MCB 3020L **Lab Experiment 4**

Examination of Natural Microbes

A four lab session experiment

It should be quite evident that the simple morphology of various bacteria, be it coccus, rod, spiral or filament and the Gram stain reaction are but two taxonomic characteristics. Many species have nearly identical morphologies. For example, a Gram negative rod could be an *Escherichia coli*, an *Enterobacter aerogenes* or any of hundreds of other species. Because of this situation, the bacteriologist must rely on getting many other traits of the bacterium to identify it. The most common traits are biochemical traits...what sort of biochemistry can this bacterium do?

From the inception of bacteriology, there has been no satisfactory taxonomic scheme. All bacteriologists utilize "the most recent edition" of Bergey's Manual of Determinative Bacteriology. The 8th Edition was published in 1974 and was contained in one volume. In the 1980s it was published in two parts: one as Bergey's Manual of Systematic Bacteriology as a four volume set, and a one volume Bergey's Manual of Determinative Bacteriology. This century, Bergey's Manual of Systematic Bacteriology is just being published as an even more expanded set. Since 1979, the bacterial taxonomic world has undergone critical reassessment due to the use of 16S RNA and other nucleic acid sequences to establish phylogenetic relationships between the various groups of bacteria. For the purposes of this experiment, you will have to rely upon the 8th or 9th Edition of Bergey's Manual of Determinative Bacteriology as the ultimate source, but use also your text and the short (and very incomplete) guide to selected genera at the end of this experiment.

In this experiment, you first have to establish (prove) that you have a pure culture. Are you sure it is? Once you are sure it is pure, then you can use it to inoculate diagnostic media and perform biochemical tests. You must continue to maintain this organism as a stock culture (grown and then kept in the refrigerator). In all of the tests below, you will inoculate your unknown into test media. The organism then must grow in the medium and produce one or more reactions. These are then either observed or tested for **after growth.** Note that if in some of your tests, the organism does not grow, you will either have to incubate it further or reinoculate. There must be growth to ascertain whether you have a positive or negative test result. There are some tests in which no growth is an acceptable result (the Citrate test), but in order for that to be valid you should have used the same inoculum to inoculate other media in which growth had occurred. Why?

BIOCHEMICAL CHARACTERIZATION

OF Medium. This is formally known as Hugh-Liefson medium and tests whether glucose is metabolized oxidatively, fermentatively or both. Some bacteria only produce acid under aerobic conditions while others will only do it under anaerobic conditions. Facultative bacteria will do it under both aerobic and anaerobic conditions. Other, non-glucose utilizing bacteria can grow on the medium and produce neutral or basic end products. This medium is a semi-solid (agar) medium containing peptone, glucose and the dye Brom-Thymol Blue. The dye is yellow in the acid and blue in the alkaline; it therefore appears green (optical illusion) at neutral conditions. **It is important that the medium be autoclaved *just prior* to use**. For this test two tubes are required, both are inoculated with the isolate. After inoculation, sterile mineral oil is layered over the medium surface of only one tube. This tube is the anaerobic tube; oxygen can diffuse into the other. After incubation, the tubes are observed for their color and presence or absence of growth. If no growth occurs, this test is **invalid**. Note that this medium utilizes only one fermentable sugar, glucose. Although bacteria have the ability to ferment many sugars and this varies from one isolate to another, if they can ferment any sugar at all it is glucose. Further, you should be able to detect gas production: this is a semisolid medium and if your unknown produces gas it should "crack" the agar.

Kligler's Iron Agar. Several facts about your organism can be determined by observing the nature of growth on Kligler's. The medium is prepared as a "deep" with a small slant at the top and is inoculated by streaking the surface and then stabbing the agar deep down to the bottom. In doing so, one inoculates down into the anaerobic-oxygen limited zone and in the aerobic surface zone. The medium contains a complex nitrogen source so most organisms can grow on it. Further, it contains 0.1% glucose and 1% lactose and the dye phenol red. Thus if the bacteria can utilize only glucose (and not lactose) only a small reaction (acid) will be produced around the growth in the deep and the slant should remain pink for facultative bacteria (the slant is yellow for aerotolerant fermenters), but if the organism can utilize lactose then the whole tube should turn yellow (acid production from lactose) because there is ten times as much lactose than glucose. Fermentation that results in gas production should result in a splitting of the agar in the deep.

Obligate aerobes will only grow on the surface of the slant whereas facultative organisms will grow both on the surface and in the deep. Sometimes aerobes will grow partly down into the deep if oxygen has diffused there, but usually not to the bottom of an inoculation that has reached the bottom of the tube.

Additionally, one can detect the production of hydrogen sulfide in this medium. Many bacteria are able to reduce sulfur in certain components of the medium (sulfur amino acids or thiosulfate) to hydrogen sulfide. If they can do that, the hydrogen sulfide so produced will react with the iron (ferrous sulfate) in the medium to produce the black precipitate, ferrous sulfide. If the medium turns black, particularly in the deep, it indicates the production of hydrogen sulfide.

Some bacteria produce alkaline endproducts from the metabolism of nitrogenous nutrients. This will be seen as a change in the color from red to a pink color. Phenol red turns a pink color in the alkaline. This reaction generally occurs under aerobic conditions.

Gelatin Hydrolysis. Proteolysis is detected with Nutrient Gelatin medium. In this medium, the solidification agent is the protein gelatin instead of agar, otherwise the medium has the same ingredients as Nutrient Agar. After growth in this medium, the medium is tested for **liquefaction**.

Gelatin is solid below 28°C. The incubation of bacteria can occur at any temperature above or below 28°C, after growth, the tubes are placed in an ice bath to test for liquefaction (gelatin breakdown). Make sure your organism has shown growth before testing liquefaction. A positive test indicates your organism produces the enzyme gelatinase.

Litmus Milk. Litmus milk is simple sterilized skim milk with litmus added. Bacteria can produce different reactions with milk proteins: coagulation, pepidtase clearing or no reaction. They can ferment lactose (acidic=pink), utilize the protein casein (alkaline=blue), and reactions creating gas (frothing). If casein is partially digested, solid curd will form (coagulation). If it is completely broken down to amino acids, it will create a clear liquid (pepidsase clearing).

Starch Hydrolysis. The ability of bacteria to produce amylase can be determined from starch containing agar. The isolate is simple streaked onto either Nutrient Agar or BHI Agar containing soluble starch. After growth, the plate is flooded with Gram's Iodine. Starch turns dark blue. If the bacteria produced amylase that has digested the starch, there will be a clear zone around the colonies.

Urease. The ability of bacteria to produce urease is tested with Urea Agar which contains peptone and glucose with 2% urea. The enzyme urease hydrolyses urea to carbon dioxide and ammonia. The medium contains phenol red as a pH indicator (yellow in acid, red near pH 7, pink in alkaline conditions). A positive test results in the medium becoming pink after growth of the bacterium on the agar.

Nitrate Reduction. Some bacteria can reduce nitrate to nitrite which is an activity found in situations when oxygen is absent or limiting. In such cases, nitrate replaces oxygen as the terminal electron acceptor in electron transport (respiration). Further, some organisms can continue to reduce nitrite to either ammonia (one pathway) or to nitrogen (another pathway). Nitrate reduction is tested in Nitrate Broth which is Nutrient Broth to which 0.2% KNO_3 is added. The broth is dispensed in tubes that contain Durham tubes. After growth in the medium, a drop is taken and tested (mixed with a drop of Nitrite Test reagent) for the presence of nitrite and the Durham tube is examined for the presence of gas (N_2). A parallel tube is inoculated that does not contain nitrate and is tested as a control.

Catalase and Oxidase Tests. Two easily performed tests (catalase test and oxidase test) are related to the use of oxygen. Cells growing in the presence of oxygen must protect themselves from super-oxide, a powerful oxygen radical that oxygen tolerant cells rapidly convert to hydrogen peroxide and water (by super-oxide dismutase). Catalase is an iron protein which avidly binds a molecule of hydrogen peroxide and then when it comes upon the next H_2O_2 it reacts one with the other to form oxygen and water:

$$H_2O_2 + H_2O_2 \quad ======> \quad 2\ H_2O + O_2\uparrow$$

thereby eliminating hydrogen peroxide. In the catalase test a portion of the colony is taken and placed in a drop of 3% H_2O_2; the generation of bubbles (O_2 gas) within a minute is a positive test.

The oxidase test tests for the presence of cytochrome-c (cyt-c). If a microbe has cyt-c it will be able to oxidize the oxidase test reagent (tetramethyl-paraphenylenediamine) to a blue color. The test reagent in the reduced state is colorless. To do this test, a part of the colony is placed into a drop of the test reagent. A positive test is the change in the color of the bacteria to dark blue. Not all

respiratory bacteria have cyt-c. Many lacking cyt-c possess other functional cytochrome electron transport systems in their respiration.

API-20 Test Strips. These strips contain twenty different tests, all of which can be inoculated with a suspension of pure culture cells in sterile saline. After growth, the reactions are read from each cupule. Some require addition of reagents. What is important is that there are two types: One type for Enteric bacteria (API20e for facultative, mixed acid fermenters) and one type for Non-Enteric bacteria (API20ne for aerobic and facultative bacteria). Directions for API strips are on the lab website as pdf files.

Materials per pair

Lab Session One

1. 1 BHI-starch Agar plates.
2. 1 Nutrient Agar plate
3. 2 tubes OF medium (3ml in a 13x100 mm tube).
4. 1 tube sterile mineral oil (2ml in a 13x100 mm tube).
5. 1 Kligler's Iron Agar Deep-Slant (10 ml in 16x150 mm tube).
6. 1 Nutrient Gelatin Deep (2 ml in a 13x100 mm tube).
7. 1 Litmus Milk tube (3 ml in a 13x100 mm tube).
8. 1 Urea Agar plate.
9. 1 Nitrate Broth tube with Durham Tube (3 ml in a 13x100 mm tube).
10. 1 Nitrate Broth tube without nitrate with Durham Tube.
11. Catalase and Oxidase test reagents.
12. 200 µl pipette tips.

Lab Session Two

1. Nitrate Test Reagent

Lab Session Three

1. API-20e Test Strip *OR* API-20ne
2. Sterile mineral oil.
3. 200 µl pipette tips.
4. 1000 µl pipette tips.
5. Known Cultures (Lab Session Two): over night slant cultures (1 per each lab section) of *Bacillus megaterium, Escherichia coli, Enterobacter aerogenes, Proteus mirabilis*, *Pseudomonas aeruginosa, Staphylococcus aureus.*
6. 1 Nutrient Agar slant

Lab Session Four

1. API-20e and API-20ne reagents

Procedure

Lab Session One

1. Streak a BHI-starch Agar plate for isolation. This will be for the starch hydrolysis test. Incubate at either room temperature or 37^{o}C.
2. Perform the oxidase and catalase test. Do this by making two smears of your culture onto a clean slide with at sterile loop, and dropping each reagent onto one of the smears.
3. Inoculate OF Medium: remember to put sterile oil over the surface of one of the OF tubes using a sterile 200 µl pipette tip.
4. Inoculate Kligler's iron agar with an **inoculating needle** rather than a loop: streak the slant surface, then stab inoculate deep down to the bottom and remove the needle so that the agar reseals

the deep portion.
5. Inoculate tubes of Nutrient Gelatin, Nitrate Reduction Broth, and a Urea Agar plate and Nutrient Agar plate.

Lab Session Two

1. Get out all the incubated test media and record the results from the plate and tubes. Make sure you know in advance how to tell the difference between positive and negative results. For the Nitrate test, you will need to mix a drop of your solution with a drop of the Nitrate Test Reagent on a slide. Determine whether your culture will need the API20 e or API20ne. You will inoculate this next lab period so your strips do not sit over the weekend.

Lab Session Three

1. Inoculate the correct API-20 strip, by suspending a loop of cells from the Nutrient plate into 5 ml of sterile saline. With a sterile 200 µl pipet, inoculate this cell suspension into each cup of the API-20 strip. Some are just filled to the bottom of the opening, others to the top...make sure you know which is which by the symbols on the strip. Add sterile mineral oil to the cups that need it.
2. The TA will inoculate known cultures into API strips.
3. Inoculate a new Nutrient Agar Slant with your pure culture. You will use this in Lab 9.

Lab Session Four

1. Add necessary reagents to certain API-20 strip cups, record the results of the API-20 strip reactions on the API form. Reduce the sets of three tests to the *profile number*. Enter these numbers into the API database for a possible identification. The lab will have a copy of the paper-database and computer database. Is this identification only tentative or is it absolutely correct?
2. Prepare a table of each trait from all the tube tests (individual tests as well as those on the API-20 strip) along with morphological and Gram stain characteristics, catalase and oxidase tests. Use these phenotypic characteristics in Bergey's Manual of Determinative Bacteriology to figure out the taxonomy of your strain.

Questions

1. Several of the biochemical tests performed in the first two lab sessions are found in the API strips. Which are these?
2. Does your results match for the tests found in question 1? Why or why not?
3. How did you determine which API strip to use?

Preparation for Experiment 5: Enrichment Culture

Experiment 5 will be in two parts: Construction of a Winogradsky column to enrich for anaerobic photosynthetic bacteria (purples and greens) and to enrich for something you decide. Each student will have to make a very short ORAL presentation on what type of bacterium you are going to enrich for and what sample you bring in that is likely to have that bacterium. Oral Presentation = 10 points. Make sure you think of something original and cool!!

A Short Guide to Selected Genera of Bacteria

All guides to genera of bacteria are flawed. The ultimate authority remains Bergey's Manual. Consult Bergey's Manual for species identifications and to verify genus.

Gram Positive Rods

I. Catalase negative. . . . . *Lactobacillus*

II. Catalase positive.

- A. Produces endospores. . . . *.Bacillus*
- B. Does not produce endospores.
  - 1. Acid Fast. . . . . *Mycobacterium*
  - 2. Not Acid Fast.
    - a. No Acid from glucose. . *.Brevibacterium*
    - b. Acid from glucose. . . *Corynebacterium*

Gram Positive Cocci

I. Catalase Negative

- A. Litmus Milk Acid Curdle . . . *Streptococcus*
- B. Litmus Milk Little or No Acid/Curdle . *.Leuconostoc*

II. Catalase Positive.

- A. Cell clusters irregular or packets of eight, aerobic. . . . . *Micrococcus*
- B. Cell clusters irregular, facultative. . . . . *Staphylococcus*
- C. Cell clusters in packets of four. . . *.Gaffkya or Micrococcus*

Gram Negative Rods

I. No Gas from glucose.

- A. Acid from glucose.
  - 1. Oxidase negative. . . . *Serratia*
  - 2. Oxidase positive
    - a. Not pigmented . . *Vibrio*
    - b. Purple pigment . . *Chromobacter*
- B. No acid from glucose.
  - 1. Gelatin hydrolysed. . . . *Pseudomonas*
  - 2. Gelatin not hydrolysed.
    - a. Oxidase positive. . . *Alcaligenes*
    - b. Oxidase negative. . . *Acinetobacter*

II. Gas from glucose.

- A. Lactose not fermented.
  - 1. Sucrose not fermented. . .*Salmonella*
  - 2. Sucrose fermented.
    - a. H_2S produced. . . *Proteus*
    - b. H_2S not produced. . . *Serratia*
- B. Lactose fermented.
  - 1. Indole positive.
    - a. Citrate utilized. . . *Citrobacter*
    - b. Citrate not utilized. . . *Escherichia*
  - 2. Indole negative.
    - a. Methyl Red positive. . *.Citrobacter*
    - b. Methyl Red negative. . *.Enterobacter*

Gram Negative Cocci

I. Oxidase positive. . . . . *Neisseria*

II. Oxidase negative. . . . . *Acinetobacter* (rod/coccus)