INQUIRY & INVESTIGATION

# Making Microbiology Even Smaller!

LINDA MULL YOUNG,
VICKI ABRAMS MOTZ

**Abstract**

*We outline protocols for producing slant-minis (SLINIs) and mini-deeps (MEEPs) and examples of their use in simple microbiology experiments suitable for high school students. The principal benefits of these protocols are decreased cost associated with significantly reduced media use; easier, less expensive disposal of waste; and increased safety by switching from glass tubes to nonbreakable plastic. Additionally, these protocols can be utilized as hands-on reinforcement of concepts of pH, metabolism, and digestion.*

**Key Words:** *Microbiology; oxidative catabolism; fermentative catabolism; selective media; differential media; pH.*

Miniaturized experiments using nonpathogenic microorganisms are an effective and inexpensive way to study cellular functions such as nutrient metabolism. If monitoring growth rather than colony morphology, slants and deeps can be scaled down. When preparing standard slants or deeps in test tubes, 5–10 mL of medium is needed. Slant-minis (SLINIs) are slants prepared with only 0.50–0.75 mL of medium in 1.5-mL microfuge tubes, which cuts the media cost to 15% that of traditional methods. Other benefits include eliminating the risk of injury associated with broken test tubes and reducing the volume of waste generated (Wood, 1990). All experiments described here have been successfully completed by high school students using only the instruction sheets. Nonpathogenic strains (as identified by the ASM 2012 guidelines) of all bacterial species suggested can be purchased inexpensively from most biological supply companies, as can various media for testing their biochemical properties (i.e., eosin methylene blue agar, MacConkey agar, mannitol salt agar, OF basal medium, peptone iron agar, and motility indole ornithine medium; available from most biological supply companies, such as Carolina Biological Supply and BD).

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Because preparation of media for SLINIs and mini-deeps (MEEPs) is simple and inexpensive, instructors can design countless protocols using this system. Encouraging students to devise their own experiments using SLINIs and MEEPs is an excellent way to foster critical thinking skills (Armbruster et al., 2009; Dale, 1969). Two easy sample experiments suitable for high school students follow. Additional media and bacteria suitable for use with SLINIs and MEEPs are presented in Table 1.

*Safety note:* Although the species of bacteria used in this laboratory are not infectious, any student who has a compromised immune system or has had a recent extended illness should talk with his or her instructor before working in the microbiology laboratory.

## ○ Directions for Instructors

### General Protocol for SLINI Preparation

- Determine the number of slants necessary for your experiment. Assuming 24 students working in pairs, you will need 40 mannitol salt agar (MSA) SLINIs and 40 MacConkey agar (MAC) SLINIs to perform Experiment 1; 10% extra was added to accommodate student errors.
- If presterilized microfuge tubes are not used, place the microfuge tubes (caps closed) and a 125-mL beaker for media prep into boiling water for 10 minutes to sterilize and transfer to a rack.
- Prepare 25 mL of each medium:
  - Boil 50 mL water for 10 minutes to sterilize.
  - Add the correct amount of sterile water and dehydrated medium (this is different for each type of medium and is always clearly stated on the label) to the sterile 125-mL beaker.
  - Swirl to mix.
  - Loosely cover the beaker with foil and briefly return the medium to a boil (ensures correct firmness when cooled).
  - Allow the sterile medium to cool slightly.
- Pouring carefully, fill each microfuge tube halfway. You don't need to measure, because approximate, not absolute, volume is needed (or you can use a large syringe without a needle to add 0.50–0.75 mL of medium to each tube).
- Quickly, close each tube and rest the tubes against a beveled edge to slant the medium as it sets (a plastic ruler works well).
- Cool tubes in this position for 20 minutes prior to use.

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**Table 1. Media and bacterial species appropriate for use with SLINI and MEEP protocols.**

| | | Positive Result | | Negative Result | |
|---|---|---|---|---|---|
| **Medium** | **Use** | **Bacterial Species** | **Response** | **Bacterial Species** | **Response** |
| MSA | Selects for salt tolerance; differentiates mannitol fermentation | *Staphylococcus saprophyticus* | Yellow medium | *Staphylococcus epidermidis* | Pink medium |
| EMB | Selects for Gram(–) species; differentiates lactose fermentation | *Escherichia coli* | Metallic green sheen to colonies | *Pseudomonas fluorescens* | Clear colonies |
| MAC | Selects for Gram(–) species; differentiates lactose fermentation | *Escherichia coli* | Pink colonies | *Pseudomonas fluorescens* | Clear colonies |
| OF glucose (aerobic) | Oxidative metabolism of glucose | *Escherichia coli* | Yellow medium | *Alcaligenes faecalis* | Blue medium (peptone use) |
| OF glucose (anaerobic) | Fermentation of glucose | *Escherichia coli* | Yellow medium | *Pseudomonas fluorescens* | Green medium |
| Peptone iron | Thiol group removal from amino acids | *Proteus vulgaris* | Black precipitate along stab line | *Escherichia coli* | No color change |
| Citrate agar | Citrate metabolism | *Enterobacter aerogenes* | Royal blue medium | *Escherichia coli* | Green medium |
| Starch agar | Amylase digestion of starch | *Bacillus subtilis* | Clear zone surrounds colonies after $I_2KI$ is added | *Escherichia coli* | No visible zone around colonies after $I_2KI$ is added |
| Urea agar | Urease degradation of urea | *Proteus vulgaris* | Fuchsia medium | *Pseudomonas fluorescens* | No medium color change |
| Phenylalanine agar | Deamination of phenylalanine | *Proteus vulgaris* | Green colonies after $FeCl_3$ is added | *Escherichia coli* | Clear-gold colonies after $FeCl_3$ is added |
| PEA | Selects for Gram(+) species | *Staphylococcus epidermidis* | Growth | *Escherichia coli* | No growth |

### General Protocol for MEEP Preparation

- Follow the presterilization and media prep as for SLINIs. Assuming 24 students working in pairs, you will need to prepare 50 mL of media to make 80 oxidative/fermentative (OF) glucose MEEPs to perform Experiment 2; 10% extra was added to accommodate student errors.
  - When preparing tubes with OF basal medium for your MEEPs, add 1% of the desired sugar (usually glucose) as an energy source for the bacteria.
- Follow filling instructions as for SLINIs, but allow tubes to cool in an upright position.
- Handle carefully once the medium has solidified, because the lower agar concentration in this medium produces only a semisolid product. Rough handling will damage the MEEPs.

### Other Pre-experiment Preparations

- Wrap packs of 10 toothpicks in foil; place in 350°F oven for 15 minutes to sterilize.
- Make fresh 10% bleach solution before each experiment (100 mL bleach in 900 mL water).

### Post-experiment Protocol

- Collect waste cups and cover toothpicks with a 10% bleach solution.
- After 2 hours, bleach can be poured off and waste disposed of in regular trash.
- Closed SLINIs and MEEPs should be placed in a sealed zip-lock bag and disposed of in the regular trash.

### Expected Results

The results for the media and bacterial species used here are presented in Table 2.

## ○ Experiments for Students

### Experiment 1: Using Selective & Differential Media SLINIs to Identify Common Bacterial Species

#### *Purpose*

The purpose of this experiment is to use pH-dependent color changes in growth media to identify common bacterial species by understanding how they perform carbohydrate metabolism.

**Table 2. Results for media and species used in Experiments 1 and 2.**

| | | Positive Result | | Negative Result | |
|---|---|---|---|---|---|
| **Medium** | **Use** | **Bacterial Species** | **Response** | **Bacterial Species** | **Response** |
| MSA | Selects for salt tolerance; differentiates mannitol fermentation | *Staphylococcus saprophyticus* | Yellow medium | *Staphylococcus epidermidis* | Pink medium |
| MAC | Selects for Gram(–) species; differentiates lactose fermentation | *Escherichia coli* | Pink colonies | *Pseudomonas fluorescens* | Clear colonies |
| OF glucose (aerobic) | Oxidative metabolism of glucose | *Escherichia coli* | Yellow medium | *Alcaligenes faecalis* | Blue medium (peptone use) |
| OF glucose (anaerobic) | Fermentation of glucose | *Escherichia coli* | Yellow medium | *Pseudomonas fluorescens* | Green medium |

### *Guiding Inquiry Questions*

After completing this lab, students should be able to answer the following questions:

(1) What is mannitol? What color does MSA agar turn when mannitol is metabolized? Which of the following bacterial species can use mannitol: *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas fluorescens*?

(2) What is the purpose of adding salt to agar? Where on the human body might you find a high-salt environment? Which of the following bacterial species can survive in a salty environment: *S. saprophyticus*, *S. epidermidis*, *E. coli*, *P. fluorescens*?

(3) What nutrient is found in MAC? What color do bacteria growing on MAC turn when this nutrient is used? What causes the color change? Which of the following bacterial species can grow on MAC: *S. saprophyticus*, *S. epidermidis*, *E. coli*, *P. fluorescens*?

### *Introduction*

There are so many different bacterial species that identifying them can be very challenging. Microbiologists solve this problem by using differential media that change color as a result of the growth of species with particular characteristics (Johnson & Case, 2009). MSA is an example of a differential medium. If a bacterial species can break down the mannitol, it will produce acidic byproducts, changing the phenol red pH indicator in the MSA from pinkish to bright yellow. The medium becomes a brighter pink if bacteria cannot utilize the mannitol (see Figure 1).

MSA contains 7.5% salt, so it also acts as a selective medium. When a mixture of bacteria is inoculated onto MSA, the medium will select *for* species that can grow in salt and select *against* others that die. Only bacterial species adapted to living in high-salt habitats like sweaty skin can survive on the MSA.

Another medium that is both differential and selective is MAC. Bacteria that are able to use the lactose in MAC for their source of energy secrete acids that interact with a pH indicator, turning the bacteria bright pink. This differentiates them from the clear appearance of species unable to use lactose. Because this medium contains bile salts and crystal violet, bacterial species commonly found in the gut thrive while most others are selected against.

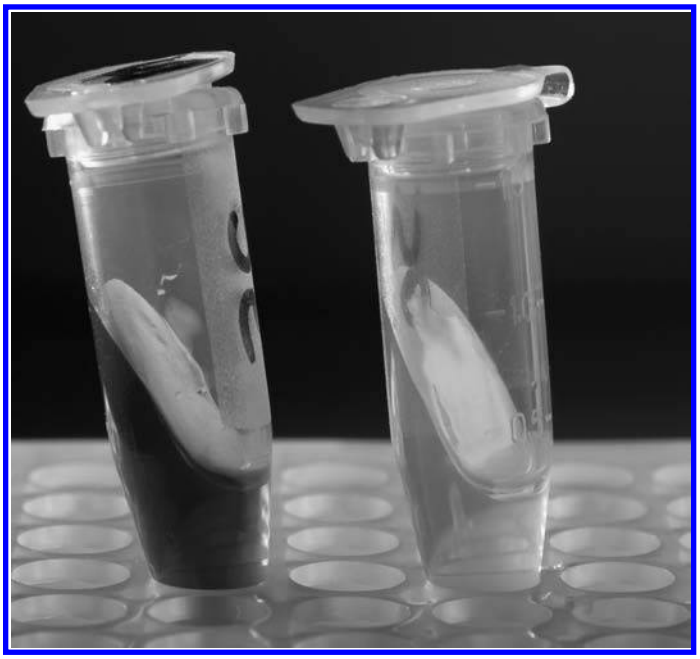

**Figure 1.** MSA SLINIs inoculated with a bacterial species that metabolizes mannitol (right) and one that cannot utilize mannitol (left).

### *Procedure*

Before coming to class, carefully read the laboratory exercise and pay particular attention to safety procedures.

### *Day 1 (approximately 30–60 minutes)*

- The following items should be available at your work station:
  - Personal protective equipment (PPE = disposable gloves, lab coats, and safety goggles)
  - Disinfectant (fresh 10% bleach solution)
  - Rack with 3 MSA SLINIs and 3 MAC SLINIs

- Packet of sterile toothpicks
- Fine-tip marker
- Small disposable cup labeled "waste"

- Wash your hands with disinfectant soap and wipe down your work station with 10% bleach solution.
- Put on your PPE.
- Label your MSA tubes as follows: *S. saprophyticus* (SS), *S. epidermidis* (SE), and *E. coli* (EC). Label your MAC tubes *S. epidermidis* (SE), *E. coli* (EC), and *P. fluorescens* (PF).
- Use the flat end of a toothpick to touch a colony of *S. saprophyticus* from the stock culture. Do not put the toothpick down or touch anything with it.
- Open the MSA tube labeled SS and gently squiggle the toothpick on the slant, starting at the bottom and working toward the top (see Figure 2).
- Carefully close the tube, write your initials on the cap, and place the tube in the rack.
- Put the contaminated toothpick in the waste disposal container.
- Follow this procedure to inoculate all of your MSA and MAC SLINIs with the appropriate bacterial species.
- Your teacher will incubate the tubes at 37°C for 24 hours or at room temperature for 48 hours.
- Disinfect your work station with bleach and wash your hands with disinfectant soap. Store your lab coat and goggles and dispose of your gloves as directed by your teacher.

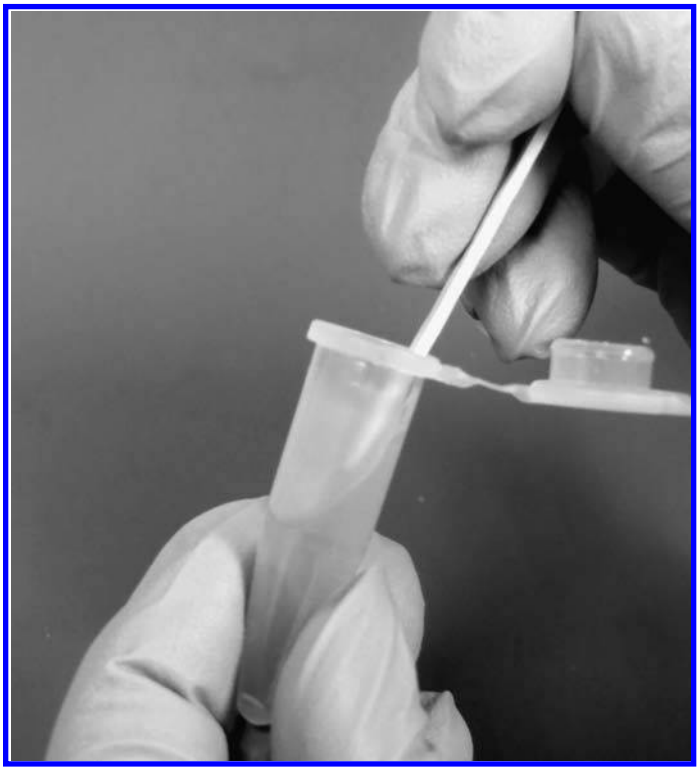

**Figure 2.** Technique for applying bacteria to a SLINI (photo is of eosin methylene blue agar).

***Day 2 (approximately 30 minutes)***

- The following items should be available at your work station:
  - PPE
  - Your incubated cultures
  - Data collection sheet
  - Disinfectant (fresh 10% bleach solution)
- Scrub in and put on your PPE.
- Examine each MSA and MAC SLINI for growth to determine the selective nature of the media.
- Record the growth data in the table provided.
- Observe color changes of the media to determine whether the bacterial species were able to utilize the mannitol (MSA) or lactose (MAC).
- Add these data to your table.
- Follow your teacher's instruction to correctly dispose of your SLINIs.

  Disinfect your work area with bleach and thoroughly wash your hands with disinfectant soap. Store your lab coat and goggles and dispose of your gloves as directed by your teacher.

## MSA Observations:

| Organism | Growth (+/–) | Media Color |
|---|---|---|
| *S. saprophyticus* | | |
| *S. epidermidis* | | |
| *E. coli* | | |

## MAC Observations:

| Organism | Growth (+/–) | Media Color |
|---|---|---|
| *S. epidermidis* | | |
| *E. coli* | | |
| *P. fluorescens* | | |

***Questions***

(1) Which bacterial species survived in the high-salt environment of the MSA? Where would you expect that these bacteria normally live?

(2) What common bacterial species is identified by a bright pink color on MAC agar? Where does this bacterial species normally live?

## Experiment 2: Using MEEPs to Compare Oxidative & Fermentative Metabolism

***Purpose***

The purpose of this laboratory is to use pH-dependent color changes to compare bacteria that use aerobic (oxidative) and anaerobic (fermentative) carbohydrate metabolism pathways.

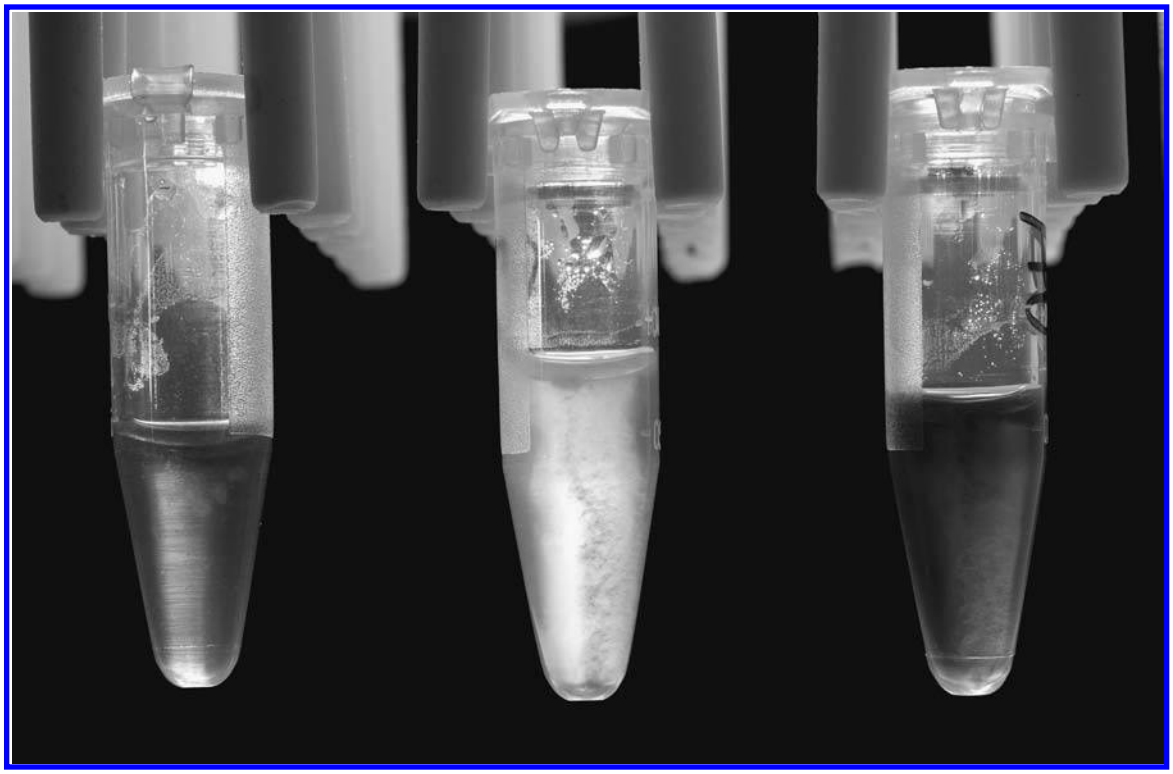

**Figure 3.** OF glucose MEEPs. Uninoculated control (left); inoculated with a glucose metabolizing bacterial species (center); and a peptone catabolizing bacterial species (right).



### *Guiding Inquiry Questions*

After completing this lab, students should be able to answer the following questions:

(1) What is the difference between oxidative and fermentative metabolism?

(2) If a bacterium breaks down glucose using oxygen (aerobic respiration), what color change will you see in the OF glucose medium? What color if it uses anaerobic respiration?

(3) What causes the color change? How do pH indicator changes determine the nature of metabolic byproducts?

(4) How does putting mineral oil on top of the MEEP tube determine what type of respiration can be used?

### *Introduction*

The process of releasing energy by chemically degrading compounds is referred to as *catabolism*. Most bacteria break down sugars such as glucose to satisfy their energy requirements. When a bacterium uses oxygen (aerobic) to degrade glucose to $CO_2$ and water, the process is known as *oxidative catabolism*. In the absence of oxygen (anaerobic), glucose is broken down by *fermentative catabolism*, which has byproducts of small organic molecules like acids and gases.

To determine whether a bacterial species performs oxidative or fermentative catabolism, the microorganisms are grown in OF glucose medium. This special growth mixture contains a high concentration of glucose and a low amount of the protein, peptone. If a microbe is unable to break down glucose, it will use the peptone. The medium also contains the pH indicator, bromthymol blue, which is green at neutrality and becomes yellow as pH decreases (acid) and blue as it increases (base), as seen in Figure 3.

When bacteria are grown in OF glucose medium, color changes indicate whether glucose or peptone was used. When fermentation occurs under anaerobic conditions, the acid byproducts turn the medium yellow. Acids produced during oxidative catabolism can also turn the medium yellow. When peptone is metabolized by fermentative catabolism, ammonia (which is basic) is a byproduct and turns the medium blue.

### *Procedure*

You will inoculate OF glucose medium with three different bacterial species to determine whether each is able to perform oxidative catabolism, fermentative catabolism, both, or neither.

### *Day 1 (approximately 30–60 minutes)*

- The following items should be available at your work station:
  - PPE
  - Disinfectant (fresh 10% bleach solution)
  - Rack with 6 OF glucose MEEPs
  - Sterilized toothpicks
  - Mineral oil in a dropper bottle
  - Fine-tip marker
  - Small disposable cup labeled "waste"

**Figure 4.** Toothpick stab technique for inoculating MEEPS.

- Wash your hands with disinfectant soap and wipe down your work station with 10% bleach solution.
- Put on your PPE.
- Label two tubes *E. coli* (EC), the next two *P. fluorescens* (PF), and the last two *Alcaligenes faecalis* (AF).
- Use the small end of a toothpick to touch a colony of *E. coli* from the stock culture. Do not put the toothpick down or touch anything with it.
- Open a MEEP labeled for *E. coli* and gently insert the toothpick down into the center, all the way to the bottom in a smooth, stabbing motion (see Figure 4).
- Put the contaminated toothpick in the waste disposal container.
- Repeat with the second EC MEEP and both of your PF and AF MEEPs.
- For *one* of the MEEPs from each species, apply a few drops of mineral oil on the surface to establish an anaerobic environment (see Figure 5).
- Carefully close each tube, write your initials on the caps, and place the tubes in the labeled rack.
- Your teacher will incubate the tubes at 37°C for 24 hours or at room temperature for 48 hours.

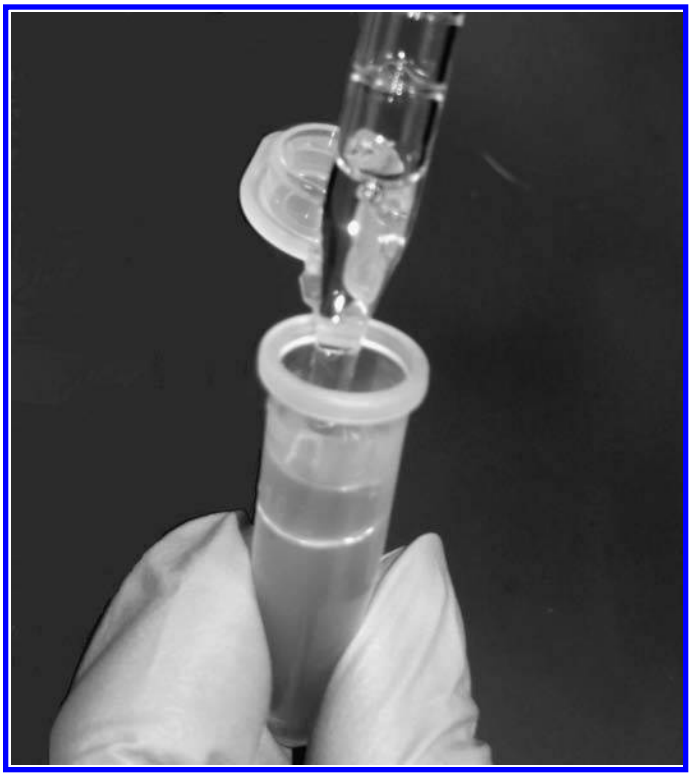

**Figure 5.** Applying oil to MEEP surface.

- Dispose of all contaminated materials in 10% bleach solution as instructed.
- Disinfect your work area with bleach and thoroughly wash your hands with disinfectant soap. Store your lab coat and goggles and dispose of your gloves as directed by your teacher.

***Day 2 (approximately 30 minutes)***

- The following items should be available at your work station:
  - PPE
  - Your incubated cultures
  - Data collection sheet
  - Disinfectant (fresh 10% bleach solution)
- Scrub in and put on your PPE.
- Examine the color of the OF glucose medium in each pair of tubes.
- Record the color changes in the data table provided and interpret the results.
- Some bacteria are motile and swim by using propeller-like flagella; these produce a cloudy region in the medium as they travel away from the stab line (see center MEEP in Figure 3). Record these motility data.
- Follow your teacher's instructions to dispose of your contaminated MEEPs.
- Disinfect your work area with bleach and thoroughly wash your hands with disinfectant soap. Store your lab coat and goggles and dispose of your gloves as directed by your teacher.

**OF Glucose Media Observations:**

| Organism | Growth | | Color | | Catabolism (O, F, B, N)* | Motile (+/–) |
|---|---|---|---|---|---|---|
| | Aerobic | Anaerobic | Aerobic | Anaerobic | | |
| *A. faecalis* | | | | | | |
| *E. coli* | | | | | | |
| *P. fluorescens* | | | | | | |
| | *O = oxidative catabolism; F = fermentative catabolism; B = both; N = neither. | | | | | |

***Questions***

(1) Can an organism be both oxidative and fermentative? Explain.

(2) Do all organisms utilize glucose?

(3) What color does OF glucose medium turn if peptone is metabolized? Why does it turn this color?

(4) What evidence do you see in the middle tube in Figure 3 to indicate that the bacteria are able to use the nutrients in the OF glucose medium for energy?

(5) Bromthymol blue turns yellow when acids are produced. Does this yellow color mean that the pH of the medium has increased or decreased?

## ○ Acknowlegment

We thank Kenneth Colwell for his photographic expertise.

## Resources for Additional Information

Further explanation of differential and selective media:

http://generalbacteriology.weebly.com/culture-media.html
http://www.scienceprofonline.com/microbiology/differential-selective-bacterial-growth-media.html

Table of different media and what each can be used to test:

http://www.highlands.edu/academics/divisions/scipe/biology/labs/rome/selectivedifferential.htm

Oxidative/fementative bacteria:

http://www.microbelibrary.org/component/resource/laboratory-test/3151-oxidative-fermentative-test-protocol

Microorganisms safety guide:

http://www.sciencebuddies.org/science-fair-projects/project_ideas/Micro_Safety.shtml

Laboratory safety:

http://www.carolina.com/teacher-resources/Interactive/nine-safe-practices-for-the-microbiology-lab/tr11085.tr?question=bacteria safety

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LINDA MULL YOUNG (l-young@onu.edu) is Professor of Biology and VICKI ABRAMS MOTZ (v-motz@onu.edu) is Assistant Professor of Biology, both in the Department of Biological and Allied Health Sciences at Ohio Northern University, 125 Mathile Center, 525 South Main St., Ada, OH 45810.