Chapter 8

Identification of Bacterial Species

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Reprinted From: Christopher, K. and E. Bruno. 2003. Identification of bacterial species. Pages 103-130, in Tested studies for laboratory teaching, Volume 24 (M. A. O'Donnell, Editor). Proceedings of the 24th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), 334 pages.

Contents

Introduction

The laboratory exercise is designed to introduce first-year cell biology students to several standard microbiological tests that can be used in an initial identification of an unknown bacterial species. In the first week, students are provided with a mixed bacterial culture containing three species, whose identity is "unknown" to the students. Students use the streaking technique to isolate individual colonies of the three species. Including the prelab talk and postlab discussion, this portion of the lab takes approximately 45 minutes for students to complete. Preparation and set up for this portion will take up to 3 hours for a lab of 20 students.

In the following week, students examine the colonies of the three "unknown" bacteria to determine colony morphology. Additionally, students perform Gram staining on cells of the isolated colonies to determine the Gram reaction, cell morphology, and cell size of the "unknown" bacteria. Students are also supplied with the test results of four morphological/biochemical/nutritional tests to learn more about the three "unknown" species. A comparison is then made with the same test results of six bacterial species, whose identities are "known," in an attempt at an *initial* identification of the three "unknown" bacteria in the mixed bacterial culture. The morphological/biochemical/nutritional tests that are included in this lab were chosen based on their relevance to the theoretical material taught in the lecture component of the course. Including the prelab talk and postlab discussion, this portion takes 3 hours for students to complete. Preparation and set up for this portion will take up to 3 hours for a lab of 20 students.

Materials

Equipment (numbers are based on a lab session of 20 students, arranged into 8 groups on 4 lab benches)

First week of lab:

(24) PCA agar plates
(16) inoculating loops
(8) Bunsen burners
(4) strikers
(4) wash bottles of Roccal (any disinfectant will work)
(4) test tubes containing 2.0 ml of a mixed bacterial culture (Stock culture is: 5 ml *E. coli*, 75 ml *Staphylococcus epidermidis*, and 75 ml *Bacillus subtilis*, all grown in TCS broth)
(2) autoclave bags (doubled bagged)
(1) test tube containing uninoculated broth for demonstration of turbidity

Second week of lab:

(6) dissecting microscopes (1 per "known" bacterial species set-up)
(8) compound microscopes with 100X oil immersion objective lenses and ocular micrometers (1 per group of students)
(1) box of lens paper
(4) Bunsen burners and strikers
(4) bottles of immersion oil
(4) boxes of glass slides
(4) Gram stain stations (1 station per bench), which includes:
  (1) Coplin jar of crystal violet
  (1) Coplin jar of Gram's iodine
  (1) wash bottle of 95% ethanol
  (1) Coplin jar of Safranin
  (2) grease pencils
  (2) pairs of forceps
  (2) inoculating loops
  (2) 600 ml beakers
(20) mixed culture agar plates (made by students; see First week of lab, above)
(1) pure culture agar plate of (PCA) *Micrococcus luteus* (ATCC 4698), labeled as such
(1) pure culture agar plate of (TCS) *Lactobacillus plantarum* (ATCC 8014), labeled as such
(2) pure culture agar plates (PCA) of *Bacillus subtilis* var. niger, one labeled as such, one labeled as "1"
(2) pure culture agar plates (PCA) of *Staphylococcus epidermidis* (ATCC 155), one labeled as such, one labeled as "3"
(3) pure culture agar plates (PCA) of *Escherichia coli* (NCIB 8666), one labeled as such, one labeled as "2", one labeled as *Proteus vulgaris*
(1) set of Test Results for the "Unknown" bacteria
(1) set of Test Results for the "Known" bacteria (1 information sheet for each "Known" station)
(2) sets of How to Read the Test Results information sheets
(6) sets of Descriptive terms of colony morphology (1 for each "Known" station)
(1) set of Gram stained photomicrographs (1 photomicrograph for each "Known" station)
(2) autoclave bags (double bagged)

Chemicals

First week of Lab:

- PCA (Plate Count Agar): either purchase pre-mixed powder and add water or use chemicals below
  - pancreatic digest of casein
  - yeast extract
  - glucose
  - agar
- TCS (Trypticase Soy broth): either purchase pre-mixed powder and add water or use chemicals below
  - pancreatic digest of casein
  - soybean peptone
  - NaCl
  - K_2HPO_4
  - glucose

Second week of Lab:

- PCA (Plate Count Agar)
- TCS (Trypticase Soya Agar)
  - pancreatic digest of casein
  - soybean peptone
  - NaCl
  - K_2HPO_4
  - glucose
  - agar
- Crystal violet
- Ammonium oxalate
- 98% ethanol
- 95% ethanol
- Iodine
- Potassium iodide
- Safranin

Student Outline

First Week of Lab

Objectives:

1. To learn the characteristics of aseptic technique
2. To learn how to streak cells from a liquid bacterial culture onto an agar plate.

Cultures of bacterial cells are kept in the lab environment in many different forms. One common form is a liquid culture -- a suspension of millions of cells in a liquid medium that contains all the nutrients required for cell growth. In this lab, you will be given a liquid culture containing a mixture of three different bacterial species. You will attempt to identify the three bacterial species in this first week using several standard microbiology identification techniques.

However, before you can attempt to identify the individual bacterial species, you will need to separate the bacteria in the mixed liquid culture from each other. The method you will use to accomplish this is a technique called streaking. The objective of this technique is to deposit single bacterial cells from the liquid culture over the surface of the agar medium in the plate. The streaking technique allows you to do this as each successive streak dilutes the number of cells from the previous streak, ideally resulting in single cells deposited within the area of the final streak (see Figure 2).

Once the cells have been streaked onto an agar plate, the plate will be placed in an incubator (at an optimal temperature) to allow the cells to grow and divide. A single cell will divide numerous times to form a small mass of cells on the plate where the original single cell was deposited. This small mass of cells is called a colony. In an isolated colony, all the cells in the colony will be genetically identical and will represent a pure culture.

When culturing microorganisms, you must practice aseptic technique (also called sterile technique). The two purposes of aseptic technique are:

- to prevent bacteria in the environment from contaminating your work
- to prevent bacteria in your work from contaminating you and the environment

To avoid contamination, it is essential to practice aseptic technique. When working with microorganisms, always follow these rules:

- ✓ Sterilize the surfaces (including working surfaces) that the medium, culture, or bacteria will contact. This kills bacteria in the environment and prevents them from contaminating your cultures. In this lab, you will use a Bunsen burner to flame the loop used to transfer the bacteria from liquid culture to agar plates. You will also flame the lip of the culture tube with a Bunsen burner. You will sterilize your bench top by wiping it with Roccal, an antiseptic. Since your hands contain bacteria, you must wash your hands with soap and water, before and after you handle microorganisms. This will prevent you from contaminating the agar plates, in addition to preventing the bacteria from infecting you. Glassware and medium are sterilized by subjecting them to high temperature and pressure in an instrument called an autoclave. Most plastic surfaces are sterilized by UV irradiation. You will be supplied with sterile glassware and plasticware so you will not have to autoclave your glassware or UV-sterilize your plasticware in this course.
- ✓ Reduce the time that sterile medium, cultures, or bacteria are exposed to the air. The air and dust particles in the air contain bacteria that can drop into your culture and contaminate it.

This also reduces the possibility of bacteria from your culture becoming airborne. You must work quickly to reduce the length of time that sterile containers are open.

✓ Work in an area with a low resident population of bacteria. Certain areas contain more bacteria than other areas. To reduce the chance of these extraneous bacteria contaminating your cultures, you should avoid these areas. You will get an impression of these areas once you have examined your Environmental Survey plates. Dusty areas, surfaces that are frequently touched, and places with a lot of "people" traffic (*e.g.*, near doorways and halls) contain a lot of bacteria and fungi.

Materials:

Per student:
- (1) PCA plate

Per group:
- (1) Bunsen burner
- (2) inoculating loops

Per bench:
- (1) tube of mixed bacterial culture
- (1) striker
- (1) wash bottle of Roccal
- (1) test tube rack

Per lab:
- (1) tube of uninoculated medium (medium with no bacterial growth)

Procedures

1. Label the outside margin of the bottom of a Plate Count Agar (PCA) plate with your name, lab day and time, and TA's name, using a waterproof marking pen.
2. Whenever bacterial cells are transferred, strict aseptic (sterile) technique must be used. Wipe your bench top with the disinfectant Roccal to sterilize the surface. Have the tube of media, the lighted Bunsen burner, the agar plate, and the inoculating loop all within easy reach on your lab bench. The agar plate should be upside down so that the lid is on the bench and the agar is facing up.
3. Flame the inoculating loop until it is red hot (see Figure 1). This will sterilize the inoculating loop.
4. Remove the cap from the culture tube by grasping the cap between your baby finger and the palm of the hand that is holding the loop. Do not place the cap on the bench. This prevents the cap from becoming contaminated (see Figure 1).

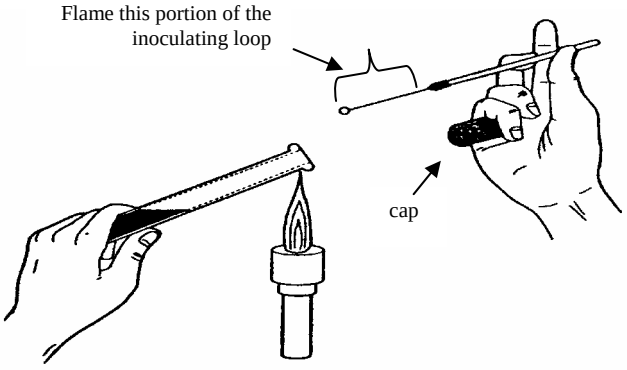


Figure 1. Removing cells from a liquid culture using an inoculating loop.

5. Flame the mouth of the tube by passing it rapidly through the Bunsen burner flame two or three times. This flaming sterilizes the mouth of the tube and heats the air around the mouth causing air currents to rise and flow away from the tube.
6. Put the hot loop into the liquid inside the tube. Remove the inoculum (a culture medium in which microorganisms are grown), re-flame the mouth of the tube, and replace the cap. The inside of the loop contains a film of medium and bacteria cells.
7. Lift the bottom of the agar plate and hold it perpendicular to the bench top. The perpendicular angle prevents particles in the air from settling on the plate.
8. Allow the loop to glide over the surface of the agar and "scribble" back and forth in the area of the plate illustrated in Step 1 of Figure 2. Place the plate back into the lid. Make sure that the loop is not plunged into the agar. Agar is very soft and will easily tear if the loop is pressed down too firmly.
9. Flame the loop until it is red hot and then cool the loop by plunging it into the agar. Since the agar is sterile, it is better to cool the loop in the agar than by waving it back and forth in the air where contaminants could be picked up. This flaming will kill all the remaining bacterial cells in the inoculating loop.
10. Touch the loop to the first set of streaks **once** and scribble the loop on the agar as illustrated in Step 2 of Figure 2. The loop only touches the first streak once thereby diluting the cells in the area of the second streak.
11. Flame the loop, cool in the agar, and make a third set of streaks as illustrated in Step 3 of Figure 2, ensuring that the loop only contacts the second set of streaks **once**. This will further dilute the number of cells in the area of the third streak.
12. After streaking, replace the lid of the Petri plate and give your PCA plate to your TA. These plates will be incubated at 30°C for 24-36 hours to permit colony growth.

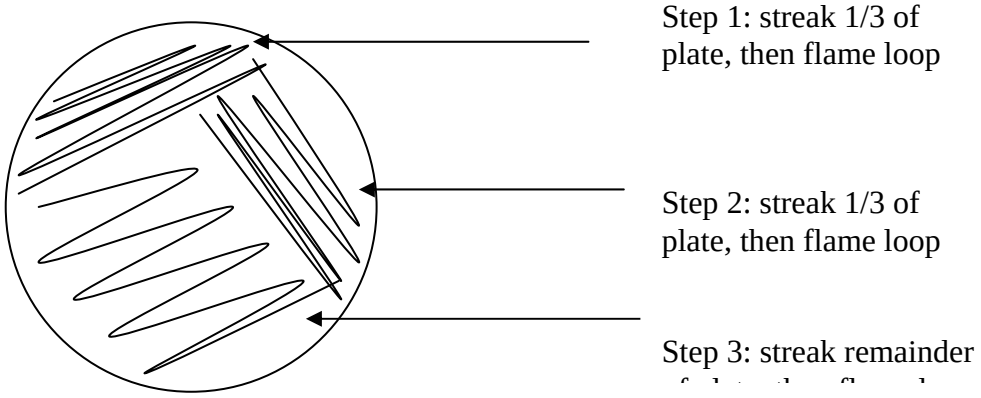


Figure 2. Streaking technique.

Clean-Up

1. Place the test tube containing the remaining mixed bacteria culture in the small test tube basket in the grey dishpan at the back of the lab. Leave the cap on the test tube. These tubes

must be autoclaved to kill the bacterial cells before the contents are disposed. Do NOT put live cultures down the drain (and into the sewer system).

2. Wipe your bench with Roccal.
3. Wash your hands.

Second Week of Lab

Objectives:

1. To characterize the three unknown bacterial species (streaked in last week's lab) using colony morphology, cell morphology, the Gram stain reaction, and several morphological, nutritional, and biochemical properties.
2. To examine colony morphology, cell morphology, the Gram stain reaction, and several morphological, nutritional, and biochemical properties of six known bacterial species.
3. To compare the unknown bacteria to the known bacteria in an *initial* attempt in identification.
4. To understand a subset of the morphological, nutritional, and biochemical tests used in bacterial identification.

The identification of a bacterial species is based on many factors, including cell and colony morphology, chemical composition of cell walls, biochemical activities, and nutritional requirements. In order to begin identifying a bacterial species, you must start with a pure culture. In last week's lab, you streaked a mixed bacterial culture containing three unknown bacterial species onto an agar plate. Recall that the purpose of streaking is to place individual cells on an agar plate so that an individual cell will divide many times to produce a colony. Once you have an isolated colony, you have millions of genetically identical cells, which can be used in identification tests.

In this exercise, you will examine the streaked plates from last week's lab and will attempt to identify the three unknown bacterial species found on these plates. Your preliminary identification will be done through a comparison of the characteristics of the three unknown bacteria to the characteristics of known bacterial species.

Gram stain reaction

An initial step in identifying a bacterial species is determining whether the cells in question are Gram-positive or Gram-negative. The Gram stain is one of the most important and widely used tools in the identification of unknown bacteria. The Gram stain reaction is dependent on the cell wall structure of the bacteria. The cell wall of Gram-positive bacteria is composed of a thick layer of peptidoglycan that surrounds the plasma or inner membrane. In contrast, a thin layer of peptidoglycan and a second phospholipid bilayer, known as the outer membrane, surround the plasma or inner membrane of Gram-negative bacteria. These characteristically different cell wall structures permit microbiologists to classify bacteria based on the color of the stain retained by cells treated with the Gram stain.

The Gram stain is a differential stain because it divides bacteria into two groups: Gram-positive and Gram-negative, where Gram-positive bacteria stain purple and Gram-negative bacteria stain pink. The procedure requires four solutions:

1. a basic dye (crystal violet)
2. a mordant (Gram's iodine)
3. a decolorizing agent (ethanol)
4. a counterstain (safranin)

Differences in cell wall structure cause the different staining reactions for Gram-positive and Gram-negative bacteria. When performing the Gram stain, the cells, which are stuck to a glass slide, are dipped into the crystal violet stain. The purple dye, crystal violet, is picked up by the cell wall of both Gram-positive and

Gram-negative bacteria. The cells are then dipped into an iodine solution; iodine is the mordant, meaning that it is a substance that increases the affinity of the cell for crystal violet so that crystal violet is more difficult to remove from the cell. Iodine forms large complexes with crystal violet and these complexes combine with the peptidoglycan in the cell wall. This crystal violet-iodine (CV-I) complex is larger than either molecule by itself. When ethanol (the decolorizing agent) is applied, the ethanol dissolves the lipids in the outer membrane of the cell wall of Gram-negative bacteria causing the CV-I complex to leave the cells. Gram-negative cells, therefore, appear colorless after the ethanol wash. However, when Gram-positive cells are washed with ethanol, their thick peptidoglycan layer prevents the large CV-I complexes from leaving the cell. Gram-positive cells therefore stain purple, which is the color of the crystal violet dye. The decolorizing step in the Gram stain is the critical step in distinguishing Gram-positive from Gram-negative bacteria because the two types of bacteria differ in the *rate* at which they decolorize when exposed to ethanol. If Gram-positive cells are decolorized too long (*i.e.,* exposed to ethanol for too long), they appear as Gram-negative cells. As Gram-negative cells are colorless after the ethanol wash, they are counterstained with safranin, a pink or reddish-colored dye. Therefore, Gram-negative cells appear pink after the Gram stain procedure, which enables comparison between those cells that decolorize with ethanol and those that do not.

Morphological characteristics

In addition to the Gram stain, microorganisms are also classified according to colony morphology and cell morphology. Bacterial colonies grow from a single cell and are composed of millions of cells. Each colony has a characteristic size, form or shape, edge, texture, degree of opacity, and color. These characteristics describe the morphology of a single colony and may be useful in the preliminary identification of a bacterial species. Colonies with a markedly different appearance (when grown on the same medium) can be assumed to contain different bacterial species. However, since many species have a similar colony morphology, the reverse (that colonies that look alike are the same species) is not always true. Figure 3 shows some of the terms used to describe colony morphology.

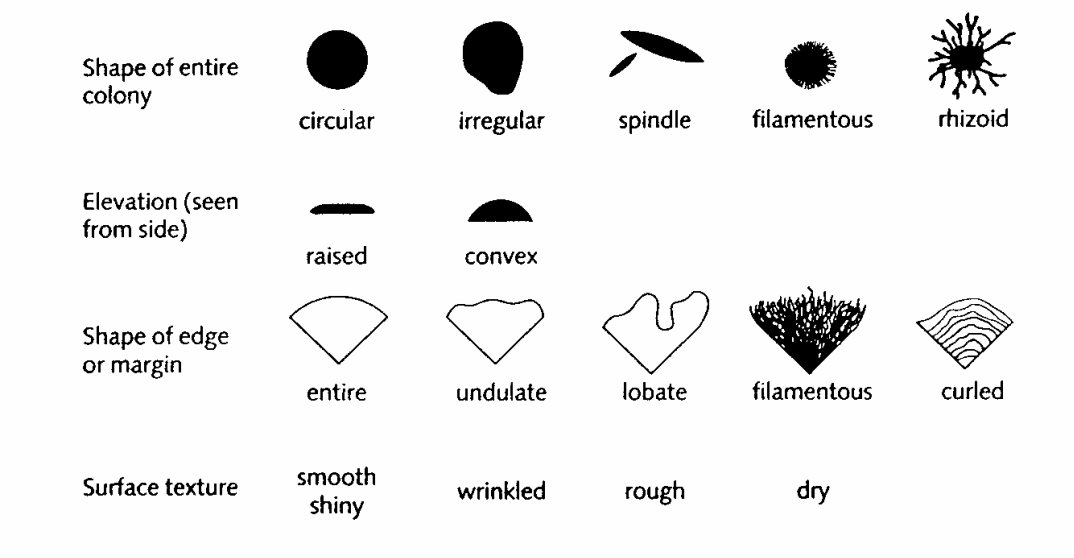


Figure 3. Some terms used to describe colony morphology.

The three most commonly recognized cell morphologies are cocci, bacilli, and spirilla. The cocci (the singular coccus means "berry") have a spherical shape. The bacilli (the singular bacillus means "little staff") are shaped like rods or cylinders. There is often great variation in the length of bacilli. Many are long and slender, while others are so short that they may resemble cocci. The spirilla (the singular spirillum means "coil") resemble a corkscrew, although the number of spirals varies considerably from species to species. In addition to cell morphology, the specific

arrangement of cells is an important identifying characteristic of many bacteria. Bacterial cells are found singly, in pairs, in clusters, or in chains when viewed under the microscope.

In addition to morphologies of the cell and of the colony, other morphological characteristics can be useful in initial identifications. Additional identifying morphological characteristics of bacteria include the presence/absence of structures external to the cell wall, such as flagella, a capsule or slime layer, and fimbriae and pili. In this lab, you will examine the bacteria for their ability to swim, which will suggest the presence of flagella. Motile bacteria that are injected into a test tube containing an agar medium will swim away from the area of injection to an area of low concentration of bacteria. The motility of a species can be examined as the medium contains a dye that turns red when oxidized by growing bacteria. The distribution of the red dye throughout the test tube will provide an indication of the swimming ability, and thus presence of flagella, of the bacteria in question.

Endospore formation

Certain Gram-positive and a single Gram-negative bacterium are capable of forming endospores when essential nutrients are depleted. Recall from lecture that endospores are highly resistant, dehydrated cells with thick walls and additional layers. The ability of bacterial cells to form endospores is another characteristic used in identification. A sample of bacterial cells that contains endospores will be able to withstand extreme conditions, such as high temperatures, and will grow when incubated at optimal conditions. In this lab, you will examine the growth of a sample of bacteria that has been previously exposed to high temperature, and from this growth, infer the presence of endospores in the sample.

Biochemical properties

Biochemical tests that investigate the enzymatic activities of cells are powerful tests in the identification of bacteria. In the research lab, a microbiologist would be begin by examining the cell morphology, colony morphology, Gram stain reaction, and environment from which the bacterium was isolated. Once these properties are known, a specific series of tests, defined by a specific flow chart, would be performed to aid in the identification of the genus and species of the bacterium. As many as 50-100 tests may have to be performed in order to positively identify the bacterium. In this lab, you will be introduced to some general tests to determine (1) the ability of the bacterial species to use glucose as a carbon source, and (2) the specific oxygen requirements for growth of each species.

Bacteria are capable of using different carbon sources to obtain the energy needed to sustain life. One main carbon source is glucose, but not all bacteria can metabolize glucose to harness energy. There are several pathways that bacteria can use to metabolize glucose. The end products and side products of each pathway provide an indication of the specific pathway the bacterium uses to metabolize glucose. In this lab, you will indirectly test the pathways the bacteria use. When bacteria are injected into a medium containing glucose and the pH indicator phenol red, the color changes that occur, and the presence of CO_2, provide evidence of the pathways used by the bacteria in question.

Bacteria are capable of growing in a variety of environmental conditions. The oxygen requirements for bacterial growth are a factor useful in identification. Bacteria are grouped into several categories based on their oxygen needs. Three of these categories are:

- obligate aerobes that require oxygen for survival and growth

- obligate anaerobes that do not tolerate oxygen and growth ceases in the presence of oxygen
- facultative anaerobes that can survive in aerobic and anaerobic conditions but prefer to grow in the presence of oxygen.

The growth patterns of bacteria injected into a medium that limits the amount of oxygen throughout the test tube will provide an indication of the oxygen requirements of the bacteria in question.

Comparisons to known bacterial species

Once you have examined the unknown bacterial species, you will then compare the characteristics of the unknowns to the characteristics of known bacterial species. These comparisons may allow you to make a preliminary identification of the unknown bacterial species streaked in last week's lab.

Materials:

Per student:

(1) PCA plate streaked last week in lab

Per group:

(1) grease pencil
(1) pair of forceps
(2) inoculating loops
- compound microscopes with 100X

Per bench:

(1) striker
(2) beakers
(1) wash bottle of 95% ethanol
(1) box of glass slides
(1) Bunsen burner
(1) bottle of 95% ethanol to clean microscope
(1) Coplin jar of crystal violet
(1) Coplin jar of Gram's iodine
(1) Coplin jar of safranin
(1) bottle of immersion oil

Per lab:

(6) dissecting microscopes
- demonstration material for unknown bacterial species
- demonstration plates and material of known bacterial species

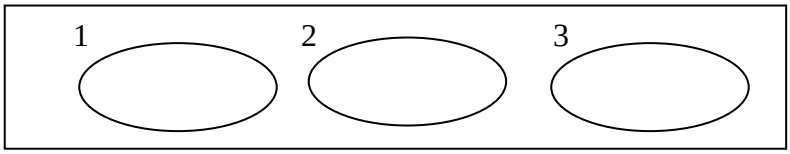
Identification of unknown bacteria

A. Colony Morphology

1. Examine the plates containing colonies of bacteria that you streaked.
2. Pick out three well-isolated colonies of different morphology. You should observe orange, cream, and white-colored colonies.
3. Using the grease pencils provided, circle and label (on the bottom of the Petri dish) a well-isolated orange colony "1," a well-isolated cream colony "2," and a well-isolated white colony "3."
4. Using the terms in Figure 3, describe the morphology of these colonies and enter your observations in Table 1.

B. Gram Stain, Cell Morphology, and Cell Size

1. The cells of each of the three circled colonies will be stained using the Gram stain to determine if the cells are Gram-negative or Gram-positive.
2. Using a grease pencil, draw 3 circles on the top of a warm, clean, grease-free slide and label the circles "1," "2," and "3."



3. Using an inoculating loop, transfer a loopful of water to the center of each circle on the slide.
4. Flame the loop, cool the loop by plunging it in the sterile agar, pick up a small sample of colony "1," transfer the sample to the center of the circle labeled "1," and spread the cells evenly while mixing them with the water. Repeat this entire step for colony "2" and colony "3."
5. Let the smears of cells and water air-dry until they don't look wet. When dry, the smear should be only faintly visible; a thick opaque film where the bacteria are piled on top of one another is useless.
6. Once the smears are dry, the cells must be fixed to the slide. Holding the side of the slide with forceps, pass it through the Bunsen burner flame until the slide is just uncomfortably warm to touch. After each pass of the slide through the flame, test the temperature on the back of your hand. If the slide is too hot to touch, then the bacteria will probably have been cooked and will be misshapen when observed under the microscope. In this case, start again at step B.2. However, if the slide is not warm, the preparation is most likely not heat-fixed adequately and the bacterial cells will wash off during the staining process. In this case, pass the slide through the flame once again and re-test for warmth using the back of your hand.
7. Holding the slide with forceps, place it into the Coplin jar of crystal violet and let it stand for 1 minute. The crystal violet dye will stain the cell wall of Gram-positive and Gram-negative cells. All cells will appear purple at this stage.
8. Gently dip the slide in and out of a beaker of water for 2-3 seconds. Remove excess water by touching the slide to the lip of the beaker. This will remove any free stain from the slide.
9. Holding the slide with forceps, place it into the Coplin jar of Gram's iodine and let it stand for 1 minute. During this minute, discard the "purple water" rinse, and refill your beaker with clean water. The Gram's iodine and crystal violet will form a crystal violet - iodine complex. All cells will appear purple at this stage.
10. Gently dip the slide in and out of a beaker of water for 2-3 seconds. Remove excess water by touching the slide to the lip of the beaker. Immediately discard the water, rinse, and refill your beaker with clean water. You will need this beaker of water ready so you can proceed immediately from step 10 to step 11.
11. Using a wash bottle, flood the slide with 95% ethanol. Agitate the slide back and forth to allow the ethanol to contact all the cells on the slide. Continue until all the "excess" color is washed out, but do not let the ethanol remain in contact with the slide for any longer than 5-10 seconds. If too much ethanol is added or the cells are in contact with the ethanol for more than 10 seconds, Gram-positive organisms may appear to be Gram-negative.

    Ethanol dissolves the lipids in the outer membrane of the Gram negative cell wall causing the crystal violet-iodine complex to leave these cells. At this stage, Gram negative cells appear colorless and Gram positive cells appear purple.

12. Immediately dip the slide in and out of a beaker of clean water for 2-3 seconds. Remove excess water by touching the slide to the lip of the beaker.
13. Holding the slide with forceps, place it into the Coplin jar of Safranin and let it stand for 1 minute.

    Safranin is a counterstain - it stains the Gram-negative cells that lost the stain during the ethanol wash. At this stage, Gram-negative cells appear pink and Gram positive cells appear purple.
14. Remove excess stain from the slide by gently dipping the slide in and out of a beaker of water for 2-3 seconds. Remove excess water by touching the slide to the lip of the beaker.
15. Let the slide air-dry.
16. Focus on the cells of circle "1" of the Gram stained slide with the 10X objective and then the 40X objective. There is no need to put a coverslip on the slide.

Note: Make sure the condenser is in focus; it is essential that the condenser be in focus, especially when using the 100X objective lens.

Note: Make sure the interpupillary distance is set for your eyes. You will be taking measurements of cell size and need to be accurate with your measurements.

17. As bacterial cells are relatively small, you will need to use the 100X objective lens with immersion oil. Using oil immersion will allow you to obtain a clear image of the cells to determine cell morphology. Rotate the turret so that the 40X objective is out of position and the 100X objective is about to be moved into position (*i.e.*, the light path should be halfway between the 40X and 100X objective).
18. Add one small drop of oil on the slide at the spot where the light from the condenser is shining on the slide.
19. Rotate the turret to click the 100X objective into place.

    Be careful not to rotate the 40X objective through the oil. Immersion oil should only be used with the 100X oil immersion objective, never with any other objective. If oil gets on any other objective lens, it must be cleaned off immediately using a piece of lens paper as oil corrodes objective lenses, even the 100X objective lens.
20. Open the condenser diaphragm as wide as possible to let more light into the field of view.
21. Adjust the fine focus to bring the specimen into sharp focus.

    Never use the coarse focus knob when viewing with the 100X objective lens. The slide could break and the glass of the objective lens could become scratched or shattered.
22. Are the cells of circle "1" Gram-positive or Gram-negative? Record the results in Table 1.
23. Describe the cell morphology of the cells in circle "1." Record your results in Table 1.
24. Measure the size of an individual cell within circle "1" using the ocular micrometer. Determine the size of an individual cell. Enter this value in Table 1.
25. Move the stage to its lowest position. Swing the 10X objective into place and using lens paper, gently wipe the oil off the 100X objective lens.

    It is important that care be taken with the objective lens. Do not rub the lens paper across the objective lens. Cleaning the objective lens harshly with lens paper, using anything but lens paper, or forgetting to wipe the oil off will ruin the lens. Oil corrodes the objective so the oil **must** always be cleaned off the objective after use. A 100X objective lens is very expensive to replace.

26. Move the stage so that the light from the condenser is shining within circle "2" of the Gram stained slide. Focus on the cells using the 10X objective and then the 40X objective. Repeat steps 17 through 25 for the cells of circle "2."
27. Move the stage so that the light from the condenser is shining within circle "3" of the Gram stained slide. Focus on the cells using the 10X objective and then the 40X objective. Repeat steps 17 through 25 for the cells of circle "3."

C. Additional Identification Characteristics

1. Examine the demonstration material regarding unknown bacteria "1," "2," and "3."
2. The first set of demonstration material examines the effect of oxygen on the growth of the microbe. Determine whether the unknowns are obligate aerobes, obligate anaerobes, or facultative. Record this information in Table 1 under "O_2 Use."

   In order to examine the effect of oxygen, bacterial cells were stabbed into a test tube containing thioglycollate. Thioglycollate lowers the redox potential of the medium, thus oxygen is present only near the surface and the remainder of the tube is anaerobic. Obligate aerobes will grow near the surface of the agar in the tube, obligate anaerobes will grow below the surface, and facultative organisms will grow throughout the tube.
3. The second set of demonstration material examines the ability of the three unknown bacteria to utilize glucose as a carbon source to obtain the energy needed to sustain life. Determine whether the unknown bacterial species can ferment glucose and, if so, whether gas is produced during fermentation. Record this information in Table 1 under "Glucose Use."

   Bacteria were inoculated in a medium containing glucose as the carbon source and phenol red as a pH indicator. Phenol red is red at neutral pH and yellow at pH <6.8. A change in color from red to yellow indicates the formation of acid and thus fermentation of glucose. Additionally, an inverted tube is placed in the inoculated test tube; if gas is produced during fermentation, it will collect in the inverted test tube.
4. Endospores are resistant to killing by high temperature, desiccation, or radiation. Determine whether the unknown organisms are capable of producing endospores. Record this information in Table 1 under "Endospore."

   In order to test whether a sample of inoculum contains endospores, the sample is exposed to high heat (80°C) for 10 minutes. This treatment would kill all cells but endospores would survive and could grow when incubated at an appropriate temperature. If endospores are present, the resultant inoculum will give rise to a turbid culture (indicating bacterial growth); if there are no endospores present, no growth is observed.
5. Most motile bacteria use flagella for motility.
   a. Using the computer in the lab, view the schematic animation of one type of bacterial movement - movement via flagella. Observe the characteristic "run and tumble" behavior of the cell. Note that there are four arrangements of flagella in bacteria. These arrangements are important factors in identifying a particular species.
   b. Examine the demonstration material and determine whether the bacteria are motile. Record this information in Table 1 under "Motile."

One way the motility of cells can be investigated is by injecting a stab of bacteria into a test tube containing a low concentration of agar (so the bacteria, if motile, can swim) and a tetrazolium salt. This salt appears red when oxidized by growing bacteria. Thus, a red color throughout the agar test tube indicates motility and a red line in the middle of the test tube indicates that the bacteria under investigation are non-motile.

Characteristics of known bacteria

A. Colony morphology

1. Examine the demonstration agar plates of the six known bacterial species found on the side bench. Examine the colony morphology using the dissecting scopes provided and record your observations in Table 2. Use the terms in Figure 3 to describe colony morphology.

B. Gram stain, cell morphology, and cell size

1. Examine the micrographs of the six known bacterial species and note the cell morphology and Gram stain reaction of each. Record your observations in Table 2. Using the scale bar found at the bottom right of each of the micrographs, estimate cell size of each species. Enter these values in Table 2.

C. Additional Identification Characteristics

1. For each of the six known bacteria, determine the requirements for oxygen in growth, their ability to ferment glucose, their ability to form endospores, and their motility. Record this information in Table 2.

D. Cleanup

1. Discard the PCA plates with your streaked culture into the autoclave bag at the back of the lab.
2. Move the stage to its lowest position, click the lowest power objective into place, and remove the slide from the stage.
3. Using a piece of lens paper, gently wipe the oil off the objective. Do not rub the lens with the lens paper; a light wipe is sufficient to fully clean the lens. If the lens is very dirty, place one drop of ethanol onto a piece of lens paper and again, gently wipe the oil off the objective lens
4. Discard glass slide into the Contaminated Glass Waste container on your lab bench.
5. Leave the inoculating loop, the forceps, grease pencil, and Gram stain set up on your lab bench so that the next group of students may use it. Empty and rinse the beakers of water you used for the Gram stain and return to your lab bench.
6. Leave dissecting microscopes, demonstration plates, and all demonstration material on the side bench.

Table 1. Colony morphology, cell morphology, Gram stain reaction, and general properties of bacterial species streaked in last week's lab.

Colony Number	Colony Morphology (from agar plates)						Cell Morphology	Gram reaction (+/-)	Cell size (μm)	O_2 Use	Glucose Use	Endospore (Y/N)	Motile (Y/N)
	Size (mm)	Shape	Elevation	Edge	Color	Surface							
1					orange								
2					cream								
3					white								

Table 2. Colony morphology, cell morphology, Gram stain reaction, and general properties of known bacterial species.

Bacteria (Genus only)	Colony Morphology (from agar plates)						Cell Morphology	Gram reaction (+/-)	Cell size (μm)	O_2 Use	Glucose Use	Endospore (Y/N)	Motile (Y/N)
	Size (mm)	Shape	Elevation	Edge	Color	Surface							
Bacillus													
Escherichia													
Lactobacillus													
Micrococcus													
Staphylococcus													
Proteus													

Notes for the Instructor

This lab was developed to introduce students to several techniques used in microbiology labs and to complement specific aspects of the microbiology content emphasized in the lecture portion of the course. As such, the tests that are included do not follow a rigorous series of taxonomic tests. Normally, bacteria are identified using a series of tests performed in an ordered manner, which function to subdivide bacteria into smaller and smaller groups. Students are clearly made aware of this fact. Our second year course in microbiology introduces students to the ordered series of taxonomic tests.

The objectives of the first week of this lab are to (1) introduce students to the streaking technique, and (2) have students prepare the mixed culture plate to be used in the next lab session. Prior to the first week of this lab, students have performed an environmental survey of bacteria in the lab. The students examine their environmental survey plates immediately prior to working with the mixed bacterial culture. This allows the TAs to talk about sterile technique and the importance of working sterilely before the students are permitted to work with the mixed bacterial culture.

The objective of the second week of this lab is to determine the possible identity of the three bacteria streaked from the mixed culture. In order to identify the unknown bacteria, students will examine a set of seven characteristics of the unknown bacteria. These seven characteristics are:

1. colony morphology, including size, shape, elevation, edge, and surface texture
2. cell morphology
3. Gram stain reaction
4. oxygen requirements for growth
5. carbon source utilization: in this lab, glucose is the carbon source
6. presence of endospores in a culture
7. motility

Once these seven characteristics are known about the unknowns, a comparison can be made with the same seven characteristics of a set of six bacteria, whose identity is known. Through this comparison, a possible preliminary identification of the unknown bacteria may be made.

The second week of lab can be taught as a "What Is It?" in a similar manner to a "Who Done It?" lab. As the majority of the material is photomicrographs, it is important for the instructor or TA to demonstrate enthusiasm for the material. Due to space and time constraints, we could not offer the lab so that students perform the tests themselves. We do not have the space to incubate the tubes, nor do we have the time to offer open labs, in which students can come to the lab to examine their tubes and record their results. The combination of students performing the Gram stain and examining results using demonstration material has worked well in our labs.

Demonstration Material

Demonstration agar plates of the six "knowns" are streaked out a week or so before the lab. The plates are kept in the refrigerator when not in use in the labs; the plates last all week. TAs are instructed to take the plates out of the fridge approximately 30 minutes prior to examination by the students. This allows the plates to warm up slightly before being examined under the lights of the dissecting microscope. We have found that if this is not done, condensation in the lid of the plates creates problems with viewing the colonies on the plates.

We use 2 plates of *E. coli* as demonstration material, one labeled as "*Escherichia coli*" and one labeled as "*Proteus vulgaris.*" Our reasons for doing this are twofold. First, we designed the lab so that after examining all the characteristics they are asked to examine, they are unable to make a preliminary identification of "unknown 2." The point of this is so they understand that these seven tests do not provide enough

information to make an identification. Additionally, our second reason is that our departmental strain of *P. vulgaris* tends to swarm across the plate during the week, making the plates of *E. coli* and *P. vulgaris* obviously different.

We also provide extra unknown plates. These plates consist of a pure streaked culture of *E. coli*, *S. epidermidis*, and *B. subtilis*, but are labeled with their respective unknown number, not their actual identification. These plates are to be used by students to perform the Gram stain if their streaked mixed culture plates do not contain all three colony types.

Students have difficulty with exactly what a colony consists of and why the morphology of the colony is different from the morphology of the cell. Students need to be reminded that one colony contains millions of cells.

Tests Used and Possible Results of Each

These tests used can be adjusted accordingly to emphasize many aspects of microbiology. As mentioned, these four were chosen for their relevance to the lecture portion of the course.

(1) Oxygen Requirements for Growth

Students will be asked to determine whether the bacterial cells in question are obligate aerobes (require oxygen to live), obligate anaerobes (are unable to use molecular oxygen for energy-yielding reactions), or facultative (both aerobic and anaerobic growth occur; greater growth in the presence of oxygen). Microaerophillic has not been included as a choice for this lab, as this term is not mentioned in lecture and we did not want to cause confusion. In order to perform this test, test tubes are made with thioglycollate in the medium. Thioglycollate lowers the redox potential of the medium thus oxygen is present only near the surface of the medium in the tube, the remainder of the tube is anaerobic. Bacteria are stabbed into the test tube and allowed to grow -- where they grow is indicative of their use of oxygen in growth. An aerobic organism will only grow at the surface so the medium in the test tube will look clear except near the top where bacteria have grown. An anaerobic organism will not grow in the presence of oxygen so the test tube looks clear only at the top of the tube where oxygen is present. Facultative organisms, when stabbed into the medium, are able to grow throughout the length of the tube but growth is greater near the surface since the oxygen concentration is greater near the surface. Notice that in the test results that facultative organisms that are non-motile will grow in the area of the stab but motile facultative organisms can grow throughout the tube.

(2) Glucose as a Carbon Source

Students will be asked to determine whether the bacterial cells in question are able to use glucose as a carbon source. In a test tube, a medium including glucose and phenol red (as a pH indicator) is inoculated with the bacteria. After incubation (within 48 hours), the tube is compared to an uninoculated tube to look for (1) a change in color from red to yellow, and (2) the presence of gas bubbles in the inverted Durham tube placed in the test tube. The change in color to yellow indicates that acid is produced and that the organism is able to use glucose as a carbon source. The presence of gas is indicative of the fermentation pathway used by the organism and can thus be used as an identifying characteristic of an unknown bacterium. Students will record whether a color change occurs and whether or not bubbles are produced.

(3) Presence of Endospores in a Culture

Students will be asked to determine whether a culture of bacterial cells in question contain endospores. Very few bacterial species are capable of producing endospores, so a positive result helps the investigator limit the number of possible identities for the unknown. Endospores are resting structures formed inside some bacteria; these structures are formed when essential nutrients are depleted and are highly durable dehydrated cells with thick walls and additional layers. In this test, a sample of inoculum that is suspected of containing endospores is exposed to 80°C for 10 minutes; this high heat will kill bacterial cells but will not harm any endospores that are present. The inoculum is then incubated and growth examined. A turbid culture

following incubation indicates that the inoculum contained endospores that were able to return to a metabolic state, and thus grow. A clear culture medium suggests that the inoculum did not contain endospores and that the high heat for 10 minutes killed all bacterial cells present in the inoculum. Students will record whether the resultant incubated tube produces a turbid culture (endospores present) or remains clear (no endospores). Also note that endospores may also be visible in Gram stain as a clear body inside cells.

(4) Motility

Students will be asked to determine whether the bacterial cells in question are motile. Bacteria normally locomote using beating flagella. Bacterial cells normally have one of four arrangements of flagella: monotrichous, amphitrichous, lophotrichous, and petrichous. We do not expect students to know these terms but they should understand that the arrangement of flagella on a bacterial cell is a characteristic that may be useful in identification. Bacteria characteristically undergo a "run and tumble" type of motion when using flagella. They need to understand that this swimming behavior is characteristic of bacterial cells. In order to test whether or not an inoculum is motile, the bacterial cells are stabbed into a test tube containing a low concentration of agar in the medium. This medium also contains a tetrazolium salt that changes color from clear to red as it is oxidized by growing bacteria. Thus, non-motile bacteria will remain at the site of the stab and the medium in the tube will appear to have a red line drawn through it; the red color indicates where the bacteria are growing. Motile bacteria will be able to grow throughout the tube and the medium in the tube will have a red color throughout. This can also be confirmed using the first test -- oxygen requirements. A non-motile facultative organism will grow in the middle of the thioglycollate-containing tube; a motile facultative organism will grow throughout the thioglycollate-containing tube. Students will also examine a departmental website with a schematic representation of the "run and tumble" swimming behavior.

Lab Setup

In Week 1, the lab is normally set up with one test tube rack on each student bench (4 benches per lab room), 2 inoculating loops and 1 Bunsen burner per group (2 groups of 2-3 students each per bench), and one test tube of mixed bacterial culture per bench. Each student streaks his or her own PCA plate. Plates are incubated at 30°C for 24-36 hours to permit colony growth and are placed in the fridge for the next 5-6 days before performing the next lab.

In Week 2, the lab is normally set up with one Gram stain station on each of the four student benches; the station is shared by two groups of students (2-3 students per group). We cover the middle portion of each student bench with bench coat to protect the bench tops from stains. On the side bench of the lab room, six dissecting microscopes are placed beside each photomicrograph of the Gram-stained bacteria and photos of the test results for each bacterial species. On the side bench, there are also two sets of photos of how to read the test results of each test. At the back of the lab room are the photos of the test results for the three unknown bacteria.

Students can perform the lab in any sequence they like. The majority of TAs divide the Gram stain stations and the known stations amongst the class so that a bottleneck does not occur at any one station.

Bacteria used

The bacteria we use for the lab are *Bacillus subtilis* var. niger, *Escherichia coli* (NCIB 8666), *Lactobacillus plantarum* (ATCC 8014), *Micrococcus luteus* (ATCC 4698), and *Staphylococcus epidermidis* (ATCC 155). These specific bacteria were used for a number of reasons. First and foremost, convenience was the reason; we had these bacteria in our department and used what was available. Secondly, the choice of using *E. coli* and *P. vulgaris* was that these species have identical characteristics in terms of the biochemical/nutritional/morphological tests that were being used. We wanted to have one unknown whose identity was "inconclusive" so that students did not think that these seven tests, and only these seven tests, were all that were necessary to perform. *L. plantarum* and *S. epidermidis* were chosen as both grow as small, white colonies. However, we grow *L. plantarum* on TCS plates and *S. epidermidis* on PCA plates to help

students understand that the appearance of colonies on a plate are dependent on the type of medium on which they are grown.

One point that we do not address in this lab is from where the bacteria would have originally been isolated. This is because the location of initial isolation would provide too much information about the bacteria in question, so much information in fact, that negative conclusions could be based solely on location, and obtaining data from all other aspects of the lab would be unnecessary.

It would be possible to have students perform the Gram stain on the "known" bacteria. However, we decided to have students perform the Gram stain on only the "unknown" bacteria and to provide them with micrographs of the "known" bacteria. The reason for this was to force students to learn how to use a scale bar to determine cell size.

Appendix A: Recipes

The Gram stain solutions can be made using the recipes below or a kit can be purchased. We make our solutions using these recipes.

Crystal Violet stain

(a) 8 g crystal violet dissolved in 80 ml 98% ethanol
(b) 3.2 g ammonium oxalate dissolved in 320 ml ddH_2O
Let stand 24 hours, mix (a) and (b), then filter.

Gram's Iodine

(a) 1.2 g iodine dissolved in 20 ml 98% ethanol
(b) 2.4 g potassium iodide dissolved in 340 ml ddH_2O
Mix (a) and (b)

Safranin

1.0 g Safranin dissolved in 40 ml 98% ethanol
Dilute with 400 ml ddH_2O, then filter

PCA and TCS can be purchased as pre-mixed powder or can be made using the recipes below. We purchase the pre-mixed powder of PCA from Fisher (Difco, catalog number 0479173) and TCS from VWR (BBL, catalog number 211768).

PCA (Plate count agar)

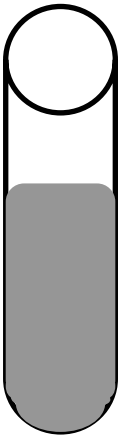
pancreatic digest of casein	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar	15.0 g

TCS (Trypticase Soya Agar)

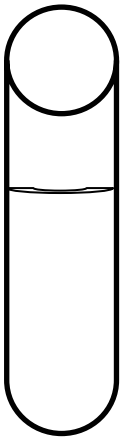
pancreatic digest of casein	17.0 g
Soybean peptone	3.0 g
NaCl	5.0 g
K_2HPO_4	2.5 g
Glucose	2.5 g
Agar	15.0 g

Appendix B: Test Results

Endospore Test

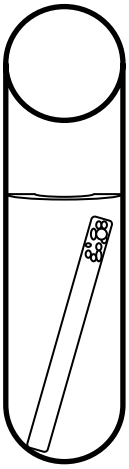


A turbid culture will form following incubation if the original inoculum contained endospores.

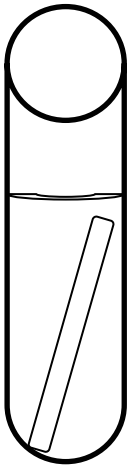


Control tube: uninoculated and Negative result: No growth occurs following extreme heat exposure if the original inoculum did not contain endospores.

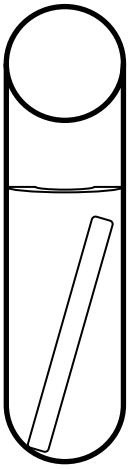
Glucose Fermentation Test



Positive result: medium is yellow in color and Positive result for gas production: bubbles collect in Durham tube.



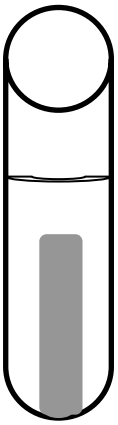
Positive result: medium is yellow in color and Negative result for gas production: no bubbles in Durham tube.



Control tube: uninoculated and Negative result. In both cases, the medium is red in color, no bubbles in Durham tube.

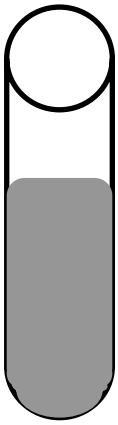
Oxygen Requirements for Growth Test

Positive test for an obligate aerobe. Growth only occurs near surface of medium.



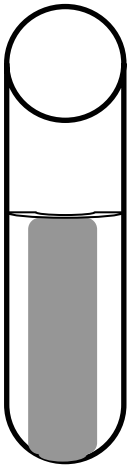
Positive test for an obligate anaerobe. Growth only occurs below the surface of medium.

Positive test for a non-motile facultative anaerobe. Growth occurs throughout the medium but is restricted to area of stab in lower portion of tube.

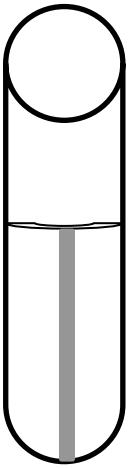


Positive test for a motile facultative anaerobe. Growth occurs throughout the tube as bacteria swim outwards from stab.

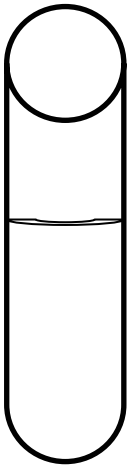
Motility Test



Positive test: growth occurs outwards from original stab. Area of growth appears red in color.



Negative test: growth occurs only in area of original stab. Area of growth appears red in color.



Control tube: uninoculated

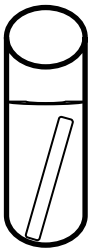
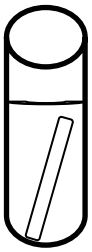
Staphyloccoccus Test Results

I. Oxygen Requirements for growth

II. Glucose Fermentation test

A: Control tube (uninoculated): medium is red

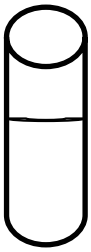
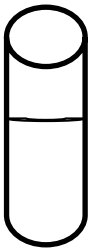
B: Glucose + *Staphylococcus*: medium is yellow

A  B 

III. Endospore Test

A: Control tube (uninoculated)

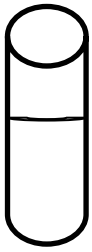
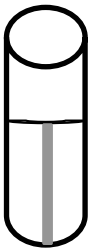
B: Growth of inoculum following heat treatment

A  B 

IV. Motility Test

A: Control tube (uninoculated)

B: Stab of *Staphylococcus*

A  B 

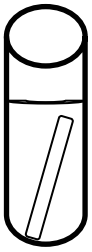
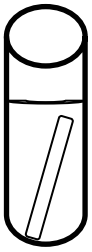
Micrococcus Test Results

I. Oxygen Requirements for growth

II. Glucose Fermentation test

A: Control tube (uninoculated): medium is red

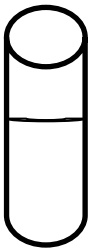
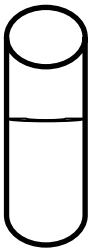
B: Glucose + *Micrococcus*: medium is red

A  B 

III. Endospore Test

A: Control tube (uninoculated)

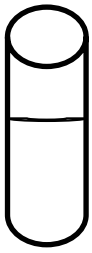
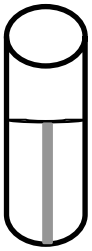
B: Growth of inoculum following heat treatment

A  B 

IV. Motility Test

A: Control tube (uninoculated)

B: Stab of *Micrococcus*

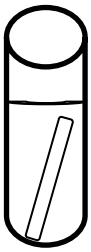
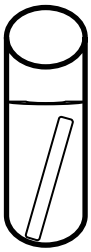
A  B 

Bacillus Test Results

I. Oxygen Requirements for growth

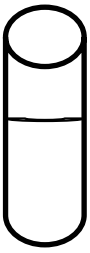
II. Glucose Fermentation test

A: Control tube (uninoculated): medium is red
B: Glucose + *Bacillus*: medium is yellow

A  B 

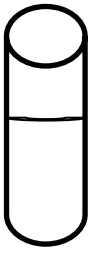
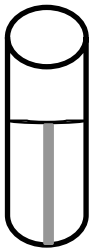
III. Endospore Test

A: Control tube (uninoculated)
B: Growth of inoculum following heat treatment

A  B 

IV. Motility Test

A: Control tube (uninoculated)
B: Stab of *Bacillus*

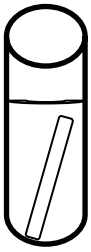
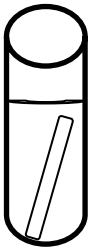
A  B 

Lactobacillus Test Results

I. Oxygen Requirements for growth

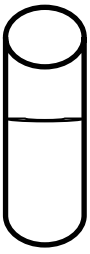
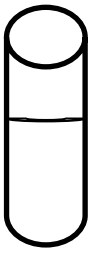
II. Glucose Fermentation test

A: Control tube (uninoculated): medium is red
B: Glucose + *Lactobacillus*: medium is yellow

A  B 

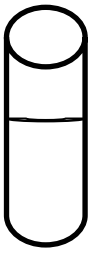
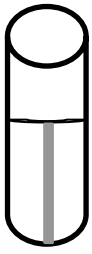
III. Endospore Test

A: Control tube (uninoculated)
B: Growth of inoculum following heat treatment

A  B 

IV. Motility Test

A: Control tube (uninoculated)
B: Stab of *Lactobacillus*

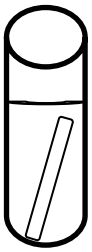
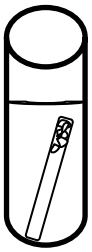
A  B 

Escherichia Test Results

I. Oxygen Requirements for growth

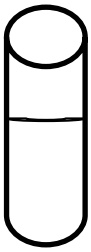
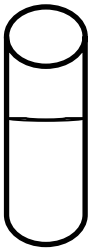
II. Glucose Fermentation test

A: Control tube (uninoculated): medium is red
B: Glucose + *Escherichia*: medium is yellow

A  B 

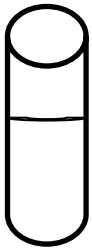
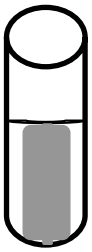
III. Endospore Test

A: Control tube (uninoculated)
B: Growth of inoculum following heat treatment

A  B 

IV. Motility Test

A: Control tube (uninoculated)
B: Stab of *Escherichia*

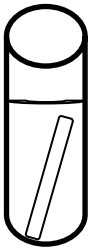
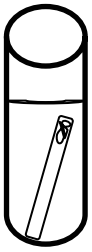
A  B 

Proteus Test Results

I. Oxygen Requirements for growth

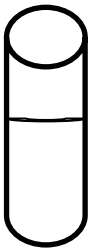
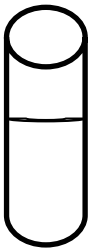
II. Glucose Fermentation test

A: Control tube (uninoculated): medium is red
B: Glucose + *Proteus*: medium is yellow

A  B 

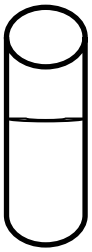
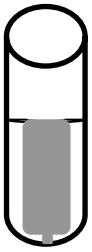
III. Endospore Test

A: Control tube (uninoculated)
B: Growth of inoculum following heat treatment

A  B 

IV. Motility Test

A: Control tube (uninoculated)
B: Stab of *Proteus*

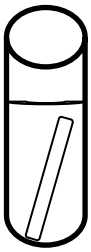
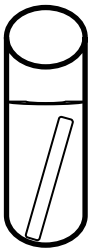
A  B 

Unknown 1 Test Results

I. Oxygen Requirements for growth

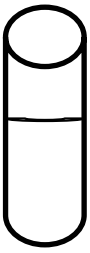
II. Glucose Fermentation test

A: Control tube (uninoculated): medium is red
B: Glucose + Unknown 1: medium is yellow

A  B 

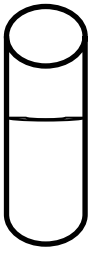
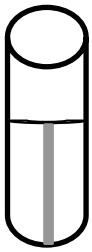
III. Endospore Test

A: Control tube (uninoculated)
B: Growth of inoculum following heat treatment

A  B 

IV. Motility Test

A: Control tube (uninoculated)
B: Stab of Unknown 1

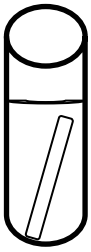
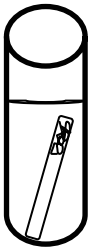
A  B 

Unknown 2 Test Results

I. Oxygen Requirements for growth

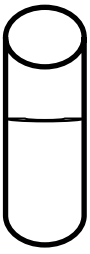
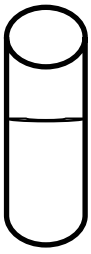
II. Glucose Fermentation test

A: Control tube (uninoculated): medium is red
B: Glucose + Unknown 2: medium is yellow

A  B 

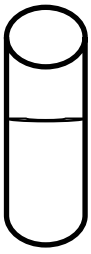
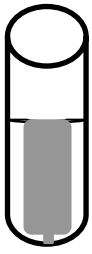
III. Endospore Test

A: Control tube (uninoculated)
B: Growth of inoculum following heat treatment

A  B 

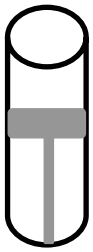
IV. Motility Test

A: Control tube (uninoculated)
B: Stab of Unknown 2

A  B 

Unknown 3 Test Results

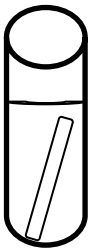
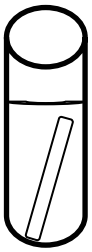
II. Oxygen Requirements for growth



II. Glucose Fermentation test

A: Control tube (uninoculated): medium is red

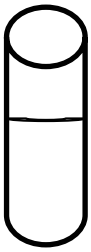
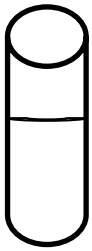
B: Glucose + Unknown 3: medium is yellow

A  B 

III. Endospore Test

A: Control tube (uninoculated)

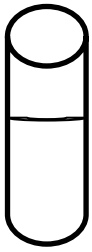
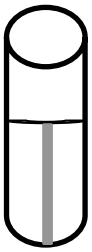
B: Growth of inoculum following heat treatment

A  B 

IV. Motility Test

A: Control tube (uninoculated)

B: Stab of Unknown 3

A  B 

Key for Table 2: Colony morphology, cell morphology, Gram stain reaction, and general properties of known bacterial species.

Bacteria (Genus only)	Colony Morphology (from agar plates)						Cell Morphology	Gram reaction (+/-)	Cell size (μm)	O_2 Use	Glucose Use	Endospore (Y/N)	Motile (Y/N)
	Size (mm)	Shape	Elevation	Edge	Color	Surface							
Bacillus	2	circular	flat	entire	orange	smooth	bacillus	+	2-5	aerobe	Y, no gas	Y	N
Escherichia	2	circular	flat	entire	cream	smooth	bacillus	-	2	facultative	Y, gas	N	Y
Lactobacillus	1	circular	convex	entire	white	smooth	bacillus	+	2-3	facultative	Y, no gas	N	N
Micrococcus	1	circular	convex	entire	yellow	smooth	cocci	+	1	aerobe	N	N	N
Staphylococcus	0.5	circular	convex	entire	white	smooth	cocci	+	1	facultative	Y, no gas	N	N
Proteus	2	circular	flat	entire	cream	smooth	bacillus	-	2	facultative	Y, gas	N	Y