Exercise 15 & 16 – Supplement A
THE SCIENTIFIC METHOD &
THE IDENTIFICATION OF UNKNOWN ORGANISMS

The Scientific Method

One goal of microbiology and science in general, is to find explanations for observed phenomena and to show interrelationships between these phenomena and related events. To achieve this aim, a type of common-sense approach referred to as the "**scientific method**" is used. Not all steps of this procedure are applicable to every aspect of microbiology, but in essence the method directs the investigator to 1) **pose pertinent questions**, and to 2) look for **testable answers**.

The steps involved in the scientific method are listed below:

1. Become aware of your surroundings by making careful observations, and by reading about what others have observed and learned through their investigations.

2. Question what you observe (what is going on, why is it happening, how does it occur, etc.) and then propose hypotheses – generalizations to account for what has been observed; i.e., well calculated guesses.

3. Test the validity of your hypotheses through experimentation. This requires making additional observations and keeping careful, accurate record of what is being done and what occurs as a result.

4. Develop a theory – an explanatory hypothesis supported by experimental data and/or observations. When a scientific theory attains universal acceptance, it becomes a **law**.

Serious students of microbiology are expected to apply the scientific method to the investigation of unknown cultures, especially careful observation and accurate record keeping, as these steps are essential to the correct identification of unknown organisms.

The Identification of Assigned Unknowns (Physiological unknowns #1 and #2) and Organisms from the Environment (Semester Project)

In this laboratory students will receive their unknown organisms as slant cultures, broth cultures, or as materials simulating clinical samples. The first physiological unknown will be a mixed culture, i.e., will contain two populations of organisms (two different genera), while the second will be a pure culture. The student's goal will be to identify each different type of unknown culture as accurately as possible. With Physiological Unknown #1 (PUNK1), this can only be accomplished after the two populations have been separated from each other, i.e., after each different type is isolated as a **pure culture**.

The Semester Project may involve various aspects of microbiology, but if you choose to identify one or more unknown types of bacteria, you will probably use methods described in association with both physiological unknown #1 and #2. Students seeking Honors credit for this course must use both. One function of these assignments is to introduce students to a variety of techniques used in the identification of bacteria. A second function is to teach students the importance of precision and accuracy in the performance and recording of procedures, data and results. **Quality control** and **quality assurance** are essential factors in microbiology.

Procedure for Physiological Unknown #1 (PUNK1):

1. Obtain a tube containing an unknown mixed culture and record the number of this container.

2. Prepare streak plates with your unknown culture (the number of plates and types of media to be used will be specified by the instructor) and incubate them at 37^{o} C (unless otherwise instructed), until the next laboratory period or until growth occurs.

3. Examine each streak plate for variation in colony morphology and **RECORD** the **data** and **results** obtained. Selective and differential media contain pH indicators and provide information about fermentation ability; be sure to **record colony color, medium color** and **what this tells you about the culture** (is the culture capable of fermenting of the carbohydrate present, or not).

4. Gram stain and run a KOH test on each of the different forms present and then observe and record the data and results obtained. Determine which media are appropriate to use, and then streak each morphologically different form onto a new plate to establish two pure cultures.

5. **After** you have obtained **pure cultures** (one kind of bacteria per plate, as indicated by well-isolated colonies with uniform characteristics), continue your investigation using the stain techniques and recommended biochemical tests as described in this manual. Review the sections on Morphological Unknown and Physiological Characteristics of Microorganisms. It will be **your** responsibility to determine which tests you should carry out (i.e., which ones will be useful in the identification of your cultures), but do not hesitate to ask your instructor for assistance. You may find it beneficial to refer to the section on Keys to Identification, and may find one or more volumes of the *Bergey's Manual of Determinative/Systematic Bacteriology* helpful.

   Please remember that the media, chemicals, etc. used in this laboratory are expensive, as is their preparation. Please do your best to work efficiently and to conserve media and materials.

6. When you have completed your identification of **Physiological Unknown #1**, you will be expected to turn in a **typed report** that includes the following:

   a. An accurate **description** of **cell and colony morphology** for both organism types present, as obtained from stained smears (Gram and indirect), KOH tests, and colonies growing on agar plates (MAC, T-7, MSA, TSA, MHA, NA, blood agar, etc.).

   b. An **explanation of the enzymatic tests used** in the identification of these cultures. This explanation must include, the **name** of each test used, **what each test is designed to show** (i.e., what is being tested for), **how the test works** (e.g., reagents used, pH indicators, presence of black precipitate, bubbles, etc.). Include selective and differential media here.

   c. **Data obtained**, i.e., **descriptions and images of the tubes, plates, slides, etc.** (this cannot be omitted as it is essential to determining results). Images of data are not required, but are recommended as they can increase your understanding, and serve as an excellent study guide.

   d. Your **results**, i.e., your interpretations of the data obtained expressed as positive or negative.

   e. The technical names of both cultures present (**genus and specific epithet**).

   f. Your Bacterial ID chart for the Gram-negative, fermentative, non-fastidious culture with last column showing your results.

Procedure for Physiological Unknown #2 (PUNK2):

1. Obtain an agar slant containing an unknown bacterial culture and record the number of this container. If Gram stain information is provided, record it, and if it is not provided, determine the cell wall composition of your culture with a KOH test.

2. Extract chromosomal DNA from the culture as instructed, and then prepare a tube containing the materials needed for a Polymerase Chain Reaction (PCR). The PCR will be used to amplify 16S ribosomal RNA genes as described in "Application of the PCR in Bacterial Identification".

3. Access and evaluate the electropherogram for your unknown, and edit the text file of this data to obtain a contiguous nucleotide sequence as described in "Automated Nucleotide Sequencing and Electropherogram Evaluation".

4. Use the BLAST algorithm to compare your nucleotide sequence to the public database of the NCBI as described in "Genomics, Proteomics and Bioinformatics – Section A".

5. Prepare a simulated digest of the 16S ribosomal DNA from your unknown using the restriction endonuclease AluI, subject the cut DNA to electrophoresis, and then apply RFLP as a means of identification. Compare band sizes (molecular weights) determined with gel data (on the webpage) to those determined from nucleotide sequence data on paper.

6. Turn in a Physiological Unknown #2 Report Form. This will include a thorough explanation of all steps used to complete the investigation and identification of PUNK2, data obtained from the NCBI BLAST and the correct technical name (**genus and specific epithet**) of your unknown.

Additional Information for Identifying Unknown Organisms (Semester Projects):

If your semester project involves the identification of unknown organisms, and you choose to use nucleic acid analysis in the identification process, you will be required to prepare a pure culture of the isolate before beginning your investigation, maintain this culture in viable form, and turn it in with your written report. DNA analysis of mixed cultures requires procedures not described in this manual, and will not be available. **YOUR CULTURE MUST BE PURE!** DNA analysis will require that you amplify a portion of the 16S ribosomal DNA from your culture using PCR, and then prepare the sample for nucleotide sequencing. Review the exercises on the PCR and Bioinformatics. The arrangements necessary to obtain nucleotide sequence information will be made by the instructor, but can be time consuming, so **projects involving DNA analysis must be initiated early in the semester**.

When identifying bacteria using nucleic acid analysis, it is not unusual to encounter species not described in any volume of the Bergey's Manual available in the laboratory. If this occurs, read the information available for the **genus** and complete staining, enzymatic and other testing applicable to the identification. In some cases you will be required to **access an original publication** in order to find a complete description for the species (use NCBI PubMed). Your unknown is not likely to be a new species (not previously described), but this is a possibility (most likely if the nucleotide sequence from your isolate shows 97% or less similarity with those recorded).

If your semester project involves the identification of unknown prokaryotes, you will be expected to include observation of cell morphology in the identification process. Prepare a Gram stain and an indirect stain, but **NOT** others.

Please DO NOT prepare acid-fast or endospore stains without the approval of your instructor. These stains are potentially messy, and generally unnecessary as endospores are readily visible with Gram and indirect stains, and most environmental isolates are not acid-fast.

If your organisms are Gram-positive rods (bacilli) and are catalase-positive, look for endospores in your Gram stain preparations since these can be useful in the identification process. Cell diameter, location and shape of endospores, and whether or not the **sporangium** (cell containing the endospore) is swollen are also important features.

If your organisms are Gram-positive cocci, one of the first tests you will need to perform in order to identify them is a catalase test. The second thing you will need to know is their type of metabolism (respiratory vs. fermentative). Although the O/F test works well for Gram-negative organisms, the MR-VP is a better test for determining the fermentation abilities of Gram-positive cultures. Members of the genus *Staphylococcus* are often positive for both MR and VP while *Micrococcus* species and other Actinobacteria are typically negative for one or both.

If you are identifying species within the genera *Bacillus* or *Staphylococcus*, the tests for acid production on various carbohydrates should be performed on **agar slants** rather than within agar deeps. These organisms vary in their abilities to produce **aerobic acid**. To make agar slants, melt the individually labeled deeps in a beaker of boiling water (being careful that water does not enter the tubes), and then lay the tubes in a slanted position (on a slant board or on the surface of a 50-tube basket with the cap propped up by one side) until the agar has solidified. To inoculate these slants, streak the agar using a "fish-tail" motion from bottom to top.

If you are working with organisms that are Gram-negative, run an O/F test to determine metabolism type and an oxidase test. If you find facultative anaerobes, that test oxidase negative, do not assume they will be found on the short identification chart provided in the laboratory. The family Enterobacteriaceae has many more species than those shown on the chart. You will need to refer to a volume of Bergey's Manual for an accurate identification. Note also that additional tests may be used in the identification of enterics including esculin and gelatin hydrolysis. If you find organisms that are Gram-negative, facultative anaerobes that are oxidase positive, you may have a species within the genera *Pasteurella*, *Vibrio*, or *Neisseria.* Again, you will need to use a volume of the Bergey's Manual for identification.

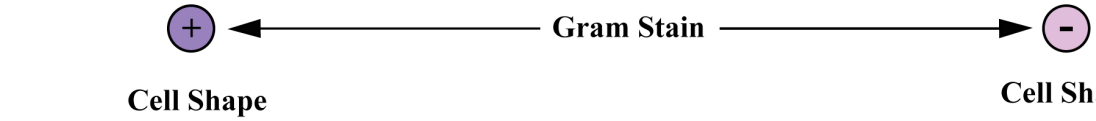
Remember – Several different versions of the **Bergey's Manual** are available in our laboratory (see Introduction to Prokaryotes). **In order to increase efficiency and reduce frustration, be sure you are looking in the right book in order to find the information you need.**

Beginning in April of 2015, a new, on-line version called the Bergey's Manual of Systematics of Archaea and Bacteria (BMSAB) is being compiled and published in association with the Wiley Online Library. This will replace and expand upon the second edition of Bergey's Manual of Systematic Bacteriology, and will provide up-to-date descriptions of the taxonomy, systematics, ecology, physiology and other biological properties of all named prokaryotic taxa. The goal of the new edition is to integrate new information about specific prokaryotic groups with existing knowledge and thereby synthesize a better, more comprehensive understanding of prokaryotic life.

Some features of this new edition are open access, and potentially very useful. Sierra student access to the complete on-line version is being investigated, and may be available.

Exercise 15 - Supplement B

GUIDE TO THE IDENTIFICATION OF UNKNOWNS (AND ADDITIONAL INFORMATION)



Cell Shape

Staphylococcus
Micrococcus
Streptococcus
Enterococcus

Group I

Bacillus
Paenibacillus
Lysinibacillus
Rothia

Group II

Cell Shape

Azotobacter
Neisseria
Acinetobacter

Note - Some short rods may appear as cocci and some are pleomorphic, i.e., can change shape.

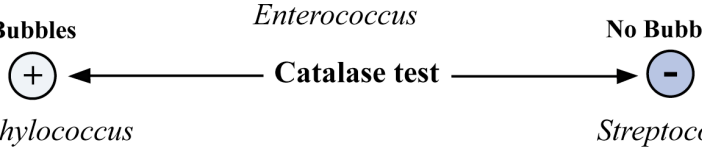
Group III

Pseudomonas
Alcaligenes
Escherichia
Salmonella
Enterobacter
Serratia
Klebsiella
Proteus
Citrobacter
Rahnella

Group IV

Key For Group I - Gram-positive Cocci

Staphylococcus
Micrococcus
Streptococcus
Enterococcus

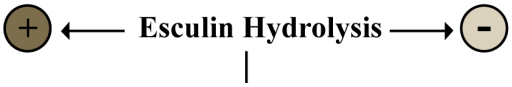


Staphylococcus
Micrococcus

Streptococcus
Enterococcus

Staphylococcus

Micrococcus lylae



Hemolysis Reaction on sheep blood agar

Enterococcus faecalis

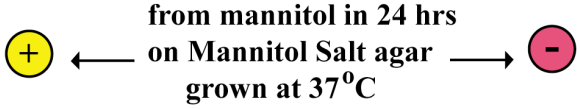
Gamma

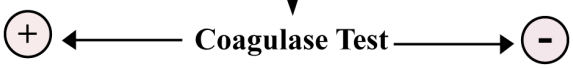
Streptococccus pyogenes

Beta

Streptococcus oralis

Alpha





Plasma is Solid

Staphylococcus aureus

Plasma is Liquid

Staphylococcus epidermidis

Note - Refer to Bergey's Manual of Systematic Bacteriology (2nd Ed.) Volume Three, Firmicutes for additional tests used to identify Gram-positive cocci.

Key For Group II - Gram-Positive Bacilli

Bacillus
Paenibacillus
Lysinibacillus
Rothia

Some Cells are Branching

Able to reduce Nitrate to Nitrite

Rothia dentocariosa

Bacillus
Paenibacillus
Lysinibacillus

Endospores Central

Endospores Terminal
Sporangium swollen

Bacillus subtilis
Bacillus cereus

Paenibacillus polymyxa
Lysinibacillus sphaericus
Bacillus psychrodurans

Streptobacilli
+
Cell Diameter
> 1 micrometer

Bacillus cereus

Bacillus subtilis

Key For Groups III and IV - Gram-Negative Rods & Cocci

Respiratory or Oxidative

Oxidation/Fermentation Test (O/F trest)

Fermentative

Pseudomonas
Alcaligenes
Acinetobacter

Enterobacteriales (organisms shown on Bacterial ID chart, require all enzymatic tests to identify)

Oxidase Test

Pseudomonas
Alcaligenes

Acinetobacter baumannii

Form aerobic acid in O/F

Form alkaline products in O/F

Pseudomonas aeruginosa

Alcaligenes faecalis

Gram-negative and Fastidious Oxidase-positive

Neisseria lactamica

Important Note – Most of the tests described under **Physiological Characteristics of Bacteria** are specifically applicable to the identification of Gram-negative species in the family Enterobacteriaceae. These tests, including TSI, SIM, citrate utilization, urea hydrolysis, MR-VP, amino acid decarboxylation and fermentation of various carbohydrates (in agar deeps) are not recommended for the identification of non-fermentative genera such as *Pseudomonas*, *Azotobacter*, *Alcaligenes* or *Acinetobacter*. Be certain that the media you choose to use are appropriate for your investigation before you begin. **DO NOT WASTE MEDIA!** Please refer to the appropriate version or volume of the *Bergey's Manual* for additional information useful in the identification of unknown bacteria.

Some additional information relative to particular organism types:

1. ***Enterococcus faecalis*** (formerly *Streptococcus faecalis)*

   Enterococcus faecalis are normally considered catalase-negative, but may show weak catalase activity. Our strain will typically yield gamma-hemolysis on sheep blood agar, although the Bergey's Manual states that these bacteria are beta-hemolytic. They will grow on MSA producing small yellowish colonies, so may be confused with *Staphylococcus aureus*. They are coagulase-negative.

2. ***Streptococcus pyogenes***

   Streptococcus pyogenes cause beta-hemolysis on sheep blood agar plates, but are catalase-negative and coagulase-negative. Colonies on blood agar are small and pale grayish-while (milky looking); those of *Staphylococcus aureus* tend to be larger, more opaque, and white or yellowish in color.

3. ***Streptococcus oralis***

   Streptococcus oralis form punctiform, somewhat grainy-textured colonies on sheep blood agar, and turn the media a greenish-yellow (show alpha-hemolysis). Like *Streptococcus pyogenes*, these organisms are catalase-negative and do not hydrolize esculin. Because they are fastidious bacteria, they will not grow readily unless cultured on enriched media such as blood agar.

4. ***Rothia dentocariosa***

   Rothia dentocariosa form circular, convex, sticky colonies that appear as opaque white or cream-colored on BHA plates. They are uniformly Gram-positive, but often appear as a mix of cocci, short irregular bacilli and branching rods. These organisms are capably of nitrate reduction (form nitrate reductase enzymes) and are normal inhabitants of the human mouth.

5. ***Bacillus subtilis***

   Bacillus subtilis form Gram-positive bacilli that are seldom in chains, and stain uniformly purple (endospores appear white). The colonies on NA are often irregular, becoming more opaque (sometimes cream-colored or brown) over time. Rods are typically 0.7-0.8 μm in diameter, 2-3 μm long, motile and catalase-positive.

6. ***Bacillus psychrodurans***

   Bacillus psychrodurans form thin, cream-colored colonies on nutrient agar. They often appear as pink-colored cells in a Gram stain, occur primarily as single cells, 0.5-0.6 μm in diameter, 2-5 μm long, and form terminal, spherical endospores within swollen sporangia when grown on NA.

7. *Bacillus cereus*

Bacillus cereus form Gram-positive bacilli that often do not stain uniformly and tend to occur in chains, the chain stability determining colony morphology. Colonies may be solid in outline with a dull or frosted glass appearance, or may develop root-like outgrowths that spread widely over the surface of the agar. Rods are typically 1.0-1.2 μm in diameter, 3-5 μm long, and catalase-positive. These organisms do not produce aerobic acid from arabinose, xylose or mannitol.

8. *Lysinibacillus sphaericus*

Colonies of *Lysinibacillus sphaericus* on nutrient agar tend to be thin (effuse), circular to irregular (sometimes spreading over moist media), pale tan-colored and have a sticky consistency. Although the Gram stain may be positive, it is often variable or negative. Cultures are catalase-positive; rods are typically 0.6-1 μm in diameter, 2-5 μm long, and show a distinct swollen appearance when endospores are present. The endospores are terminal and spherical.

9. *Paenibacillus polymyxa*

Paenibacillus polymyxa form thin, pale tannish colonies on nutrient agar plates. They have Gram-positive type cell walls (thick peptidoglycan), but often appear as pink-colored cells in a Gram stain. They occur primarily as single cells, and form ellipsoidal endospores within swollen sporangia. Because they can fix nitrogen, these organisms will grow readily on nitrogen-free media. They are the source of polymyxin, an antibiotic effective against Gram-negative pathogens.

10. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa form translucent colonies that may appear somewhat iridescent. When grown on Mueller-Hinton agar they form a blue-green, water-soluble pigment called pyocyanin, that diffuses into the medium. These bacteria are Gram-negative bacilli that form aerobic acid in O/F media (turn the surface yellow), and are oxidase-positive.

11. *Alcaligenes faecalis*

Colonies of *Alcaligenes faecalis* may appear pinkish on Mueller-Hinton agar, but do not form water-soluble pigments. The cells are Gram-negative bacilli and the cultures form alkaline end products (turn the pH indicator blue) when grown on O/F media exposed to air. They are oxidase positive.

12. *Acinetobacter baumannii*

Acinetobacter baumannii form sticky, cream-colored, convex colonies on Mueller-Hinton agar and TSA. The cultures are respiratory, but will form aerobic acid in O/F media (turn the surface yellow). They are oxidase-negative. Although these bacteria cannot ferment lactose, they will often accumulate neutral-red within their colonies (turning them pink) when grown on Mac Conkey's agar plates, especially if they are sharing the medium with lactose fermenting organisms.

13. *Neisseria lactamica*

Neisseria lactamica appear as Gram-negative cocci in pairs, and are fastidious (grow only on BHA or blood agar), forming tan-colored colonies. They are oxidase-positive.