Lab VII-3

Culturing Bacteria

Equipment and Materials

You'll need the following items to complete this lab session. (The standard kit for this book, available from *www.thehomescientist.com*, includes the items listed in the first group.)

Materials from Kit

- □ Goggles
- □ Coverslip
- □ Inoculating loop
- □ Microscope slide, flat
- □ Pipettes
- □ Stain, Gram's iodine
- □ Stain, Hucker's crystal violet
- □ Stain, safranin O
- □ Stirring rod
- □ Test tube rack

Materials You Provide

- □ Gloves
- □ Alcohol (ethanol or isopropanol)
- □ Alcohol lamp, candle, or butane lighter
- □ Agar Petri dishes (from preceding lab)
- □ Agar slant tubes (from preceding lab)
- □ Bleach, chlorine laundry
- □ Broth culturing tubes (from preceding lab)
- □ Clock or watch with second hand
- □ Large container with lid (for bleach bath)
- □ Microscope
- □ Mixed bacteria culture (see introduction)
- □ Normal saline tubes (from preceding lab)
- □ Paper towels
- □ Sanitized work area (see preceding lab)
- □ Tape (transparent, masking, or similar)

Background

> If you or any member of your family is immuno-suppressed, do not do this session or any of the remaining lab sessions in this group unless your physician tells you that it is safe to do so.

In the preceding lab session, we made up culturing media and vessels. In this lab session, we'll use those materials to culture bacteria.

Obtaining bacteria to culture is certainly no problem. We're surrounded by them. Unfortunately, although most environmental bacteria are harmless or actually beneficial to humans, a small

percentage of them are pathogenic (disease-causing). The normal human immune system can deal with the small numbers of pathogenic bacteria normally present in the environment, but culturing those bacteria multiplies their numbers exponentially, presenting a potentially serious hazard.

For example, MSRA (methicillin-resistant *Staphylococcus aureus*) bacteria are widely distributed in the environment; there are probably millions of them in and on your body right now. Your immune system can deal with those relatively small numbers, but culturing *S. aureus*, intentionally or unintentionally, multiplies those numbers by orders of magnitude, producing a concentrated colony of *S. aureus*, which is most definitely hazardous.

For that reason, we'll do this lab using a purchased mixed bacteria culture that contains three species of relatively innocuous bacteria. We chose a mixed culture that includes all three shapes of bacteria and examples of both Gram-positive and -negative bacteria:

- *Bacillus subtilis* – Gram-positive rod-shaped bacteria
- *Micrococcus luteus* – Gram-positive sphere-shaped bacteria
- *Rhodospirillum rubrum* – Gram-negative spiral-shaped bacteria

You can purchase this mixed suspension of living bacteria as item #154760 from Carolina Biological Supply (*www.carolina.com*). As we wrote this, the price was $16.90 plus shipping.

To ensure freshness, do NOT order this living culture until you have scheduled this lab session. Carolina Biological allows you to specify an arrival date in the shopping cart. Schedule this arrival date for the day of (or the day before) you intend to start this lab session.

If you decide to do this lab session using environmental bacteria rather than the purchased bacterial culture, we strongly recommend that you use extreme caution in handling and disposing of cultures. Work in a sanitized area, as described in the preceding lab session. Wear an N100 particulate mask, goggles, and gloves while handling the culture vessels, and spray the area thoroughly with Lysol or a similar disinfectant before and after handling the cultures. Disinfect your gloves with an alcohol-based hand sanitizer before removing them, and wash your hands thoroughly with soap and water after you de-glove.

If you spill or break a culturing vessel, immediately spray disinfectant liberally into the air of the room to kill and precipitate out any airborne bacteria. Flood the spill or breakage area with disinfectant, and leave the room for at least one hour before commencing clean-up.

Dispose of grown gel or broth cultures by autoclaving (for glass or polypropylene vessels) or by placing the vessel in a bucket or similar container that is filled with a solution of one part of chlorine laundry bleach to four parts tap water. Make sure the vessel is fully immersed in the solution, and then open the vessel. Make sure that all parts of the vessel and media are exposed to the bleach solution (watch out for air bubbles) and leave the contaminated vessels in the bleach solution overnight. At that point, anything other than spores has been killed. Place disposable culture vessels in a plastic bag and discard them with the household trash. Wash re-usable vessels in soap and hot water, and allow them to dry. If necessary, agar gel can be re-liquefied by placing the vessel in a container of very hot water.

In this lab session, we'll inoculate two Petri dishes with the purchased mixed culture. (We'll use two Petri dishes in case one is contaminated by environmental bacteria.) We'll then incubate the

Petri dishes at room temperature, observe the growth of our colonies, and thereby determine which of the three bacteria species can be cultured successfully on the nutrient medium we're using.

Because we don't want to have to purchase the mixed culture more than once, we'll also inoculate one broth tube and two normal saline tubes with the purchased culture and store the broth tube in the refrigerator. This procedure, called *re-culturing*, allows us to extend the useful life of the original culture.

Because they are made up of living things, cultures pass through life stages. In the *juvenile stage*, relatively few bacteria are present in the culturing medium. They have essentially unlimited food and other resources, and are free to reproduce rapidly. In the *mature stage*, large numbers of bacteria are present, and competition for resources starts to become an issue. In the *senescent stage*, the culture is approaching its death. The bacterial population is much too large to be supported by the available resources, and bacteria must compete for those limited resources. Poisonous waste products are accumulating, and mutations may develop.

> Under ideal conditions, bacteria can reproduce very rapidly, some in as little as 20 minutes or less per generation. In other words, if you start with one bacterium, after 20 minutes you have two bacteria, after 40 minutes you have four, after 60 minutes you have eight, and so on. That might not seem impressive until you realize that after 10 generations (3 hours and 20 minutes) you have about a thousand bacteria, after 20 generations (6 hours and 40 minutes) you have about a million, and after 30 generations (10 hours) you have about a billion.
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> Of course, this exponential growth can never last for long, at least in a lab, because the bacteria quickly run out of food and space to grow. But it does illustrate how a juvenile culture can quickly become a mature culture and then a senescent culture.

By re-culturing, we can produce a juvenile culture from a mature culture. By refrigerating the broth tube, we slow the reproduction rate of the bacteria, essentially putting them into hibernation. Because the broth tube contains nutrients, the bacteria will continue to reproduce in the refrigerated broth, but at a greatly reduced rate. (This is why non-sterile food stored in the refrigerator remains edible much longer than non-sterile food stored at room temperature.)

The saline tubes contain no nutrients. We expect (or at least hope) that the bacteria in the saline medium will remain alive indefinitely but cease reproduction. Some species of bacteria can be maintained in “suspended animation” for months, years, or even decades in phosphate-buffered saline. We'll determine if any or all of our three species can be maintained for at least short periods in the simpler normal saline solution.

And, of course, we'll want to look at our bacteria, so we'll do a smear mount of the original mixed suspension and use Gram staining to differentially stain the various bacteria before observing them under the microscope.

Procedure VII-3-1: Staining and observing the original culture

1. If you have not already done so, put on your goggles and gloves.
2. In your sanitized work area, lay out your alcohol lamp (or other flame source), the inoculating loop, a microscope slide, the stirring rod, and the original culture tube. Make sure your gloves are sterile before proceeding.
3. Ignite the flame source, and hold the tip of the inoculating loop at the top of the flame until the metal loop glows red. Remove the loop from the flame and allow it to cool. Do not put down

the loop; continue holding it in your hand until you complete the transfer of the bacterial culture to the slide.

4. As the loop cools, pass the mouth of the bacterial culture tube quickly through the flame. A second or so suffices. Your goal is not to heat the tube and cap, but to kill any surface bacteria present on the tube or cap.
5. Carefully remove the cap from the culture tube just enough to allow you to insert the cool inoculating loop. (Hold the cap in your hand; placing it on the work surface risks contamination.) Dip the inoculating loop into the culture liquid, remove the loop, and recap the tube immediately.
6. Transfer the drop of liquid culture to the center of the microscope slide and then flame-sterilize the loop before placing it aside.
7. Use the stirring rod or a second microscope slide to make a smear mount by spreading the drop of culture liquid into a thin smear on the slide.
8. Holding the slide by the edges at one end, pass the central section of the slide through the flame several times, culture side up. The goal is not to “cook” the culture liquid, but to dry it out gently and cause the bacteria present to adhere to the slide. When heat-fixing is complete, the central area of the slide should have a dry, slightly hazy appearance. Place the slide aside and extinguish the flame source.
9. Place the heat-fixed slide on a paper towel on a clean, flat surface.
10. Use a clean pipette to place a drop or two of Hucker's crystal violet stain on the smear. Use the tip of the pipette gently to spread the stain until it covers the entire smear. Do not touch the smear with the tip of the pipette.
11. Allow the crystal violet stain to remain in contact with the smear for 1 minute, and then rinse the slide, smear-side down under a trickle of cold tap water, tilting the slide back and forth to flood the smear with water. Rinse for at most a second or two, and don't allow the water to fall directly on the smear. Drain the slide and place it flat on the paper towel.
12. Use a clean pipette to place a drop or two of Gram's iodine stain on the smear. Again, use the tip of the pipette carefully to spread the stain over the entire smear.
13. Allow the Gram's iodine stain to remain in contact with the smear for 1 minute.
14. Fill a clean pipette with alcohol (drugstore 70% ethanol or 70% isopropanol is fine). Hold the slide at an angle over the sink and gently flood the smear with the alcohol. Continue until the alcohol runs colorless.
15. Repeat step 11 to rinse all of the alcohol from the slide. (It's important to remove all of the alcohol, because the following step won't work if alcohol is still present.) Drain the slide and place it flat on the paper towel.
16. Use a clean pipette to place a drop or two of safranin O stain on the smear. Use the tip of the pipette carefully to spread the stain over the entire smear.
17. Allow the safranin O stain to remain in contact with the smear for 1 minute.
18. Repeat step 11 to rinse excess safranin O stain from the slide and then allow the slide to air-dry.
19. You'll probably want to keep this slide for reference, at least until you complete this related group of lab sessions, so place a drop of mounting fluid in the center of the smear. You can use glycerol to make a mount that will last for a week or two, or a permanent mounting fluid if you want to keep the slide indefinitely.

20. Carefully lower a coverslip onto the mounting fluid, beginning with one edge of the coverslip in contact with the slide and then tilting the coverslip downward until it is in full contact with the slide. Make sure to eliminate any air bubbles by pressing gently with the tip of a pipette or forceps to expel the bubble.
21. Position the slide on the microscope stage and use medium magnification to center an area of the smear that shows a large number of bacteria in the field of view. Switch to high-dry magnification, adjust the diaphragm and illuminator brightness to reveal the maximum detail in the bacteria, attempt to identify each of the three types of bacteria that should be present in the smear, and record your observations in your lab notebook.
22. If you have a 100X (oil-immersion) objective, put one drop of immersion oil on the center of the coverslip and carefully rotate the oil immersion objective into position, making sure it comes into contact only with the oil drop. Observe the bacteria at 1000X, and record your observations in your lab notebook.

Procedure VII-3-2: Inoculating plates and tubes

Allow the culturing dishes and tubes to warm to room temperature before proceeding.

1. If you have not already done so, put on your goggles and gloves.
2. In your sanitized work area, lay out your alcohol lamp (or other flame source), the inoculating loop, the original culture tube, both prepared Petri dishes (gel-side down), and the test tube rack with both slant tubes and one of the broth tubes. Ignite the flame source, and make sure your gloves are sterile before proceeding.
3. Hold the tip of the inoculating loop at the top of the flame until the metal loop glows red. Remove the loop from the flame and allow it to cool. Do not put down the loop; continue holding it in your hand until you complete the transfer of the bacterial culture from the original tube.
4. As the loop cools, pass the mouth of the original bacterial culture tube through the flame for a second or two to kill any surface bacteria present on the tube or cap.
5. Carefully remove the cap from the culture tube just enough to allow you to insert the cool inoculating loop. Holding the cap in your hand to prevent contamination, dip the inoculating loop into the culture liquid, remove the loop, and recap the tube immediately.
6. Carefully lift the lid of the first Petri dish just enough to allow access for the inoculating loop. Place the tip of the inoculating loop into gentle contact with the agar gel surface, and move the tip of the loop in a zig-zag pattern across the surface of the agar. The goal is not to gouge a groove in the agar, but simply to move the inoculating loop across its surface to allow bacteria to be transferred from the loop to the agar.
7. Repeat the streaking at a 90° angle to the first streak. If you are using the two-section Petri dishes included in the kit, repeat the streaking in the second section. After you finish streaking, immediately lower the Petri dish lid back into place and place the Petri dish aside for now.
8. Repeat steps 3 through 7 to inoculate the second Petri dish.
9. Repeat steps 3 through 5 to load the inoculating loop.
10. Flame-sterilize the mouth of the broth tube and inoculate the tube with the mixed bacteria culture. Replace the cap and place the inoculated tube in the rack.
11. Repeat steps 9 and 10 to inoculate each of the two slant tubes. Inoculate the slant tubes in the same manner you inoculated the Petri dishes, just touching the loop to the surface of the agar.

12. Repeat steps 9 and 10 to inoculate each of the two normal saline tubes. Simply dip the loaded loop into the tube and swirl gently to transfer the bacterial culture to the saline medium.

    Obviously, you must take care when flame-sterilizing polypropylene tubes to avoid melting the tube and/or lid. Simply rotate the tube quickly in the flame for a second or so to flame-sterilize it.

13. Refrigerate the broth tube and both normal saline tubes. To isolate the inoculated vessels from the food storage area, we recommend standing the tubes in a beaker or similar container inside a sealed wide-mouth jar or plastic food-storage container.
14. Place both Petri dishes (gel side on top) and one slant tube in a dark area to incubate. Allow the second slant tube to incubate where it will be exposed to direct sunlight.
15. Observe the Petri dishes and slant tubes at least daily and record the progress in your lab notebook. (Do not open the containers; observe the growth through the glass.) If you can, shoot images to record the progress. At room temperature some changes should be evident in some or all of the containers after a day or two, with discrete colonies starting to develop after two to three days.

Procedure VII-3-3: Producing pure cultures

You can begin this procedure any time after discrete bacterial colonies appear in your Petri dishes. Allow the remaining three broth tubes to warm to room temperature before proceeding. Label each of the tubes.

1. If you have not already done so, put on your goggles and gloves.
2. Choose one of the Petri dishes that contains discrete colonies of all three bacterial species. Ideally, you want large, widely-separated colonies of each of the three
3. In your sanitized work area, lay out your alcohol lamp (or other flame source), the inoculating loop, the Petri dish you selected (gel-side down), and the test tube rack with the three remaining broth tubes. Ignite the flame source, and make sure your gloves are sterile before proceeding.
4. Hold the tip of the inoculating loop at the top of the flame until the metal loop glows red. Remove the loop from the flame and allow it to cool. Do not put down the loop; continue holding it in your hand until you complete the transfer of the bacterial culture from the Petri dish to the first broth tube.
5. As the loop cools, pass the mouth of the first broth tube through the flame for a second or two to kill any surface bacteria present on the tube. Hold the cap in the same hand as the tube.

    Yes, biologists often need four to six hands. Rather than grow more hands, you might ask someone to assist you in procedures that require more than two hands.

6. When the loop is cool, lift the lid of the Petri dish just enough to insert the tip of the loop. Touch the loop to the first colony type. Immediately withdraw the loop and replace the lid on the Petri dish.
7. Dip the loop into the first broth tube and swirl slightly to transfer the bacteria from the loop to the broth tube. Replace the cap on the tube and place the tube back in the rack.
8. Repeat steps 4 through 7 to inoculate each of the two remaining broth tubes with bacteria from one of the two remaining colony types. When you finish, flame-sterilize the loop before putting it away.

9. Place all three broth tubes upright in a beaker or similar container and put them in a dark area to incubate.
10. Observe the broth tubes every few hours and record the progress in your lab notebook. We'll retain these broth cultures for the next lab session.

As bacteria grow in the broth, the broth gradually changes from transparent to cloudy. (Or not so gradually, depending on species and incubation conditions.) In a senescent broth culture, clumps may actually become visible, but we don't want to let things get to that point. We'd like to have young-mature to mature cultures for the following lab session, so keep a close eye on the broth cultures. Once the broth becomes at least somewhat cloudy in appearance, you can refrigerate the broth tubes to slow bacterial reproduction until you're ready to begin the next lab session. (Use the steps mentioned in the preceding procedure to isolate your cultures from the food in your refrigerator.)

Also retain the original mixed culture tube, the normal saline tubes, and the mixed culture broth tube for later use. You can keep all of these refrigerated to slow bacterial growth and the inevitable senescence of the cultures.

Destroy the remaining cultures by immersing the Petri dishes and slant tubes in a deep vessel filled with a mixture of one part chlorine laundry bleach to four parts tap water. Wear gloves, and open the Petri dishes and tubes only after they are fully submerged. Make sure that there are no air bubbles, and that the bleach solution can reach all parts of the vessels. Allow the vessels to soak in the bleach solution overnight, by which time everything except perhaps a few spores has been killed.

We do not recommend reusing the plastic Petri dishes. Simply rinse them with tap water and discard them with the household trash. The tubes can be washed with hot (if necessary, boiling) soapy water to clean them. Make sure to remove all traces of the agar gel, which can be persistent.

Review Questions

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1. Why did we bother doing the first procedure, rather than simply starting with the second procedure and observing the bacteria after we'd re-cultured them?

   Doing the first procedure gave us a control. We knew which three bacteria species were supposed to be present, but checking the original culture allowed us to verify that the three species actually were present. Doing that check eliminates some future uncertainties. For example, if when we re-cultured on agar plates only two types of colonies grew, we wouldn't know for sure if only two species were actually present in the original culture or if one of the species was present in the original culture but did not grow successfully on the nutrient mixture we used.

2. Based on your observation of the Gram-stained mixture culture, which of the three bacteria species were Gram-negative, and which were Gram-positive?

   Rhodospirillum rubrum (spirals) are stained pink, indicating that they are Gram-negative. *Bacillus subtilis* (rods) and *Micrococcus luteus* (spheres) are stained violet, indicating that they are Gram-positive.

   Note: depending on how successful your staining procedure was, there may be variation in the colors of the various bacteria. For example, different individual bacteria of the same species may stain different colors or may be partially stained one color and partially the other. This is not because these bacteria are either Gram-indeterminate or Gram-variable

species, but simply because inexperienced students often do not perform the protocol correctly.

The most common error is under- or over-decolorizing. Decolorize only until alcohol flowing off the slide is no longer obviously violet, and then rise with water. If you under-decolorize, Gram-negative bacteria may appear violet; if you over-decolorize, Gram-positive bacteria may appear pink. We specify alcohol as a decolorizer rather than acetone for this lab session because alcohol is a much slower decolorizer than acetone. If you will be doing many Gram staining procedures, you may wish to substitute an alcohol-acetone mixture or pure acetone once you have gained experience in using the protocol.

3. In the second procedure, we carefully observed aseptic technique for both the original culture tube and the destination vessels. In the first procedure, we observed aseptic technique for the original culture tube, but transferred the culture medium to a non-sterile microscope slide. Why the difference?

   In the second procedure, we were transferring the original culture to vessels where we intended to grow (or store) the bacteria, so it was important to avoid contamination from environmental microorganisms. In the first procedure, we were simply transferring a small amount of the original culture to a slide in order to observe it. None of the microorganisms present would have a chance to reproduce, so the presence of a few extraneous microorganisms was of little concern.

4. Which of the three bacteria species were you able to culture successfully on agar and in broth?

   Answers may vary (science is never entirely predictable), but all three of the species should culture successfully on agar and in broth with the nutrient mixture we used.

5. What differences, if any, did you observe in the slant tube incubated in the dark versus the one exposed to direct sunlight? Propose an explanation for any differences you observed and suggest an experiment to verify or falsify your proposed explanation.

   Answers may vary, but the slant tube incubated in the dark should show a nice growth of mixed colonies, while the one exposed to direct sunlight should show slower growth in the colonies, if any growth at all is visible. The most likely explanation for the slower growth in the tube exposed to direct sunlight is that the ultraviolet wavelenths in sunlight hindered or stopped reproduction of the bacteria. Students may also suggest other possible explanations, such as temperature variations between the tubes or the fact that in the sunlit tube the agar might have been dried out or the atmosphere rendered more humid.

   To determine if ultraviolet was the controlling factor, students might propose making up two more slant tubes and exposing one to a fluorescent ultraviolet lamp and the second to a similar intensity of incandescent light (which contains very little ultraviolet). Other proposed explanations could be verified or falsified by exposing freshly inoculated slant tubes to the temperatures measured in the dark and sunlit incubation areas and comparing growth rates or by re-culturing the bacteria at controlled levels of humidity and agar moistness.