcellulose, 0.5 g of proteose peptone, 0.2 g of K_2HPO_4, 0.2 g of magnesium sulfate, 0.4 g of potassium carbonate, 0.02 g of $CaCl_2$, 0.02 g of ferrous sulphate, 0.02 g of NaCl, 15 g of agar, 1 L of deionized water)
- Congo red stain (0.01 g of Congo red, 100 ml of deionized water)
- 1 N NaCl solution (5.84 g of NaCl, 100 ml of deionized water)

I.7. Agar Digesters

- Water agar plates (15 g of agar, tap water)

I.8. Starch Digesters

- Starch agar plates (3 g of beef extract, 10 g of soluble starch, 15 g of agar)
- Gram's iodine

I.9. Nitrogen Fixers

- Calcium carbonate
- Mannitol
- Dibasic phosphate

II.2. *Desulfovibrio* and *Desulfotomaculum*

- 0.9% sterile NaCl solution
- Tubes of *Desulfovibrio* medium (5.0 g of yeast extract, 0.3 g of ammonium phosphate dibasic, 3.0 g of sodium lactate, 2.0 g of sodium sulfite, 1.7 g of dipotassium phosphate, 0.5 of sodium thioglycollate, 3.0 g of agar, 1,000 ml of pond or river water)
- 0.5% sterile ferrous ammonium sulfate

II.4. Myxobacteria

- Petri dishes containing water agar (15 g of agar, 1 L of deionized water, no added nutrients!)
- Spread a heavy suspension of virtually any gram-negative bacterium onto the plate

II.5. *Photobacterium*

- 3% NaCl (3 g of NaCl, 100 ml of deionized water)
- Glycerol-peptone agar (nutrient agar with 3% NaCl, 1% glycerol, and 0.5% calcium carbonate)

II.6. Azotobacter

- Soil
- Mannitol (0.75 g/50 g of soil)
- Calcium carbonate (0.1 g/50 g of soil)
- 5 ml of 3% dibasic sodium phosphate (Na_2HPO_4) solution

II.7. *Rhizobium* from legume roots

- Crystal violet stain

II.8. *Serratia* species

- Nutrient agar plates containing 50 μg/ml cyclohexamide

II.9. Streptomycetes

Any of these typically work well:

- Actinomycete isolation agar plates (15 g of agar, 4 g of sodium proprionate, 2 g of sodium caseinate, 0.5 g of K_2HPO_4, 0.1 g of asparagines, 0.1 g of $MgSO_4$-$7H_2O$, 1 mg of $FeSO_4$-$7H_2O$, adjust to pH 8.1)
- Starch agar plates (3 g of beef extract, 10 g of soluble starch, 15 g of agar)
- Nutrient agar plates containing 50 μg/ml cyclohexamide