

## Chapter 6

# Isolation of an Unknown Bacterium from Soil

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## Introduction

In an undergraduate microbiology lab class consisting of both majors and non-majors (health-related fields), one hopes to teach the student standard microbiology tests used to identify and characterize various microbes. To stimulate those students who may eventually conduct research, or actually work in a microbiology lab, I have incorporated the identification and characterization of an unknown bacterium.

Each student begins the procedure of isolating an unknown bacterium from soil during our first laboratory session. The students tend to get quite involved in trying to identify their unknown bacterium. The most frequently isolated genus has been *Bacillus*, but we have also seen *Streptococcus*, *Staphylococcus*, and *Escherichia*. Other less frequent bacteria are *Arthrobacter* and *Actinomyces*.

Soil is an excellent source for unknown microorganisms, since bacteria, algae, protozoans, yeasts, molds, and microscopic worms are routinely found in this environment. Students initially isolate bacteria, yeast, and molds on their primary culture plates. This variety allows an early exposure of the student to staining, and microscopically observing some obvious differences between prokaryotes and eukaryotes.

The lab experiments are arranged into four exercises: (a) the isolation of an unknown bacterium from soil; (b) identification an unknown bacterium utilizing various staining techniques; (c) determining the motility of an unknown bacterium; and (d) determining the physiologic characteristics of an unknown bacterium.

Each student turns in a lab report on his/her unknown bacterium which includes the following: (1) a brief introduction describing the purpose of the experiments; (2) summary tables of all unknown results; (3) a tentative identification of the isolated bacterium using flow charts (Bensen, 1990), and *Bergey's Manual of Systemic Bacteriology* (Volumes I–IV); and (4) a conclusion discussing the basis on which the unknown was tentatively identified.

## Exercise A: Isolation of an Unknown Bacterium from Soil

### Experiment 1: Primary Cultures from Soil Extracts

*Objective:* In this experiment students will obtain soil samples, and isolate colonies on primary culture plates using the T-streak.

#### *Materials*

Bunsen burners	30°C incubator
Strikers with flint	4°C refrigerator
Test tube racks	Trypticase Soy Agar (TSA)
Inoculating loops	TSA plates
Disinfectant (10% clorox)	5 ml tubes of Trypticase Soy
Paper towels	Broth (TSB)
Wax pencils	Sterile wooden popsicle sticks

#### *Procedure*

1. Obtain a tube containing 5 ml of TSB and a sterile wooden popsicle stick. Label tube with name and lab number.
2. Aseptically transfer a small amount of soil into the tube of TSB. A greater variety of microorganisms can be found around plants.
3. Mix tube to suspend any organisms from the soil into the broth. Return to lab, and let tube sit at 25°C (room temperature) for about 30 minutes until the most of the soil particles have settled.
4. Use upper liquid broth to streak primary streak plates.

5. Students in a group of two will practice the T-streak on a practice plate. Use one practice plate for two students; these are at your benches labelled “practice.”
6. Obtain two TSA plates.
7. Label bottom of plates (not the top) along the edge. Label in small letters name, lab number, and soil extract.
8. Inoculate each plate with the soil extract from step 4 (above) using the T-streak method; steps 1 to 7 on page 122 in Claus (1989).
9. Follow steps 3, 5, and 6 on pages 121–124 in Claus (1989).
10. Put plates in bin to be incubated at 30°C.
11. We will pull plates after 48 hours of incubation and store at 4°C until next lab.
12. These plates will be used in Experiment 2.
13. Place original soil extract tube in rack labelled soil extract to be stored at 4°C in case we need them.

### **Experiment 2: Secondary Cultures from Primary Cultures**

*Objective:* In this experiment students will pick out three different colonies from their primary culture plates, and streak three secondary culture plates from them. One of the secondary pure cultures will serve as their unknown bacterium.

#### *Materials*

Bunsen burner	Paper towels
Striker with flint	Wax pencils
Test tube rack	30°C incubator
Inoculating loop	4°C refrigerator
Lens paper	TSA plates
Immersion oil	Primary cultures
10% clorox	

#### *Procedure*

1. Examine the two streak plates done on your soil extract. Notice the different types of colonies. Pick out three different colonies which might be of interest (we are looking for a bacterium to serve as the unknown) due to their color, texture, shape, or frequency. Do not pick a colony which is obviously filamentous suggesting a fungus. Ask your instructor if you need help picking three colonies.
2. Obtain three TSA plates label them with name, lab #, and isolate #1, 2, or 3.
3. Follow procedure for T-streak in Experiment 1 to streak three secondary plates.

### Experiment 3: Identification of an Unknown Bacterium from Secondary Cultures

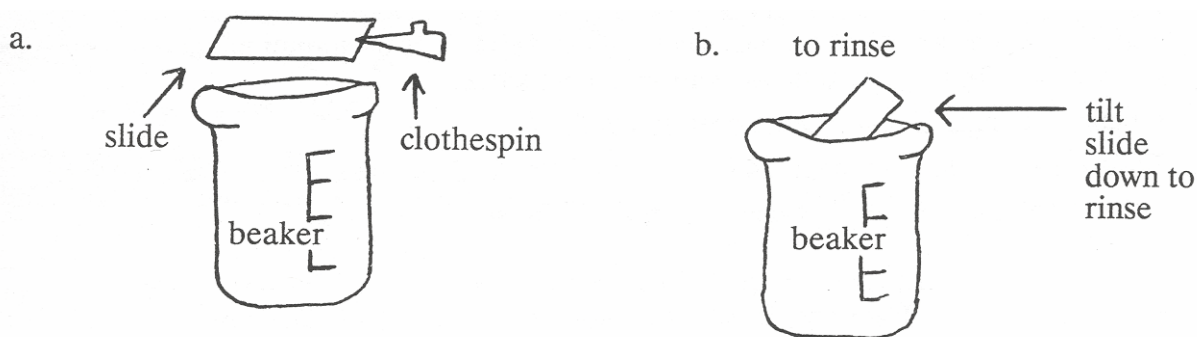
*Objective:* In this experiment students will record colony characteristics of the three pure cultures from their three secondary plates. They will pick their unknown bacterium from one of the plates by looking at crystal violet stained smears.

#### Materials

Bunsen burner	Immersion oil
Striker with flint	Glass slides
Test tube rack	Clothespin
Inoculating loops	Beakers, 400 ml (2 for every 2 students)
Wax pencils	Crystal violet
10% clorox	Wash bottles with dH <sub>2</sub> O
Lens tissue	Paper towels
Windex bottles	Secondary cultures
Microscopes	

#### Procedure

1. Observe your three secondary pure cultures. Record colony characteristics of each pure culture on Table 6.1.
2. Make one smear preparation of a colony from each of your secondary pure cultures. You will make three slides.
3. Follow steps 2 to 10 on pages 29–31 in Claus (1989).
4. Omit step 4b on page 30 in Claus (1989).
5. To stain cells with crystal violet use the following set-up for step 5 on pages 30–31 in Claus (1989).



6. Fill in last two columns of Table 6.1.
7. Discard all slides when done in autoclave can.
8. If you have a slide you want to look at again, gently blot oil off with tissue and reuse.

**Table 6.1.** Colony characteristics and morphology of unknown microorganism.

Colony	Colony			Surface		Prokaryote/ Eukaryote	Shape
	Color	Texture	Edge	Elevation	Appearance		
1							
2							
3							

**Experiment 4: Stock Agar Slants of Unknown Bacterium**

*Objective:* In this experiment students will make four stock agar slants of their unknown bacterium to use in the remaining 23 experiments for the identification, determination of motility, and physiological characteristics of their unknown.

*Materials*

- |                    |                  |
|--------------------|------------------|
| Wax pencils        | 30°C incubator   |
| Bunsen burner      | 4°C refrigerator |
| Striker with flint | 10% clorox       |
| Test tube rack     | Paper towels     |
| Inoculating loops  | Agar slants      |

*Procedure*

1. From the results in Experiment 3 determine which pure culture secondary plates from you soil extract contains an unknown bacterium. Pick one of your bacterium unknown secondary plates to inoculate four agar slants for your unknown stock.
2. Obtain four agar slants. Label agar slants #1–4.
3. Follow steps 1 to 5 on page 144 and steps 12 and 13 on page 145 in Claus (1989).
4. After incubation at 30°C, the agar slants will be stored at 4°C.

**Exercise B: Identification of Unknown Bacterium  
Using Various Staining Techniques**

**Experiment 5: Gram Stain**

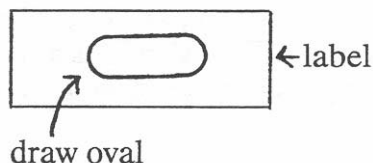
*Objective:* In this experiment students will determine if their unknown bacterium is gram positive (Gm<sup>+</sup>) or gram negative (Gm<sup>-</sup>).

*Materials*

Bunsen burner	Basic fuchsin
Striker with flint	dH <sub>2</sub> O
Test tube rack	Paper towels
Inoculating loop	Beakers
Clothespin	10% clorox
Lens tissue	4°C refrigerator
Immersion oil	Microscopes
Crystal violet	Wax pencils
Gram's iodine	Slide
95% ethanol	Unknown agar slant #1

*Procedure*

1. Obtain two slides.
2. Obtain unknown agar slant #1.
3. Follow procedure for preparing smears just as you did in Experiment 3.
4. *Remember:*



5. Use beaker set up as for Experiment 3.
6. After the slides are heat fixed follow procedure for gram staining steps 4 to 11 on pages 52–53 in Claus (1989). Do step 4, F-2 (dripping ethanol onto the smears) until no more crystal violet comes off; *do not* do step 4, F-1.
7. Fill in Table 6.2 under “gram.”
8. Autoclave slides.
9. Store unknown agar slant #1 at 4°C.

**Table 6.2.** Staining characteristics of unknown bacterium.

Stain	(+/-)	Shape
Gram		
Endospore		
Acid-Fast		
Capsule		

### Experiment 6: Endospore Stain

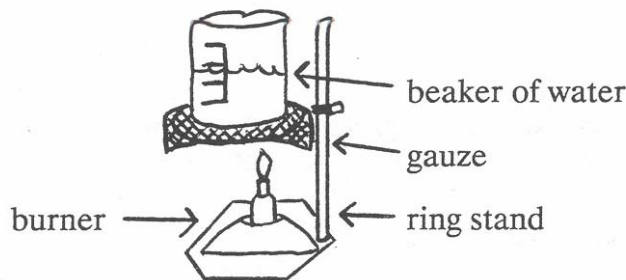
*Objective:* In this experiment students will determine if their unknown bacterium is an endospore former.

*Materials*

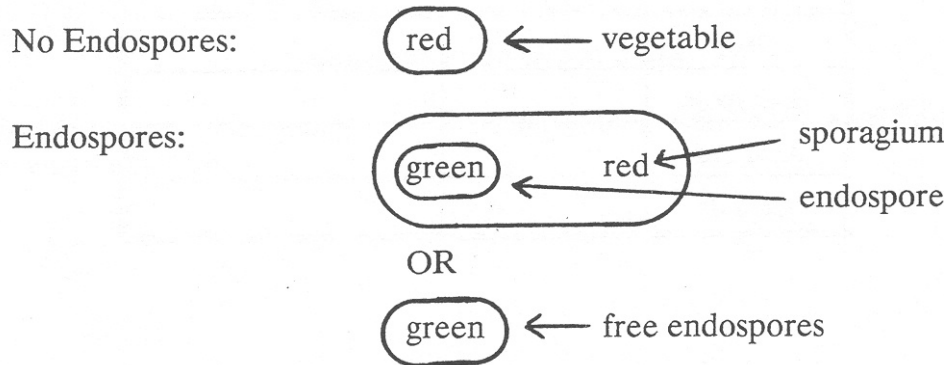
- |                               |                       |
|-------------------------------|-----------------------|
| Bunsen burner                 | Paper towels          |
| Striker with flint            | Malachite green       |
| Test tube rack                | Basic fuchsin         |
| Inoculating loop              | Beakers               |
| Clothespin                    | 10% clorox            |
| Lens tissue immersion oil     | Microscopes           |
| 4°C refrigerator              | Wax pencils           |
| dH <sub>2</sub> O             | Slides                |
| Metal tripods with wire gauze | Unknown agar slant #1 |

*Procedure*

1. Prepare two smears from your agar slant stock unknown #1.
2. For this experiment *do not* use step 4 (a-d) on page 71 in Claus (1989). Instead, set up a beaker water bath and set the slide(s) on top. They should heat a full 5 minutes over the water bath when staining with malachite green. Add more stain as needed.



3. Follow steps 4 to 8 on page 71 in Claus (1989). (*Remember* use water bath; set-up as shown.)
4. *Remember:* Malachite green is hard to get off from skin (it will eventually wear off or alcohol may remove). On clothing, only scissors will remove stain.
5. What you will see:





6. Fill in Table 6.2 under “endospore.”
7. Autoclave slides.
8. Store unknown agar slant #1 at 4°C.

### Experiment 7: Acid-Fast Stain

*Objective:* In this experiment students will determine if their unknown bacterium is acid-fast.

#### *Materials*

Bunsen burner	Methylene blue
Striker with flint	Metal tripods with wire gauze (16 set-ups with beakers)
Test tube rack	Carbol fuchsin
Paper towels	4°C refrigerator
Inoculating loops	Microscopes
Clothespin	Slides
Lens tissue	Wax pencils
Immersion oil	Unknown agar slant #1
dH <sub>2</sub> O	
Acid alcohol	

#### *Procedure*

1. Prepare two smears from your agar slant unknown #1.
2. After the slides are heat fixed follow procedure for acid-fast staining using the water bath set-up used in Experiment 6 (endospores).
3. Follow steps 3, 5, 6, and 7 on pages 60–62 in Claus (1989) using the water bath set-up. Stain with carbol fuchsin over boiling water for 5 minutes *adding additional stain* as needed. Then wash, decolorize, wash, counter-stain, and wash as indicated in lab manual.
4. You are *not doing* step 4 on page 62 in Claus (1989).
5. *Note:* The presence of mycolic acid in acid-fast bacteria often makes the bacteria clump, so when checking for shape try to find single unclumped cells.
6. Fill in Table 6.2 under “acid-fast.”
7. Autoclave slides.
8. Store unknown agar slant #1 at 4°C.

### Experiment 8: Capsule Stain

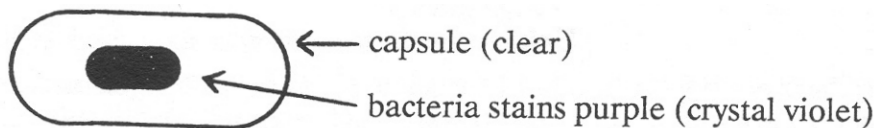
*Objective:* In this experiment students will determine if their unknown bacterium has a capsule.

*Materials*

- |                    |                       |
|--------------------|-----------------------|
| Bunsen burner      | India ink             |
| Striker with flint | dH <sub>2</sub> O     |
| Test tube rack     | 4°C refrigerator      |
| Paper towels       | Microscopes           |
| Inoculating loops  | Slides                |
| Lens tissue        | Wax pencils           |
| Immersion oil      | Unknown agar slant #1 |
| Crystal violet     |                       |

*Procedure*

1. Obtain four slides. You will do a capsule stain using the dry smeared method. Follow steps 2 to 8 on page 93 in Claus (1989). Your instructor will demonstrate the technique.
2. Remember heat will destroy the capsule. *Do not heat fix*. Simple stains will not adhere to the capsule which appears clear against a black (india ink-negative stain) background.



3. Make two capsule stains from your unknown agar slant #1.
4. Make sure india ink is mixed, and that it is coming out of the bottle.
5. Observe under oil. Fill in Table 6.2 under “capsule.”
6. Discard slides to be autoclaved.
7. Autoclave unknown agar slant #1.

**Exercise C: Determination of Motility of Unknown Bacterium**

**Experiment 9: Wet Mounts**

*Objective:* In this experiment students will determine motility of their unknown bacterium by microscopic observation of wet mounts.

*Materials*

- |                    |                       |
|--------------------|-----------------------|
| Bunsen burner      | dH <sub>2</sub> O     |
| Striker with flint | Microscopes           |
| Test tube rack     | 4°C refrigerator      |
| Paper towels       | Wax pencils           |
| Inoculating loops  | Slides                |
| Lens tissue        | Cover slips           |
| Immersion oil      | Unknown agar slant #2 |

*Procedure*

1. Obtain two slides and make wet mounts from your unknown agar slant #2.
2. Follow procedure for agar cultures: steps 1 to 3, and then steps 6 to 8 on pages 41–42 in Claus (1989).
3. *Note:* Make sure the bacterium is truly motile not just exhibiting brownian movement (vibration of bacteria caused by collisions with water molecules; bacteria will appear to shake or vibrate in one spot. This is *not motility*).
4. Record results on Table 6.3 under “wet mounts.”
5. Autoclave slides.
6. Store unknown stock agar slant #2 at 4°C.

**Table 6.3.** Motility determination of unknown bacterium.

	Motility (+/-)
Wet mounts	
Soft agar deeps	
Soft agar plates	

### Experiment 10: Soft Agar Plates; Soft Agar Deep

*Objective:* In this experiment students will confirm their motility observations from their wet mounts (Experiment 9) of their unknown bacterium.

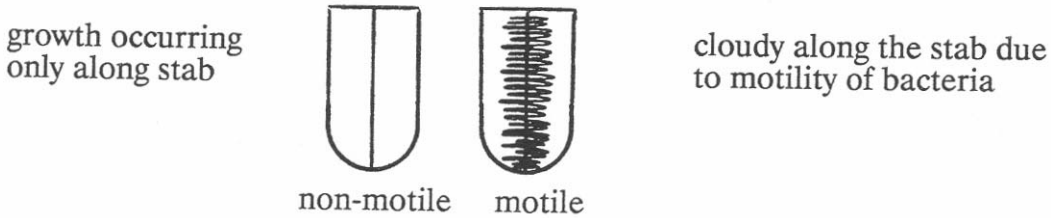
#### Materials

Bunsen burner	Vortexes
Striker with flint	4°C refrigerator
Test tube rack	30°C incubator
Paper towels	Soft agar deeps
Inoculating loops	Soft agar plates
Inoculating needles	TSB, 5 ml tubes

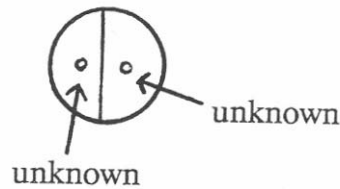
#### Procedure

1. Make a broth of your unknown bacterium.
2. Take a 5 ml sterile tube of TSB. Inoculating with an inoculating loop some bacteria from your unknown agar slant #2. Vortex and use this suspension for your soft agar deep and soft agar plate.
3. Obtain two soft agar deeps. Inoculate each deep with an inoculating needle. Follow steps 7 to 11 on pages 144–145 in Claus (1989).

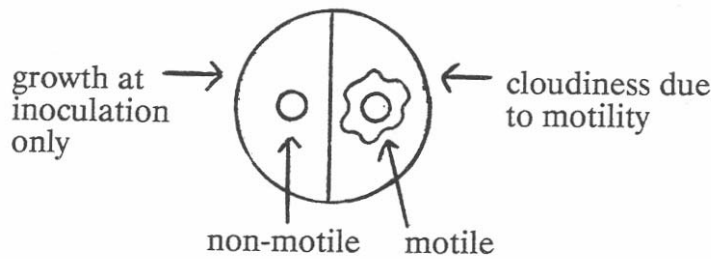
4. Bacteria in soft agar deeps:



- 5. For soft agar plates work in groups of two. Each student will inoculate one side of a soft agar plate using an inoculating loop with their unknown broth.
- 6. Follow steps 4 and 5 on page 83 in Claus (1989).



7. Bacteria on soft agar plates:



- 8. Record results on Table 6.3 under “soft agar deeps plates.”
- 9. Autoclave tubes and plates.
- 10. Store unknown agar slant #2 at 4°C.

**Exercise D: Physiological Characteristics of Unknown Bacterium**

**Experiment 11: Oxygen Requirements**

*Objective:* In this experiment students will determine the oxygen requirements of their unknown bacterium.

*Materials*

Bunsen burner	Anaerobic jars
Striker with flint	30°C incubator
Test tube rack	4°C refrigerator
Inoculating loops	TSB (5 ml tubes)
Paper towels	Thioglycollate (6 ml tubes)
Vortexes	TSA
10% clorox	Unknown agar slant #2
Wax pencils	

*Procedure*

1. Obtain two TSA plates.
2. You will need to make a broth culture of your unknown. Inoculate 5 ml of TSB with a loopfull of your unknown vortex.
3. Work in groups of two. Each student will inoculate each plate with their unknown broth using a continuous streak.



unknown      unknown

4. Place one plate inverted in the bin to be incubated at 30°C aerobically for 48 hours and then store at 4°C.
5. Place the second plate inverted in the bin to be incubated at 30°C anaerobically for 48 hours and then stored at 4°C.
6. Obtain one tube of thioglycollate broth.
7. Without shaking the tube, inoculate with a loopfull from your unknown broth.
8. Place the tubes in the rack. The tubes will be incubated at 30°C for 48 hours, then stored at 4°C.
9. Store unknown agar slant #2 at 4°C.
10. Obtain your two plates and tubes. Record results in Table 6.4.
11. Autoclave plates and tube.

**Table 6.4.** Oxygen requirements of unknown bacterium (obligate aerobe, facultative anaerobe, or obligate anaerobe).

Medium	Oxygen requirement
TSA	
Thioglycollate	

### Experiment 12: Growth on Selective and Differential Media

*Objective:* In this experiment students will determine if their unknown bacterium will grow on nutrient agar, high-salt agar, and mannitol-salt agar.

*Materials*

- |                    |                              |
|--------------------|------------------------------|
| Bunsen burner      | Wax pencils                  |
| Striker with flint | 30°C incubator               |
| Test tube rack     | 4°C refrigerator             |
| Paper towels       | TSB (5 ml tubes)             |
| Inoculating loops  | Nutrient agar plates         |
| Paper towels       | High-salt (7.5%) agar plates |
| Vortexes           | Mannitol-salt agar plates    |
| 10% clorox         | Unknown agar slant #2        |

*Procedure*

1. Obtain three plates: nutrient agar, high-salt agar, and mannitol-salt agar.
2. Make a broth of your unknown by inoculating 5 ml of TSB, and vortex.
3. Work in groups of three. Each group will streak three plates: nutrient agar, high-salt agar, and mannitol-salt agar. Each student will streak one-third of the latter plates with their unknown broth.
4. Divide the plates into thirds, and inoculate one-third of the plate with your broth unknown. Label as shown below.



3 different unknowns from your group

5. Inoculate with a continuous streak, invert, tape, and incubate at 30°C for 48 hours, and then stored at 4°C.
6. Store unknown stock agar slant #2 at 4°C.
7. Obtain your three plates. Record results on Table 6.5.
8. Autoclave plates.

**Table 6.5.** Growth of unknown bacterium on selective and differential media.

Medium	Growth (+/-)	Mannitol fermentation (+ yellow/- red [orange])
Nutrient		not applicable
High-salt		not applicable
Mannitol-salt		

### Experiment 13: Temperature Optimum

**Objective:** In this experiment students will determine the temperature optimum of their unknown bacterium.

#### Materials

Bunsen burner	Wax pencils
Striker with flint	30°C incubator
Test tube rack	4°C refrigerator
Paper towels	TSB (5 ml tubes)
Inoculating loops	TSA plates
Paper towels	Unknown agar slant #2
Vortexes	37°C, 55°C incubator
10% clorox	

#### Procedure

1. Make a broth of your unknown by inoculating 5 ml of TSB, and vortex.
2. Work in groups of three. Each group will streak three TSA plates. Each student in the group will inoculate one-third of one plate (25°C, 37°C, or 55°C) with their unknown broth.
3. Divide each plate into thirds, and inoculate one-third of the plate with your broth unknown. Label as shown below.



4. Inoculate with a continuous streak.
5. Invert, and place plates in the right incubator (37°C, 55°C). Place the 25°C plate on shelf. These will be incubated for 48 hours, and then stored at 4°C.
6. Store unknown stock agar slant #2 at 4°C.
7. Obtain your three plates. Record results on Table 6.6.
8. Autoclave plates.

**Table 6.6.** Temperature optimum of unknown bacterium.

Temperature (°C)	Growth*	Color of colony
25		
37		
55		

\*Growth = - no growth; +/- faint growth; + definite growth.

### Experiment 14: Osmotic Effects

*Objective:* In this experiment the students will determine their unknown bacterium's tolerance for increasing concentrations of NaCl.

*Materials*

- |                    |                            |
|--------------------|----------------------------|
| Bunsen burner      | Wax pencils                |
| Striker with flint | 30°C incubator             |
| Test tube rack     | 4°C refrigerator           |
| Paper towels       | TSB (5 ml tubes)           |
| Inoculating loops  | TSA plates with 0.5% NaCl  |
| Paper towels       | TSA plates with 5.0% NaCl  |
| Vortexes           | TSA plates with 20.0% NaCl |
| 10% clorox         | Unknown agar slant #2      |

*Procedure*

1. Make a broth of your unknown by inoculating 5 ml of TSB, and vortex.
2. Work in groups of three. Each group will streak three plates. Each student in the group will inoculate one-third of the plate (0.5 [regular TSA], 5.0, or 20% NaCl) with their unknown broth organism.
3. Divide each plate into thirds, and inoculate one-third of the plate with your broth unknown. Label as shown below.



4. Inoculate with a single streak.
5. Invert and tape the three plates and incubate at 30°C for 48 hours and then stored at 4°C.
6. Autoclave unknown stock agar slant #2.
7. Obtain your three plates. Record results on Table 6.7.
8. Autoclave plates.

**Table 6.7.** Unknown bacterium's tolerance for increasing concentrations of NaCl.

NaCl	Growth*
0.5%	
5.0%	
20%	

\*Growth = - no growth; +/- faint growth; + definite growth.



## Experiment 15: pH Optimum

*Objective:* In this experiment the students will determine the pH optimum for the growth of their unknown bacterium.

### *Materials*

Bunsen burner	TSA plates with 0.5% NaCl
Striker with flint	TSA plates with 5.0% NaCl
Test tube rack	TSA plates with 20.0% NaCl
Paper towels	Unknown agar slant #2
Inoculating loops	Parafilm squares
Paper towels	Spectrophotometers
Vortexes	TSB (5 ml tubes)
10% clorox	TSB (5 ml tubes), pH 5
Wax pencils	TSB (5 ml tubes), pH 7
30°C incubator	TSB (5 ml tubes), pH 9
4°C refrigerator	Unknown stock agar slant #3
TSB (5 ml tubes)	

### *Procedure*

1. Make a broth of your unknown by inoculating 5 ml of TSB, and vortex.
2. Each student will inoculate three tubes (pH 5, pH 7, and pH 9) with their broth unknown.
3. Incubated tubes at 30°C for 48 hours, then stored at 4°C.
4. Obtain tubes.
5. You will be using a spectrophotometer to measure the amount of turbidity in a suspension. Remember turbidity indicates the total number of bacteria in a suspension (both viable and non-viable). A beam of light is transmitted through a bacterial suspension, and a light sensitive detector measures the amount of light transmitted or absorbed by a bacterial suspension.
6. Remove all caps from your tubes and place a parafilm square on top and completely seal the tube. This is to prevent leaking when you invert each tube to mix.
7. Make sure wavelength is set at 600 nm.
8. Adjust to 0% T (transmittance) without a blank. No light is being transmitted to the phototube.
9. Adjust to 100% T with a blank (sterile TSB). Wipe the outside of tube with a Kimwipe and invert to mix. Place cuvet into the sample holder and shut lid. Adjust to 100%. Now 100% of the light leaving the light source is being transmitted to the phototube.
10. Remove blank, the T should go back to 0%. If not, do steps 6 and 7 again.
11. Read each of your tubes:
  - (a) Invert to mix by placing finger over parafilm and inverting.
  - (b) Wipe outside of tube with Kimwipe.
  - (c) Place in holder and close the lid.
  - (d) Push button that says absorbance this will give you a direct idea of the concentration of bacteria in your suspension, since the greater the number of bacteria there is in a suspension the more light is absorbed (less transmitted).

- (e) Check periodically to see that the % T still goes back to zero when no cuvette is in sample holder.
- (f) Record results on Table 6.8.
- 12. Store agar slant unknown #3 at 4°C.
- 13. Autoclave tubes.

**Table 6.8.** pH optimum of unknown bacterium.

pH	Absorbance
5	
7	
9	

**Experiment 16: Degradation of Polysaccharides**

*Objective:* In this experiment students will determine if their unknown bacterium hydrolyzes starch.

*Materials*

- |                    |                       |
|--------------------|-----------------------|
| Bunsen burner      | Vortexes              |
| Striker with flint | Wax pencils           |
| Test tube rack     | TSB (5 ml tubes)      |
| Inoculating loop   | 30°C incubator        |
| Paper towels       | 4°C refrigerator      |
| dH <sub>2</sub> O  | Starch agar plates    |
| 10% clorox         | Unknown agar slant #3 |

*Procedure*

1. Make a broth of your unknown by inoculating 5 ml of TSB, and vortex.
2. Work in groups of three. Obtain one starch agar plate. Divide plate into thirds; see step 1 on page 243 in Claus (1989). Each student will inoculate one-third of plate with their unknown broth.
3. Follow steps 1 to 3 on pages 243–244 in Claus (1989).
4. Incubate 30°C for 48 hours, and then store at 4°C.
5. Store unknown stock agar slant #3 at 4°C.
6. Obtain your plate. Record results on Table 6.9 under “starch hydrolysis”.
7. Autoclave plates.

**Table 6.9.** Physiological characteristics of unknown bacterium (Part 1).

	Hydrolysis (+/-)
Starch <sup>1</sup>	
Casein <sup>2</sup>	
Gelatin <sup>3</sup>	
Phospholipid <sup>4</sup>	

1. + clear zone after iodine; - brown color after iodine.
2. + clear zone; - no change.
3. + remain liquid on ice after 30 minutes; - solid on ice after 30 minutes.
4. + cloudy zone; - no change.

### Experiment 17: Degradation of Proteins

*Objective:* In this experiment the students will determine if their unknown bacterium hydrolyzes casein, and gelatin.

#### Materials

Bunsen burner	Wax pencils
Striker with flint	4°C refrigerator
Test tube rack	Beakers
Inoculating loop	Ice
Paper towels	Gram's iodine
dH <sub>2</sub> O	Skim milk agar
10% clorox	TSB + 4% gelatin tubes (9 ml)
Vortexes	Unknown agar slant #3

#### Procedure

1. You will do Part A and B.
2. Make a broth of your unknown by inoculating 5 ml of TSB, and vortex.
3. *Part A:* Work in groups of three. Obtain one skim milk agar plate. Divide plate into thirds; see step 2 on page 249 in Claus (1989). Each student will inoculate one-third of the plate with their unknown broth.
4. Follow steps 1 to 3 on pages 249–250 in Claus (1989).
5. Incubate plates at 30°C for 48 hours and then stored at 4°C.
6. *Part B:* Each student will inoculate one TSB + 4% gelatin tubes with their unknown broth.
7. Follow steps 1 and 2 on page 252 in Claus (1989).
8. Place tubes in rack and incubate at 30°C for 48 hours, and then stored at 4°C.
9. Store unknown stock agar slant #3 at 4°C.
10. Obtain your skim milk agar plate, and your TSB + 4% gelatin tubes.
11. For plates: Follow steps 1 and 2 on page 250 in Claus (1989).

12. Fill in Table 6.9 under “casein hydrolysis.”
13. For tubes: Follow steps 1 to 3 on page 252 in Claus (1989). Use a beaker of ice to put your tubes for 30 minutes.
14. Record results in Table 6.9 under “gelatin hydrolysis.”
15. Autoclave tubes and plates.

### Experiment 18: Degradation of Lipids

*Objective:* In this experiment the students will determine if their unknown bacterium hydrolyzes phospholipids.

#### *Materials*

Bunsen burner	10% clorox
Striker with flint	Vortexes
Test tube rack	Wax pencils
Inoculating loop	4°C refrigerator
Paper towels	Egg yolk agar plates
dH <sub>2</sub> O	Unknown agar slant #3

#### *Procedure*

1. Make a broth of your unknown by inoculating 5 ml of TSB, and vortex.
2. Work in groups of four. Obtain one egg yolk agar plate. Divide plate into fourths; see step 1 on page 259 in Claus (1989). Each student will inoculate one-fourth of the plate with their unknown broth by placing a loopfull of each of the following organisms in the center of each of the following organisms in the center of each section; see step 1c on page 259 in Claus (1989).
3. Follow steps 1 and 2 on page 259 in Claus (1989).
4. Invert plates and incubate at 30°C for 48 hours, then stored at 4°C.
5. Store unknown stock agar slant #3 at 4°C.
6. Obtain your egg yolk agar plates.
7. Follow steps 1 and 2 on pages 259–260 in Claus (1989).
8. Record results in Table 6.9 under “phospholipid hydrolysis.”
9. Autoclave plates.

### Experiment 19: Utilization of Citrate

*Objective:* In this experiment students will determine if their unknown bacterium metabolizes citrate.

*Materials*

- Bunsen burner
- Striker with flint
- Test tube rack
- Inoculating loop
- Paper towels
- dH<sub>2</sub>O
- 10% clorox

- Vortexes
- Wax pencils
- 4°C refrigerator
- Simmon's Citrate Agar Plates
- 0.85% NaCl (5 ml tubes)
- Unknown agar slant #3

*Procedure*

1. *Note:* We will use Simmon's citrate agar plates, not slants.
2. Make a *slightly turbid suspension* from your unknown slant. Follow steps 1 to 3 on pages 264–265 in Claus (1989).
3. *Note:* You will use sterile 0.85% NaCl not distilled H<sub>2</sub>O to make a slightly turbid suspension of organisms; step 1 on page 264 in Claus (1989). Work in groups of two. Each group will inoculate one Simmon's citrate agar plate. Each student will inoculate one-half of the plate with their unknown using a single streak.



4. Incubate inverted plates at 30°C for 48 hours, then stored at 4°C.
5. Store unknown stock agar slant #3 at 4°C.
6. Obtain your plate.
7. Record results on Table 6.10 under “citrate utilization.”
8. Autoclave plates.

**Table 6.10.** Physiological characteristics of unknown bacterium (Part 2).

	(+/-)
Citrate utilization <sup>1</sup>	
Indole production <sup>2</sup> (1% tryptone)	
Indole production <sup>2</sup> (1% tryptone + 1% glucose)	
Urea hydrolysis <sup>3</sup>	

1. + blue; - green.
2. + red in upper level; - other colors in upper level.
3. + red; - orange (or any other color).

### Experiment 20: Indole Production from Tryptophan

*Objective:* In this experiment students will determine if their unknown bacterium metabolizes tryptophan.

*Materials*

Bunsen burner	Wax pencils
Striker with flint	4°C refrigerator
Test tube rack	Pasteur pipet (0.5 ml)
Inoculating loop	0.85% NaCl (5 ml)
Paper towels	Kovac's reagent
dH <sub>2</sub> O	1% tryptone broth (5 ml)
10% clorox	1% tryptone broth + 1% glucose (5 ml)
Vortexes	

*Procedure*

1. Obtain a tube of 1% tryptone broth, and a tube of 1% tryptone broth + 1% glucose. (*Note:* We are using 1% glucose, not 5%.)
2. Make a slightly turbid suspension from your unknown agar slant by inoculating 5 ml of 0.85% NaCl. Vortex.
3. Follow step 1 on page 293 in Claus (1989).
4. Place tubes in rack to be incubated at 30°C for 48 hours, then stored at 4°C.
5. Store unknown stock agar slant #3 at 4°C.
6. Obtain your tubes of 1% tryptone broth and 1% glucose.
7. Follow steps 1 and 2 on pages 293–294 in Claus (1989).
8. Record your results in Table 6.10 under “indole production.”
9. Autoclave tubes.

### Experiment 21: Urea Hydrolysis

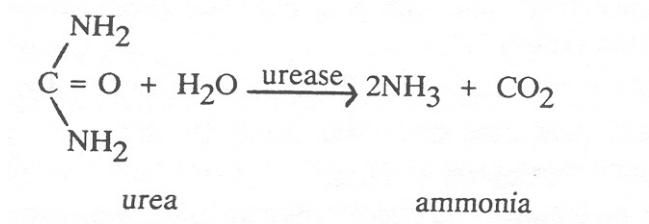
*Objective:* In this experiment students will determine if their unknown bacterium hydrolyzes urea.

*Materials*

Bunsen burner	10% clorox
Striker with flint	Vortexes
Test tube rack	Wax pencils
Inoculating loop	4°C refrigerator
Paper towels	Urea broth (3 ml tubes)
dH <sub>2</sub> O	Unknown agar slant #4

*Procedure*

1. Make a broth of your unknown by inoculating 5 ml of TSB, and vortex.
2. To distinguish between the Gm<sup>-</sup> pathogens and non-pathogens bacteria of the intestines (*Proteus*), one looks for the production of urease an enzyme which splits ammonia off of the urea molecule. Urease is *not* produced by Gm<sup>-</sup> pathogens, but *is* a characteristic of *Proteus* (non-pathogenic):



3. Urea broth contains yeast extract, and urea. To indicate pH changes phenol red has been added.
4. Obtain one tube of urea broth inoculate with your unknown broth.
5. Place tubes in rack and incubate at 30°C for 48 hours and stored at 4°C.
6. Store unknown stock agar slant #4 at 4°C.
7. Obtain your tubes.
8. If an organism produces urease, the NH<sub>3</sub> released raises the pH. As the pH becomes higher, the phenol red changes from yellow (pH 6.8) to red (pH > 8.1).
9. Examine your tubes for a red color indicating, the hydrolysis of urea. Any color other than red is negative for the hydrolysis of urea (orange is negative).
10. Record results on Table 6.10 under “urea hydrolysis.”
11. Autoclave tubes.

**Experiment 22: Sugar Fermentation**

*Objective:* In this experiment students will determine if their unknown bacterium will ferment sucrose, lactose, or glucose with production of acid or both acid and gas.

*Materials*

Bunsen burner	30°C incubator
Striker with flint	4°C refrigerator
Test tube rack	Inoculating loops
Paper towels	0.85% NaCl (2 ml tubes)
dH <sub>2</sub> O	Tubes of glucose fermentation (9 ml)
10% clorox	Tubes of sucrose fermentation (9 ml)
Wax pencils	Tubes of lactose fermentation (9 ml)
Vortex	Unknown agar slant #4

*Procedure*

1. Obtain your agar slant unknown stock #4.
2. Inoculate 2 ml of 0.85% NaCl to make a *broth* of your agar slant unknown stock-#4, and vortex.
3. Obtain three fermentation tubes of each type: one glucose, one lactose, and one sucrose. *Be careful* not to *shake* tubes since this will introduce bubbles into the Durham tubes.
4. Inoculate each fermentation tube with your unknown broth from step 1. Follow steps 1 and 2 on page 273 in Claus (1989).
5. Place tubes in racks to be incubated at 30°C for 48 hours, and stored at 4°C.
6. Store unknown stock agar slant #4 at 4°C.
7. Obtain your unknown fermentation tubes.
8. Follow step 2 to 4 on page 273 in Claus (1989), also read interpretation of results on page 273, and test summary on page 274.
9. Record results in Table 6.11.
10. Autoclave tubes.

**Table 6.11** Physiological characteristics of unknown bacterium (Part 3).

Sugar	Growth (+/-)	Acid* (+/-)	Gas (+/-)
Glucose			
Lactose			
Sucrose			

\* Acid = + yellow; - any other color (orange).

**Experiment 23: Mixed-Acid Fermentation; Butylene Glycol Fermentation**

*Objective:* In this experiment students will determine the type of glucose fermentation their unknown bacterium exhibits (if it ferments glucose).

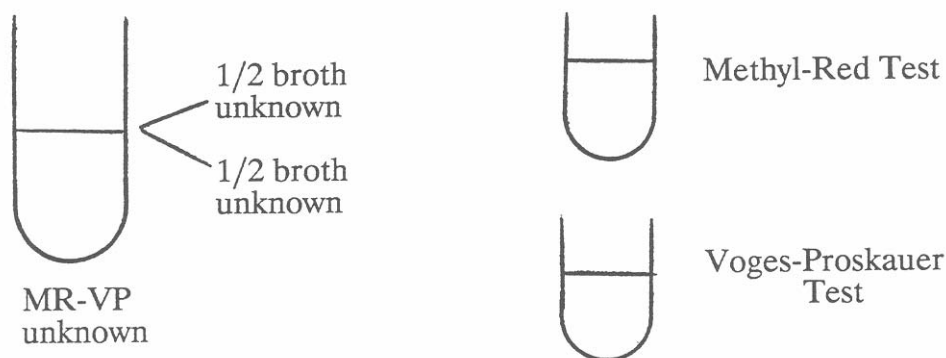
*Materials*

- |                    |                                   |
|--------------------|-----------------------------------|
| Bunsen burner      | Methyl-red reagent (droppers)     |
| Striker with flint | Pasteur pipettes (non-sterile)    |
| Test tube rack     | Alpha-naphthol reagent (droppers) |
| Paper towels       | KOH solution (0.2 ml droppers)    |
| dH <sub>2</sub> O  | Inoculating loops                 |
| 10% clorox         | Clean test tubes (non-sterile)    |
| Wax pencils        | 0.85% NaCl (2 ml tubes)           |
| Vortex             | MR-VP broths (9 ml)               |
| 30°C incubator     | Unknown agar slant #4             |
| 4°C refrigerator   |                                   |



### Procedure

1. Obtain your agar slant unknown stock #4.
2. Inoculate 2 ml of 0.85% NaCl to make a broth of your unknown and vortex.
3. Inoculate a tube of MR-VP broth with a loopfull of inoculum from your unknown broth.
4. Place tube in rack to be incubated at 30°C for 48 hours, and stored at 4°C.
5. Store unknown stock agar slant #4 at 4°C.
6. Obtain your unknown MR-VP broth.
7. Obtain one clean test tubes follow step 1 on page 279 in Claus (1989) and using a pasteur pipet and bulb to transfer half of the MR-VP broth inoculated with your unknown. Instructor will demonstrate. Also, obtain two uninoculated MR-VP broth to serve as controls.
8. *Note:* Use only one tube containing half of the MR-VP broth (inoculated with unknown) for methyl-red test, and the other tube with half of the MR-VP broth (inoculated with unknown) for the Voges-Proskauer test.



9. Methyl-red test: Follow steps 2 and 3 on pages 279–280 in Claus (1989). *Remember* to run control tube at the same time.
10. Record results in Table 6.12 under “methyl-red.”
11. Voges-Proskauer test: Follow steps 4 to 8 on pages 280–281 in Claus (1989) using the other one-half of MR-VP broth (inoculated with unknown). *Remember* to run a control tube at the same time.
12. Record results in Table 6.12 under “acetoin production.”
13. Autoclave tubes.

**Table 6.12.** Physiological characteristics of unknown bacterium (Part 4).

	(+/-)
Methyl-red <sup>1</sup>	
Acetoin production <sup>2</sup>	
H <sub>2</sub> S production <sup>3</sup>	
Oxidase <sup>4</sup>	
Catalase <sup>5</sup>	
Nitrate reduction <sup>6</sup>	

1. + red; - yellow (or any other color).
2. + red; - no red.
3. + black precipitate; - no black precipitate.
4. + deep violet/purple; - no color/light color.
5. + bubbles of oxygen; - no bubbles of oxygen.
6. + red; - clear.

### Experiment 24: H<sub>2</sub>S Production

*Objective:* In this experiment students will determine if their unknown bacterium metabolizes sulfur containing compounds to produce H<sub>2</sub>S.

#### *Materials*

Bunsen burner	Vortex
Striker with flint	30°C incubator
Test tube rack	4°C refrigerator
Paper towels	Inoculating needles
dH <sub>2</sub> O	Peptone iron agar deeps (9 ml)
10% clorox	Unknown agar slant #4
Wax pencils	

#### *Procedure*

1. Inoculate a peptone iron agar deep with your agar slant unknown stock #4. Inoculate using an inoculating needle picking a small amount of your unknown off your slant, and stabbing into the deep; follow steps 1 to 3 on page 288 in Claus (1989).
2. Place tube in rack to be incubated at 30°C for 48 hours, and stored at 4°C.
3. Store unknown stock agar slant #4 at 4°C.
4. Obtain your unknown peptone iron agar deep.
5. Follow steps 1 to 3 on page 288 in Claus (1989).
6. Record results in Table 6.12 under “H<sub>2</sub>S production.”
7. Autoclave tubes.

### Experiment 25: Oxidase and Catalase Activity

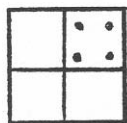
*Objective:* In this experiment students will determine the presence of cytochrome C (oxidase +) and catalase in their unknown bacterium (which carry out aerobic respiration).

#### Materials

Bunsen burner	30°C incubator
Striker with flint	4°C refrigerator
Test tube rack	Inoculating loop
Paper towels	Sterile tooth picks
dH <sub>2</sub> O	3% H <sub>2</sub> O <sub>2</sub> with droppers
10% clorox	Oxidase cards
Wax pencils	Slides
Vortex	Unknown agar slant #4

#### Procedure

1. Obtain one oxidase card to be used by 16 students.
2. Use a sterile toothpick (false positive if use metal loops). Inoculate and smear your unknown bacterium (from slant #4) in one corner of one of the four squares.



4 different unknowns

3. Check color of smear exactly 20 seconds after rubbing the cells on the card.
4. See interpretation of results “Cytochrome C (oxidase) Test Summary”; page 306 in Claus (1989).
5. Record results in Table 6.12 under “oxidase.”
6. Catalase test: Inoculate a slide with your unknown agar slant #4.
7. Follow steps 2 to 4 on page 307 in Claus (1989); we *will not* use a microscope.
8. Record results in Table 6.12 under “catalase.”

### Experiment 26: Reduction of Nitrate

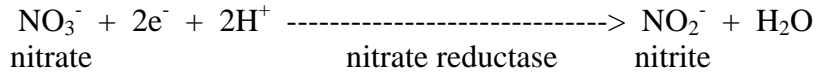
*Objective:* In this experiment students will determine if their unknown bacterium reduces nitrate.

#### Materials

Bunsen burner	30°C incubator
Striker with flint	4°C refrigerator
Test tube rack	Inoculating loop
Paper towels	Solution A (with droppers)
dH <sub>2</sub> O	Solution B (with droppers)
10% clorox	Nitrate broth (2 ml)
Wax pencils	Unknown agar slant #4
Vortex	

*Procedure*

1. A number of facultative bacteria can use oxygen in nitrate as a H<sup>+</sup> acceptor, thus converting nitrate. The reaction requires the enzyme nitratase.



2. Obtain 2 ml of nitrate broth inoculate broth with your agar slant unknown stock #4 and vortex.
3. Place tubes in rack. Incubate at 30°C for 24 hours, then stored at 4°C.
4. Autoclave unknown stock agar slant #4.
5. Obtain nitrate tubes.
6. Add 2–3 drops of nitrate test solution A (sulfanilic acid) and an equal amount of solution B (dimethyl alpha naphthalamine) to the nitrate broth of your unknown. A red color should appear immediately to indicate a positive reaction. If no red color develops, your unknown is negative for the nitratase enzyme necessary for nitrate reduction.
7. Record results in Table 6.12 under “nitrate reduction.”
8. Autoclave tubes.

**Experiment 27: Litmus Milk Test**

*Objective:* In this experiment students will interpret a number of results from the metabolism of various compounds found in milk.

*Materials*

Bunsen burner	Vortex
Striker with flint	30°C incubator
Test tube rack	4°C refrigerator
Paper towels	Inoculating loop
dH <sub>2</sub> O	0.85% NaCl (2 ml tubes)
10% clorox	Litmus milk (9 ml)
Wax pencils	Unknown agar slant #4

*Procedure*

1. Obtain you agar slant unknown stock #4.
2. Inoculate 2 ml of 0.85% NaCl to make a broth of your unknown vortex.
3. Inoculate a tube of litmus milk with a loopfull of your unknown broth.
4. Place tube in rack to be incubated at 30°C for 1 week.
5. Store unknown agar slant #4 at 4°C.
6. Obtain your unknown litmus milk tube and one uninoculated control tube to compare results to.
7. Follow step 1 on page 299 in Claus (1989).
8. Record your results in Table 6.13.
9. Autoclave tubes.

**Table 6.13.** Physiological characteristics of unknown bacterium (Part 5).

	(+/-)
Acid <sup>1</sup>	
Litmus reaction <sup>2</sup>	
Curd formation <sup>3</sup>	
Alkaline <sup>4</sup>	
Proteolysis <sup>5</sup>	
No change <sup>6</sup>	

1. + pink; - light blue/gray.
2. + white; - light blue/gray.
3. + solid chunks; - no solid chunks.
4. + dark amber color; - light blue/gray.
5. + decrease turbidity (watery); - no decrease in turbidity.
6. + similar to uninoculated tubes; - not similar to uninoculated tubes.

### Notes for the Instructor

Most of the students really enjoy trying to identify their unknown bacterium. I always keep extra media and often times students come in on their own time to repeat tests on their unknown to confirm results.

I run 11 3-hour labs during the semester (not including two labs set aside for the practicums). In these 11 lab sessions all of the aforementioned experiments are run along with nine additional experiments (which are not done with their unknown bacterium).

#### Experiment 1

Student's primary cultures contain a majority of *Bacillus* colonies spreading often times over other types of colonies. I try to look at all the primary plates with my students, and suggest picking other colonies (beside *Bacillus*) that look bacterial. Some students have no choice but to use some of the *Bacillus* type colonies for their secondary cultures.

#### Experiment 2

Student's secondary cultures should be examined individually for purity. If you have room, you may want to keep their secondary plate that they have chosen for their unknown. (Store at 4°C.) At least one or two students will find they have a mixed culture after doing several experiments. If they have their original secondary plate, then they can make new agar slants.

#### Experiment 3

Controls: *Staphylococcus epidermidis*, *Saccharomyces cerevisiae*, *Bacillus cereus*, *Pseudomonas fluorescens*, *Micrococcus luteus*. I have had a few students with pleomorphic-shaped unknown bacterium. These cultures appear to be pure and have proven to be quite challenging.

*Experiment 5*

Controls: *Escherichia coli* (Gm<sup>-</sup>), *Bacillus cereus* (Gm<sup>+</sup>), *Mycobacterium smegmatis* (variable), *Staphylococcus epidermidis* (Gm<sup>+</sup>).

*Experiment 6*

Controls: *Bacillus subtilis* (endospores), *Escherichia coli* (no endospores).

*Experiment 7*

Controls: *Mycobacterium smegmatis* (acid-fast), *Bacillus cereus* (non-acid-fast), *Mycobacterium phlei* (acid-fast).

*Experiment 8*

Controls: *Flavobacterium capsulatum* (capsule), *Staphylococcus epidermidis* (no capsule).

*Experiment 9*

Controls: *Proteus mirabilis* (motile), *Staphylococcus epidermidis* (non-motile). I find that students have a hard time finding their organisms on wet mounts using the oil immersion lens (100X). The more they do wet mounts the better they get. It just takes practice.

*Experiment 10*

Controls: *Proteus mirabilis* (motile), *Staphylococcus epidermidis* (non-motile). If the soft agar plates are incubated too long, *P. mirabilis* will spread over the entire plate.

*Experiment 11*

Controls: *Escherichia coli* (facultative anaerobe), *Micrococcus luteus* (obligate aerobe), *Clostridium sporogenes* (obligate anaerobe). As the thioglycollate tubes get handled, and the microorganisms tend to settle, the results may appear misleading.

*Experiment 12*

Controls: Mannitol-salt: *Staphylococcus aureus* (+ growth, + acid); *Staphylococcus epidermidis* (+ growth, - acid); *Escherichia coli* (- growth, - acid); *Pseudomonas fluorescens* (- growth, - acid). Nutrient: All organisms grow. High-salt: Only *S. epidermidis* and *S. aureus* grow.

*Experiment 13*

Controls (optimal temperature): *Escherichia coli* (37°C), *Bacillus stearothermophilis* (55°C), *Serratia marcescens* (25°C, red; 37°C, white).

*Experiment 14*

Controls: *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*. Note: No organisms grow at 20% NaCl; best growth at 0.5% NaCl. A few students have had unknown halophilic bacterium that grew well at 20% NaCl.

*Experiment 15*

Controls (optimal pH): *Lactobacillus brevis* (pH 5), *Escherichia coli* (pH 7), *Alcaligenes viscolactis* (pH 9).

*Experiment 16*

Controls: *Escherichia coli* (- zone of hydrolysis), *Bacillus cereus* (+ zone of hydrolysis).

*Experiment 17*

Controls: *Escherichia coli* (- casein hydrolysis/gelatin hydrolysis), *Bacillus cereus* (+ casein hydrolysis/gelatin hydrolysis).

*Experiment 18*

Controls: *Bacillus cereus* (+ phospholipid hydrolysis), *Staphylococcus epidermidis* (- phospholipid hydrolysis).

*Experiment 19*

Controls: *Escherichia coli* (- citrate utilization/growth), *Enterobacter aerogenes* (+ citrate utilization/growth).

*Experiment 20*

Controls: *Escherichia coli* (+ 1% tryptone; - [1% tryptone + 1% glucose]), *Enterobacter aerogenes* (- both tubes).

*Experiment 21*

Controls: *Proteus vulgaris* (+ urea hydrolysis), *Staphylococcus epidermidis* (- urea hydrolysis).

*Experiment 22*

Controls:

Bacteria	Sugar	Growth	Acid	Gas
<i>Escherichia coli</i>	Glucose	+	+	+
	Lactose	+	+	+
	Sucrose	+	-	-
<i>Streptococcus faecalis</i>	Glucose	+	+	-
	Lactose	+	+	-
	Sucrose	+	-	-
<i>Proteus mirabilis</i>	Glucose	+	+	+
	Lactose	+	-	-
	Sucrose	+	-	-

*Experiment 23*

Controls: *Enterobacter aerogenes* (- methyl-red; + acetoin production), *Escherichia coli* (+ methyl-red; - acetoin production).

*Experiment 24*

Controls: *Escherichia coli* (- H<sub>2</sub>S), *Proteus mirabilis* (+ H<sub>2</sub>S).

*Experiment 25*

Controls: *Escherichia coli* (- oxidase), *Pseudomonas fluorescens* (+ oxidase), *Staphylococcus epidermidis* (+ catalase), *Lactobacillus brevis* (- catalase).

*Experiment 26*

Controls: *Escherichia coli* (+ nitrate reduction), *Micrococcus luteus* (- nitrate reduction).

*Experiment 27*

Controls:

Bacteria	A	LR	CF	Alkaline	P	No change
<i>Lactobacillus brevis</i>	+	+	+	-	-	-
<i>Escherichia coli</i>	+	-	-	-	-	-
<i>Bacillus cereus</i>	-	-	-	+	+	-
<i>Proteus mirabilis</i>	-	-	-	-	-	+

**Acknowledgments**

I would like to thank Dr. Charles Deutch for his help and expertise in developing this course. I greatly appreciate the time and effort spent by Karen Chodikov in typing and editing this manuscript, and to Bryant Hess for all media preparation and laboratory set up.

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APPENDIX  
*Media, Stains, and Reagents*

**Media** (Claus, 1989)

Egg Yolk Agar  
Glucose Broth with Phenol Red  
High-Salt (7.5%) Agar  
Lactose Broth with Phenol Red  
Litmus Milk Medium  
Mannitol Salt Agar  
Methyl-Red/Voges-Proskauer (MR-VP Broth)  
Nutrient Agar  
Peptone Iron Agar  
Simmon's Citrate Agar  
Skim Milk Agar  
Starch Agar  
Sucrose Broth with Phenol Red  
Trypticase Soy Agar (TSA)  
Trypticase Soy Agar with NaCl (0.5%, 5%, and 20%)  
Trypticase Soy Broth (TSB)  
Tryptone (1%) Broth  
Tryptone (1%) Broth with Glucose (1%)

**Media** (Benson, 1990)

Nitrate Broth  
Urea Broth

**Stains** (Claus, 1989)

Acid Alcohol  
Basic Fuchsin  
Carbol Fuchsin  
Crystal Violet  
India Ink  
Iodine Solution (Gram's)  
Malachite Green  
Methylene Blue

**Reagents** (Claus, 1989)

Alpha-Naphthol Solution  
95% Ethyl Alcohol  
3% Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>)  
Iodine Solution  
Kovac's Reagent  
Methyl Red Reagent  
Potassium Hydroxide (KOH)  
0.85% Saline (NaCl)

**Reagents** (Benson, 1990)

Nitrate – Solution A  
Nitrate – Solution B