SCHEDULE

Wednesday:

Pet Investigations:

Phenol Red Broth with Durham tubes (pg. 3-4)
Oxidation/Fermentation Agar (pg. 5-6)
Anaerobic Growth (pg. 7)
Growth in Liquid Culture (pg. 8-9)

Friday:

Pet Investigations:

Plate counts - how to know how many clones of your pet you have (pg. 9-10)

Introduction to Bacteriophage (viruses of bacteria)

plaque titers - how to kill bacteria with viruses and count viral particles

* see Lab Notes for Bacteriophage for background information and methods

Pet Investigations

This week we will start with more assays to investigate the properties of your pet microbes. These assays will test your pet's ability to ferment various carbon sources, to swim, and to grow without oxygen. Although you have already tested some of these properties, an important part of doing science is to get evidence to address a hypothesis using different methods. Doing so ensures that the results from one assay were actually a true measure of the biology of the system and not artifacts specific to a particular assay. We will use assays that provide additional ways to test for motility and fermentation of lactose, properties that you already have collected some data on. We will also use new assays to detect metabolism of other sugars and the ability to grow without oxygen. Finally, you will also learn a new technique for growing your pet: liquid culture. So far we have only grown pets on solid growth medium. However, many methods in microbiology use microbes growing in liquid media.

Key Concepts

Why do microbiologists care about the various growth and metabolic properties of microbes? On the surface, these experiments may seem esoteric and difficult to apply to "real life" situations. However, these properties can be extremely important to explore for a wide range of applications, a few of which are listed below.

- Metabolic properties are a key way to identify an unknown microbe (just like you are doing with your pets). Similar tests are used in health clinics to determine which pathogen is causing an infection, in water quality management to identify contaminants in municipal water supplies, and in environment microbiology to identify particular microbes in particular ecosystems.
- An extremely active and promising area of research is to gain a detailed understanding of metabolic processes so they can be exploited in bioengineering applications to generate pathways for the production of high value biofuels and biochemicals. For example, scientists are engineering *E. coli* to ferment cellulose from plants into biofuels. Others are engineering *E. coli* to make drugs to treat malaria.
- The metabolic properties of microbes have profound effects on their surroundings. For example, specific bacteria can metabolize hydrocarbons found in oil, and these bacteria played a large role in detoxifying the ocean in the Gulf of Mexico following the Deep Horizons oil spill.

Challenge

> You will be provided with materials to perform new tests on your pet microbe that assess its ability to metabolize different sugars, grow in the absence of oxygen, and swim. Design experiments using these materials to further explore the capabilities of your pet. Since, you have already used other assays to examine motility and fermentation lactose and mannitol, you can use results from those experiments when you formulate hypotheses and predictions for this series of experiments.

We've used three different plate assays to examine fermentation of sugars, two for fermentation of lactose (MacConkey and EMB agar) and one for fermentation of mannitol (Mannitol Salt agar). These media were both selective (containing components that prevented the growth of particular bacteria) and differential (containing indicators that caused different colony phenotypes). In addition to plate assays, there are liquid assays for fermentation that aren't selective, i.e. most bacteria can grow. We will have materials for two of these assays available for you - Phenol Red Broth + Durham tube and Oxidation/Fermentation Agar. You can use these assays to get additional information about the fermentation capabilities, or lack thereof, of your pet microbe, even if your pet did not grow on MacConkey, EMB, or Mannitol salt agar. If your pet was able to grow on MacConkey, EMB, or Mannitol salt agar, these assays will provide more information about your pet's metabolic capabilities.

Phenol Red Broth + Durham tube

tests for:

1) fermentation of sucrose, lactose, and glucose

2) nature of the end-products of fermentation (organic acids and gases)

The fermentation capabilities of microbes can vary widely. A given bacterial species can often ferment some sugars, but not others. The fermentation products also vary. They can be organic acids or gases (think of why yeast causes dough to rise, if you don't know, a quick google search will suffice). In this assay, phenol red is added to a nutrient-rich growth medium containing beef and meat extracts - yum! yum! A single sugar or carbohydrate is added to the medium as a substrate for fermentation. We will provide media with sucrose, glucose, and lactose.

Here is how the assay works. Phenol red is a pH indicator that is yellow at acidic pH, when it is fully protonated. At basic pH, phenol red is not protonated and is red. At pH values between these extremes, the color of phenol red depends on the concentration of protons, making it shades of orange depending on the pH. Therefore, the color of the broth will reflect the amount of organic acids produced by fermentation.

pH	phenol red color
< 6.3	yellow
6.8	yellowish orange
7.3	orange
7.8	reddish orange
> 8.3	red

To detect any gases produced by fermentation, a small tube is placed upside down into the culture tube. If gases are produced, some will collect in the tube and a gas bubble will form at the top of the inverted tube.

Some potential outcomes are:

red color - no gas = negative for fermentation and gas production If the broth is cloudy, the bacteria grew by respiration, not fermentation.

yellow color - no gas = positive for fermentation and negative for gas production, end-products of fermentation are both organic acids.

yellow color - gas = positive for fermentation and gas production, end-products of fermentation are both organic acids and gases

Procedure:

1. Take some bacteria from a slant with an inoculating loop using sterile technique.
2. Dip the loop with the bacteria in the broth.

Oxidation/Fermentation (O/F) Agar

tests for:

1) metabolism of glucose to produce acids oxidatively

2) metabolism of glucose to produce acids by fermentation

3) motility

4) anaerobic growth

The growth medium in the O/F agar tubes contains glucose and peptone as carbon and energy sources. The medium also has the pH indicator bromothymol blue. Bromothymol blue is yellow at acidic pH (≤ 6.0) when it is protonated and blue at basic pH (≥ 8.0) when it is deprotonated. At neutral pH, there are an equal number of protonated and deprotonated molecules, so the color is green (yellow + blue = green).
In this medium, there is more glucose than peptone. Bacteria that are able to use glucose for energy will use it primarily, instead of using the amino acids in peptone.

Glucose can be used as a an energy source both oxidatively and by fermentation. Some bacteria that oxidize glucose aerobically produce a small amount of organic acids during glycolysis and the TCA cycle. The acids turn the growth medium yellow in the region exposed to oxygen at the top of the tube. Bacteria that can ferment glucose will cause the broth in the entire tube to be yellow, because fermentation does not require oxygen so the bacteria producing organic acids aren't localized to the top of the tube. Bacteria that cannot use glucose as an energy source, but can use amino acids, will release ammonia while metabolizing the amino acids. Ammonia increases the pH and turns the growth medium blue.

For this assay, you will use two tubes, one of which will be topped with a layer of sterile mineral oil. The mineral oil prevents oxygen from entering the tube, essentially creating an anaerobic environment. Evidence of bacterial growth means that the bacteria can survive in the absence of oxygen. Since fermentation can occur without oxygen, bacteria that can ferment glucose will release acids and the growth medium will be yellow.

Finally the O/F agar has a low percentage of agar (0.2 %), similar to the motility plates we used earlier this semester. Therefore, motile bacteria will swim away from the area of inoculation, while non-motile bacteria will only grow in the area where they were added to the tube.

Some potential outcomes are:

- *medium in both tubes is yellow* = fermentation of glucose
- *medium is yellow at the top of the tube but green at the bottom* = oxidative production of acids
- *medium in tube with mineral oil is green with no growth* = bacterium can't grow anaerobically
- *medium is blue* = inability to metabolize glucose, metabolism of amino acids releasing ammonia

- *turbid growth (a) throughout tube or (b) at top of open tube* = (a) bacterium is motile, (b) bacterium is motile and swims to areas with higher oxygen concentrations

Procedure:

1. Take some bacteria from a slant with sterile technique using an inoculating loop.
2. Stab the loop into the semi-solid agar in the tube to a depth of about 1 inch from the top of the tube (or about half-way to the bottom of the tube).
3. Pull the loop straight out of the tube - don't wiggle it around.
4. Inoculate 2 tubes and put a layer of sterile mineral oil about 1 cm deep on the top of the agar in one of the tubes.

Growth in a sealed box without oxygen

tests for:

1) ability to grow anaerobically

In this assay, bacterial cultures on liquid or solid growth media are placed in a sealed box with a GasPak which is a specially-made pouch that contains inorganic carbonate, activated carbon, ascorbic acid, and water. When exposed to air in the sealed box, the pouch reduces the oxygen concentration within the box and the inorganic carbonate produces CO_2. Within 2.5 hours, there will be anaerobic conditions within the box, with less than 1% oxygen. Only bacteria that can grow anaerobically will grow.

Procedure:

1. Streak out your pet on your preferred growth medium.
2. Place the plate in the box. The instructors will add the pouch and seal the box.

Growth of bacteria in liquid culture

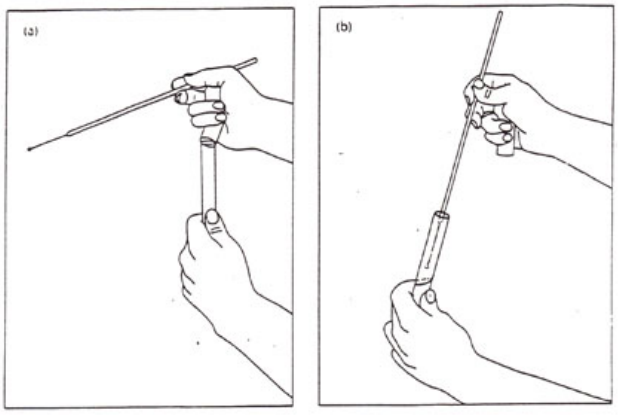
For many experiments in microbiology, bacteria are grown in liquid culture. To do so, the bacteria are transferred to a tube or flask containing sterile liquid growth medium (often the same medium that is used in plates without the agar). When the bacteria grow the broth will become turbid or cloudy. As with streaking bacteria, it is critical to use sterile technique. When you are growing microbes on a plate, it is usually easy to see if the growth medium in the plates was contaminated. For liquid cultures, it can be more difficult to know if your culture medium is contaminated, because there is little diversity in the appearance of liquid cultures with growing microbes. Many microbes will make liquid growth medium uniformly turbid. Therefore, it is important to use an uninoculated tube of broth as a control.

Transferring a Pure Culture on a plate to broth

Transfer of culture from agar plates to tubes, or from tube to tube, is a common, simple procedure. It is important to perform these transfers in a consistent and rapid manner.

Procedure:

1. Place the Bunsen burner in front of you and assemble all necessary equipment with in arms reach. Position everything so that you will not burn yourself while trying to inoculate your tubes.
2. Label the tube of broth or agar to be inoculated with identifying marks. The culture, the date, and your initials for example. Place it in a rack in front of you.
3. Holding the inoculating loop handle, flame the entire wire to redness.
4. When the wire cools (about 15-30 seconds) remove the lid of the plate with your other hand and obtain an inoculum by removing a small portion of the surface growth on the agar plate. In most cases you will be picking an isolated colony. Choose a well isolated one. Do not dig into the agar. Replace the lid of the plate immediately.
5. Hold the tube to be inoculated with the free hand. Remove the cap of the tube with the little finger of the hand holding the needle holder as in panel (a). Pass the mouth of the tube briefly through the flame to singe off anything that may fall into the tube and contaminate the medium.
6. Introduce the inoculum into the tube (see panel B). When inoculating a tube of broth, rub the wire against the glass just above the fluid level and then tip the tube slightly to wash the inoculum into the broth. The wire should not be rattled against the



sides of the tube to shake an inoculum into the broth; this is unnecessary and may create a dangerous aerosol.

7. Reflame the mouth of the tube then replace the cap.
8. Flame the inoculating wire again to redness, slowly to avoid spattering. Put the loop holder down after the wire cools.

Spreading cells from a broth culture onto a plate:

In addition to transferring cells from plate to plate, plate to broth, broth to broth, it is important to be able to transfer cells from broth to a plate. The method is straightforward and can be easlily mastered with careful, consistent technique.

Procedure:

You will need a reusable, glass spreader, a petri dish of 95% alcohol, your broth culture(s), and agar plates.

1. Place the Bunsen burner in front of you and assemble all necessary equipment with in arms reach. Position everything so that you will not burn yourself or anything else
2. Sterilize the spreader by briefly dipping it in the alcohol and passing it through the flame of your Bunsen burner. (The Bunsen burner is not used to heat sterilize the spreader but only for igniting the ethanol.)
3. After the spreader has been flamed, it should be moved through the air as little as possible. The flame will burn out quickly. BE CAREFUL! DON'T IGNITE ANYTHING ON YOUR LAB BENCH!
4. In order to cool the spreader, lift the cover of a petri dish just enough to get the spreader under it. Briefly touch the spreader to the agar avoiding the dispensed dilution (touching the hot spreader to the microbial solution may kill cells.)
5. Use the sterilized spreader to gently push the dispensed sample two or three times clockwise around the dish, and then several times counterclockwise. Don't press too hard as force will cause the microorganisms to collect at the edge of the spreader, resulting in uneven distribution.

Serial Dilution Technique :

Often bacterial or bacteriophage cultures are highly concentrated, with 10^9 bacterial cells per ml or 10^{10} bacteriophage per ml. Simply plating a 100 µL aliquot of these cultures would result in way too many colonies or plaques to count (10^8 colonies or 10^9 plaques), because each colony and plaque is formed by a single bacterium or bacteriophage respectively. This technique is useful for obtaining countable numbers of bacterial colonies or bacteriophage plaques by diluting a highly concentrated culture. The protocol described here is for serial 1:10 dilutions and can be modified for any dilution series desired.

Procedure:

1) Label tubes 10^{-1}, 10^{-2} etc. through 10^{-7} or carry out to whatever dilutions are appropriate.

2) Label destination plates of appropriate solid medium with the dilution and identifying information. Because your goal is to obtain 30-300 well isolated colonies on a plate, generally only the 10^{-5} through 10^{-7} dilutions are plated; however, the choice of dilutions to plate will vary.
3) Pipet 0.9 ml of sterile water or broth into the appropriate number of tubes labeled in step 1.
4) Using a P200 micropipettor, transfer 100 µL of a 1:100 dilution to the 0.9 ml diluent in the tube labeled 10^{-1}, mix well by tapping the tube with your finger.
5) Using a new tip, transfer 100 µL ml of the 10^{-1} dilution to the 0.9 ml diluent in the tube labeled 10^{-2}. Mix well. This makes a 10^{-2} dilution of the original culture.
6) Continue sequentially in this manner until you have carried the dilution to an appropriate end, 10^{-7} in this case.
6) Label your plates with the appropriate dilution, your name, and the date.
7) Use your P200 µL pipet and a new tip to transfer 100 µL of the most concentrated dilution that you want to plate (most often this is the 10^{-4} dilution)and dispense it to the center of an appropriately labeled agar plate. Spread the bacteria on the plate using the glass spreader and sterile technique.

Protocols adapted from:
http://openwetware.org/wiki/BISC209/S12
http://www.microbelibrary.org/library/laboratory-test/3151-oxidative-fermentative-test-protocol