

MICROBIOLOGY 481 SCHEDULE - Spring 2018

SECTION	INSTRUCTOR	TEACHING ASSISTANT
Diversity	Kelly Bender	
Medical Microbiology	Vjollca Konjufca	Nita Shillova
Immunology	Vjollca Konjufca	

DIVERSITY SECTION

Week 1

Tuesday (January 16):

- General introduction
- Media preparation: Basic principles
- Exercise 1 - Prepare media
- Exercise 2 - Prepare media

Thursday (January 18):

- Introduction to Exercises 1, 2 and 4
- Exercise 4 – Prepare media
- Exercise 5 – Prepare media
- Exercise 1 - Set up enrichments (bring a soil sample to class)
- Exercise 2 - Parts I and II - Set up enrichments

Week 2

Tuesday (January 23)

- Introduction to Exercises 3 and 5
- Exercise 1 - Examine enrichments/streak plates
- Exercise 2 - Parts I and II (continued)
- Exercise 3 - Set up enrichments
- Exercise 4 – Set up enrichments
- Exercise 5 – Set up enrichments for Parts I and II

Wednesday (January 24)

- Exercise 3 - Measure pH two times (early and late)

Thursday (January 25)

- Introduction to Exercise 6
- Exercise 1 - continued
- Exercise 2 - Part I
- Exercise 3 - Measure pH early and in afternoon lab
- Exercise 6 - Set up enrichments

Friday (January 26)

- Exercise 3 - Measure pH early and late

Saturday (January 27)

- Exercise 3 - Measure pH early and late (Your TA will do this)

Week 3

Monday (January 29)

- Exercise 3 - Measure pH any time

Tuesday (January 30)

- Exercise 1 - Transfer to liquid media
- Exercise 2 - Parts I and II (continued)
- Exercise 3 - Examine enrichments

- d. Exercise 4 - Examine enrichments; streak plates
- e. Exercise 5 - Examine enrichments; streak plates Parts I and II
- f. Exercise 6 – Examine enrichments; streak plates

Thursday (February 1)

- a. Exercise 1 - Set up growth experiment
- b. Exercise 2 - Complete Part I
- c. Exercise 3 - Examine enrichments, set up Gibson tubes
- d. Exercise 5 – Pure cultures for Parts I and II
- e. Exercise 6 – Examine enrichments, streak for isolation
- f. Introduction to Exercise 7 – Set up enrichments

Friday (February 2)

- a. Exercise 4- Restreak plates any time *after noon*
- b. Exercise 7- Streak EMB plates

Week 4

Tuesday (February 6)

- a. Exercise 1 - Complete
- b. Exercise 2 - Continue Part II
- c. Exercise 3 - Complete
- d. Exercise 4 – Restreak plates
- e. Exercise 6 - Transfer isolate to TS broth
- f. Exercise 7- Examine isolates/restreak for isolation

Wednesday (February 7)

- a. Exercise 7 – Establish liquid cultures

Thursday (February 8)

- a. Exercise 2 – Continue Part II
- b. Exercise 4 – Set up for antimicrobial activity
- c. Exercise 5 – Pure cultures continued for Parts I and II
- d. Exercise 6 – Set up for metabolic diversity
- e. Exercise 7 – Characterization
- f. Notebooks Due - Complete through Exercise 3

Week 5

Tuesday (February 13)

- a. Exercise 2 – Complete Part II
- b. Exercise 4 – Counterstreak plates
- c. Exercise 5 – Complete Parts I and II
- d. Exercise 6 – Score for growth, complete
- e. Exercise 7 - Complete
- f. Clean up

Thursday (February 15)

- a. EXAM I - Microbial Diversity
- b. Exercise 4 – complete

Friday (February 16)

- a. Notebooks Due - Complete through Exercise 7

MEDICAL MICROBIOLOGY SECTION

Week 6

Tuesday (February 20):

- a. Exercise 8- Staphylococcus/Streptococcus, Part II

Thursday (February 22):

- a. Exercise 8- Staphylococcus/Streptococcus, Part III

Week 7

Tuesday (February 27)

- a. Exercise 9 - Enterobacteriaceae, Part II

Thursday (March 1)

- a. Exercise 9 - Enterobacteriaceae, Part III

Week 8

Tuesday (March 6)

- a. Exercise 10 - Molecular Techniques, Part II

Thursday (March 8)

- a. Exercise 10 - Molecular Techniques, Part III

Friday (March 19)

- a. Notebooks Due - Complete through Exercise 10

March 12-16: SPRING BREAK

Week 9

Tuesday (March 20)

- a. Exercise 11 - Campylobacter/Clostridium, Part II

Thursday (March 22)

- a. Exercise 11 Campylobacter/Clostridium, Part III

Week 10

Tuesday (March 27)

- a. Exercise 12 - Neisseria/Haemophilus, Part II
- b. Exercise 13 – Unknown Identification

Wednesday (March 28)

- a. Exercise 13 – Continue Unknown Identification

Thursday (March 29)

- a. EXAM II - Medical Microbiology Section
- b. Exercise 12 - Neisseria/Haemophilus, Part III
- c. Exercise 13 – Continue Unknown Identification

Friday (March 30)

- a. Exercise 13 – Continue Unknown Identification
- b. Notebooks Due - Complete through Exercise 12

IMMUNOLOGY SECTION

Week 11

Monday (April 2)

- a. Exercise 13 – Unknown Identification Report Due

Tuesday (April 3)

- a. Exercise 14: Preparation of blood smears.
- b. Exercise 15: Demonstration of the murine lymphoid organs and freezing of the lymphoid tissues.
- c. Exercise 16: Leukocytes – differential count in whole blood.

Thursday (April 5)

- a. Exercise 17: Preparation of single cell suspensions from spleens and MLNs

- b. Exercise 18: Determination of live/dead cell concentrations in cell suspensions using a hemocytometer.

Friday (April 6)

Notebooks Due - Complete through Exercise 17

Week 12

Tuesday (April 10)

- a. Exercise 19: In situ staining of the frozen sections with fluorescent antibodies.

Thursday (April 12)

- b. Exercise 20: Mounting of the stained sections, imaging by fluorescent microscope and analysis of the acquired images.

Week 13

Tuesday (April 17)

- a. Exercise 21: Preparation of single cell suspensions from the spleen
- b. Exercise 22: Red blood cell lysis and staining with fluorescent antibodies.

Thursday (April 19)

- a. Exercise 23: Analysis of cell population profiles of the spleen by Flow Cytometry.

Friday (April 20)

- a. Notebooks Due - Complete through Exercise 20

Week 14

Tuesday (April 24)

- a. Exercise 24: Enzyme-Linked Immunosorbent Assays
- b. Exercise 24: continued: Enzyme-Linked Immunosorbent Assays

Thursday (April 26)

- a. Exercise 25: Titration of a serological reagent
- b. Exercise 26: Two-dimensional gel diffusion and precipitation

Week 15

Tuesday (May 1)

- a. Exercise 26 continued: check precipitation after overnight incubation

Wednesday (May 2)

- a. Exercise 26 continued: check gel slides

Thursday (May 3)

- a. Exercise 26 continued: check gel slides.
- a. EXAM III – Immunology Section

Friday (May 4)

- a. Notebooks Due - Complete through Exercise 26

MICROBIOLOGY 481 GENERAL INFORMATION

LABORATORY SAFETY RULES

GENERAL PRECAUTIONS - observe for ALL laboratories

- A. Wear a lab coat while in the laboratory. You must wear a lab coat in order to work in the lab. You must also provide eye and face protection (i.e. goggles) for all experiments requiring Universal Precautions.
- B. Place books, etc. on the shelves above the bench or coat rack.
- C. Do not work with an uncovered open cut.
- D. Upon entering the lab and after finishing work with bacteria, fungi or viruses, wash down your bench space with the disinfectant provided.
- E. Keep all sources of possible contamination out of your mouth---hands, pencils, laboratory ware, etc. **DO NOT EAT OR APPLY COSMETICS OR LIP BALM IN THE LABORATORY.**
- F. Discard contaminated equipment such as pipettes into the disinfectant tray provided on the bench. Petri dishes, test tubes, and similar items go into the large plastic pans provided in Room 113.
- G. Wash hands with soap and water or disinfectant before leaving the laboratory.
- H. Report accidents, such as a spilled culture or a cut, to the laboratory instructor. Do not pick up any pieces of broken or contaminated glassware.
- I. Observe aseptic technique at all times when dealing with live cultures.
- J. On regularly scheduled lab days, no work should be started until you have received pre-lab directions from your instructor.

UNIVERSAL PRECAUTIONS - observe for SPECIFIC laboratories

Universal precautions are prudent practices that are designed to prevent infectious disease transmission through body fluids, particularly the spread of hepatitis and HIV through blood. In this course, universal precautions will be used in all laboratories in which body fluids such as human or animal blood or serum or other body-derived materials from any source (including experimental animals) are used as experimental material. Universal precautions will also be used when handling known or suspected human pathogens or hazardous chemicals. As you will notice, many of the features of universal precautions are already employed in the microbiology laboratory. These precautions, based on recommendations from the Centers for Disease Control and Prevention, must be used by all persons and on all items contaminated by body fluids or infectious agents, or when using hazardous chemicals.

- A. *Handwashing.* Handwashing is the single most effective means of preventing the spread of infections. Hands and other skin must be washed thoroughly and immediately with soap and water if they accidentally become contaminated with blood, body fluids, excretions, secretions or cultures of infectious agents. Hands must be thoroughly and immediately washed with soap

and water after removal of gloves. Further, mucous membranes must be flushed with water immediately after contamination.

B. *Sharps disposal.*

1. Used sharp items (needles, scalpel blades, glass pipettes, slides and other sharp instruments) should be considered as potentially infectious and must be handled with extraordinary care to prevent accidental injuries.
2. Disposable syringes and needles, scalpel blades, glass pipettes, slides and other sharp items should be placed in the puncture-resistant red containers designated specifically for this purpose. These containers must be located as close as practical to the area where the sharps are used. **Needles SHALL NOT be recapped, purposefully bent, or removed from disposable syringes, or otherwise manipulated by hand. Shearing or breaking of contaminated needles is prohibited.**

C. *Anticipated potential exposure.* All treatments and procedures must be performed in such a manner as to reduce the possibility of direct exposure to an experimental subject's mucous membranes, broken skin (including rashes), blood or other body fluids, secretions or excretions, or potentially infectious laboratory cultures or hazardous chemicals. Anticipated exposure may require gloves, as when having potential contact with blood or other body fluids, secretions or excretions, or when handling soiled items, contaminated equipment, or hazardous chemicals. Gloves are required and are always available to students who perform venipunctures and routine injections.

Gloves are mandatory for:

1. Direct contact with skin or mucous membranes at all times and especially when the student has cuts, scratches, or other breaks in the skin.
2. Venipunctures.
3. Situations where hand contamination with blood may occur, for example, when transporting collected blood.
4. Persons who are receiving training in venipuncture procedures.
5. Persons handling body fluids or potentially infectious laboratory cultures or hazardous chemicals.

D. *Anticipated direct exposure.* Masks (provided), eye coverings, and lab coats are required, in addition to gloves, if aerosolization or splashes are likely to occur when performing procedures involving predictable contact with blood or other body fluids, secretions or excretions, or in handling infectious cultures or hazardous chemicals.

E. *Blood spills.* If a spill of blood occurs, spray the spill with full-strength chlorine bleach (5.25% sodium hypochlorite) or any disinfectant certified to be effective for use against bloodborne pathogens. Wearing gloves, wipe up the spill with paper towels. Spray the area again with disinfectant and then wipe again. Discard paper towels and gloves in plastic bag and tie securely prior to disposal.

G. *Laboratory restrictions.* Routine laboratory restrictions for students who are pregnant or who have chronic illness are not necessary for purposes of infection control.

H. *Open wounds.* No student who has exudative lesions or weeping dermatitis should perform or assist in invasive procedures or other procedures which are likely to result in contamination of the student.

I. *Pipetting.* Mouth pipetting of any materials is prohibited.

- J. *Specimen handling.* Specimens of blood, laboratory cultures, or other potentially infectious materials, or laboratory chemicals, shall be placed in a container which prevents leakage during collection, handling, processing, storage, transport, or shipping.
1. The container for storage or transport shall be labeled or color-coded appropriately with universal biohazard and/or chemical hazard warnings and closed prior to being stored or transported.
 2. If outside contamination of the primary container occurs, the primary container shall be placed within a second container which prevents leakage during handling, processing, storage, transport, or shipping and is labeled or color-coded with universal biohazard or chemical hazard warnings.
 3. If the specimen could puncture the primary container, the primary container shall be placed within a secondary container which is puncture-resistant in addition to the above characteristics.
- K. *Equipment decontamination.* Equipment which may become contaminated with blood or other potentially infectious materials shall be examined and shall be decontaminated as necessary by the Teaching Assistants or instructors. Please notify the Teaching Assistants or instructors if you have reason to believe that equipment (i.e. micropipettes, incubators, microscopes, etc.) may have been contaminated in the course of a laboratory exercise.

GRADES

Your final grade in this course will be based on the following criteria:

- 20% EXAM I – Diversity
- 20% EXAM II – Medical Microbiology
- 20% EXAM III – Immunology
- 10% Unannounced quizzes (two 10-pt. quizzes per each 5 week segment, 1 drop)
- 30% Laboratory Notebooks (50 points per each 5 week segment)

REQUIRED SUPPLIES

- Lab Coat
- Protective Eye Wear
- Sharpie Pens
- Laboratory Notebook (bound book)
- ProPipettor (that green thing)
- Microscope slides (one box; cover slips will be provided)

ATTENDANCE

Attendance is required. Each absence beyond one will result in a loss of 50 laboratory points. In addition, we expect you to come to the laboratory well prepared for the day's exercise. Many of the quizzes will be based on the laboratory exercise to be performed that day. Moreover, the time you have to spend in the lab as well as the chances of accidental contamination (of your experiment, yourself, or a lab-mate) can be significantly diminished **simply by being prepared.**

MICROBIOLOGY 481 ADDITIONAL INFORMATION

I. THE LABORATORY NOTEBOOK

Scientists keep their daily records of procedures, observations and data in their laboratory notebook. The notebook should be a **bound notebook**, and every page should have a number. For each period, record the date, all operations performed, all observations made and all conclusions reached. **The date is critically important.** Also, save the first couple of pages in your notebook for a table of contents. Add to your table of contents as you finish one exercise and start another, detailing things as much as possible, so that you (and your instructor) can find them later.

A convenient way of keeping track of enrichments and cultured isolates is to *use the page numbers of the notebook as the prefix for the culture numbering system.* For example, if an enrichment is set up as described on page 17 of your notebook, then the number 17 can be written on the tube, flask or bottle, and this number can be continued through subsequent isolation steps. If more than one enrichment is set up on page 17, then a series of numbers can be used: 17-1, 17-2, 17-3 etc. Since each number is unique and something about it is described in the notebook, there will be no subsequent confusion about cultures or enrichments.

Colored pens or pencils are useful for notebook drawings of things observed in the laboratory. However, for data recording, **ink is required** for recording even tentative observations. If something in the notebook must be changed, draw a single line through it. It is often important to know **when** you changed your mind and **why**. Accordingly, *date each page of notes* and write yourself little notes to refresh your memory, lest you forget an idea or conclusion.

Your notebook should be logically organized both for your benefit and for that of the instructor who will grade them. Notebooks will be collected on six occasions during the semester and graded on a scale of 0 (worthless) to 25 (excellent). Late notebooks lose 10 points. For each group of organisms enriched, the following *minimal* information should be included in the notebook:

1. **Experiment Title:** Name of group, for example: Sulfate-reducing bacteria
2. **General Characteristics of the Group:** from text or other readings, lab lectures etc.
3. **Enrichment and Isolation:** the steps from the initial inoculation to the attainment of axenic (pure) cultures
 - 3A. **Principles:** exploitation of certain unique properties of the bacterial group in question by special manipulations and/or the use of appropriate selective agents in the media
 - 3B. **Procedure:** the use of a **flow chart** may help here and is encouraged if possible; observations on the enrichments and isolation procedures may be included where applicable
4. **Characterization of Isolates:** a table or chart showing isolate number, source, morphological and cultural observations and results of any physiological tests performed.
5. **Conclusions:** success obtained, difficulties encountered or overcome, relation of enrichment strategy to isolation of the organism.
6. **Answers to the Review Questions:** The questions are designed to get you to think about the exercise. Write as complete answers as you can.

II. PREPARATION OF CULTURE MEDIA

On several occasions in MICR 481 you will have the opportunity to prepare some of your own culture media for use in enriching and isolating specific organisms. Since it is assumed that most of you have had little experience in this area, we will start from first principles and go into detail in how to prepare various media. Although students frequently take media preparation for granted (because they're usually supplied with sterile media in other classes), culture media preparation should not be taken lightly. After all, if you go through the trouble of setting up an enrichment, you might as well start off with media that you can trust. Accuracy and reproducibility are important aspects of media preparation and, like learning a foreign language, there are lots of exceptions, special cases and the like concerning media preparation that only come from practice and experience. In all class experiments you will work in pairs, so choose a partner now that you can trust to be as careful as you will be. Above all, ***in each step of media preparation be sure you know what you are doing and why.***

A. Operation of Equipment

Things such as electronic weighing balances, magnetic stirring motors, pH meters and autoclaves will be described and instructions for their use given in the laboratory. The balances are expensive and, like microscopes, should be handled with care.

B. Weights and Measures

The basic units of measurements in the Metric System are as follows:

1. Mass. The basic unit is the gram (g)
2. Volume. The basic unit is the liter (l)
3. Length. The basic unit is the meter (m).

Generally, the prefix "milli-" (m) means 10^{-3} of the basic unit; "micro-" (μ) means 10^{-6} of the basic unit; "nano-" (n) means 10^{-9} of the basic unit. Other units are derived from the basic units of the metric system. For example:

4a. Concentration (mass of substance per volume of water) - g/ml or "%"

4b. Concentration (amount of substance per volume of water) - mol/l

5. Content (mass of substance per mass of water) - no unit; often given in %

A one **molar** (1 M) solution is a solution containing 1 mol ($N_A = 6.023 \times 10^{23}$ atoms or molecules) of a substance dissolved in one liter of solvent. The **atomic** or **molecular weight** (Mr) of a substance refers to the mass of 1 mol of that substance; accordingly its unit is g/mol. For example, 1 l of a 1 millimolar (1 mM) solution of NaCl contains 58 mg NaCl [1 l x 1 mmol/l x 58 g/mol = 58 mg].

C. Preparing Media

In most cases you will weigh out solid chemicals and add them to a stirred liquid. Formulations of complex media often give concentrations in "%" instead of molarity. Moles are meaningless here, because complex ingredients, such as yeast extract, are not pure substances. For instance, if a solid chemical is required at a 1% (w/v) final concentration, then 1 g of that chemical would be added to the solvent (presumably distilled water) and the solution made up to 100 ml final volume.

EXAMPLE: Prepare one liter of Tryptone Yeast Extract Glucose (TYEG) broth from the following recipe:

1% (w/v) tryptone

0.2% (w/v) yeast extract

0.5% (w/v) glucose

A 1% (w/v) solution is prepared from 10 g in 1000 ml (1g per 100 ml) as discussed above; a

0.2% solution from 2 g/1000 ml; a 0.5% solution from 5 g/1000 ml. Thus our TYEG broth would be made up as follows:

Add 800–900 ml distilled water to a beaker containing a magnetic stirring bar. Add 10 g of tryptone and **let it dissolve completely**. Then add 2 g of yeast extract. After the yeast extract has dissolved, add 5 g of glucose. After the glucose has dissolved, pour the medium into a graduated cylinder and make it up to volume (1 liter) with distilled water. Return the medium to the beaker and mix well before distributing to tubes or bottles for autoclaving. Frequently, smaller volumes of media will be needed and you will have to calculate the amount of a particular reagent needed to obtain a certain percentage. In that case, scale down your recipe proportionally.

D. Rules and Exceptions

***RULE 1** - always stir your medium as it is being prepared and use a fresh spatula for each new chemical weighed out (if you cross-contaminate a chemical, you can't trust it again for precision work).

***RULE 2** - always add chemicals **in the order they are listed** in the recipe (although rule #2 can sometimes be violated without any problems arising, if you fail to follow this rule for certain media, you will end up with a precipitate or have some other problem). Precipitates are almost always bad.

***RULE 3** - Always use *distilled water* unless the recipe specifies otherwise. Tap water varies considerably in quality and may contain components toxic to cells (like metal ions).

***RULE 4** - When making a medium with many salts in it, always dissolve each chemical *completely* before adding the next (chemicals that don't react to form insoluble precipitates in dilute solutions, might do so in concentrated solutions).

***RULE 5** - Be aware of components that cannot be autoclaved (most vitamins, animal sera, some amino acids like cysteine, glutamine, asparagine and tryptophan, some sugars, some dyes, most nucleotides, almost all antibiotics). These compounds should be *filter-sterilized* (see later discussion of filter sterilization). To prepare the solution for filter sterilizing, make up a 100X or 1000X solution of the compound, so you only need to add a small amount to the final medium. For example, if you have 1 liter of mineral salts medium that has been autoclaved and needs 1 mg/l of thiamine, make up a 1 mg/ml [1 g/l] solution of thiamine, filter sterilize it, then add 1 ml to the liter of medium.

***RULE 6**- Be aware of components that cannot be autoclaved *together*, because they will react at the high temperature of the autoclave and form precipitates or soluble but altered (and possibly toxic) products.

Examples:

1. High concentrations of **divalent cations** (Mg^{2+} , Ca^{2+}) should not be autoclaved *in a mineral salts medium* with high (or even relatively low) concentrations of phosphates, because they will interact and form insoluble precipitates. When this occurs, the cation is rendered unavailable to the organisms, and the pH of the medium is frequently altered. How can this be avoided? If high concentrations of a divalent metal are required, sterilize the divalent cation in a concentrated solution and add it later. For example, $MgSO_4 \cdot 7H_2O$ can be made up in a 10 % solution and autoclaved. If the medium calls for 0.1 g/l (0.01 %) $MgSO_4 \cdot 7H_2O$, 1 ml of the 10 % (100X) solution can be added to the medium base *after* autoclaving. The reaction between divalent cations and phosphates usually does not occur in the presence of "protecting" agents such as organic compounds (peptone or yeast extract) present in concentrations greater than 0.1%, so you usually do not have to be concerned with phosphate problems unless you are making mineral salts media.

2. **Sugars** (glucose, lactose, sucrose) should *not* be autoclaved with high concentrations of phosphates in a mineral salts medium, because sugars and phosphates interact at high temperatures, altering the sugar. This results in both a lower concentration of the unaltered

sugar (which was probably added to the medium to be a carbon source and electron donor) and the formation of toxic products that can actually inhibit growth. These products (the result of "caramelization") are brown, so if your uncolored medium comes out of the autoclave brown, you may well have growth problems later on. How can this be remedied? If possible, make up a more concentrated solution, e.g., 25% glucose, so you can still add a volume to your medium that will not significantly change the volume of your autoclaved medium base. With a 25% glucose solution, only 40 ml per liter must be added to the base medium to give a 1% w/v glucose final concentration. Concentrated sugar solutions (in distilled water) are generally autoclavable. However, lactose is not soluble as a 25% solution. If you prepare a 10% solution, you must add 100 ml to your base medium to give a 1% final solution. This can be compensated for by dissolving the designated quantity of your salts for a one liter volume in only 900 ml (instead of 1000 ml) of distilled water.

***RULE 7** - Be aware of the hydration state of the chemicals that you add. If a recipe calls for 0.3 g of MgSO₄ and you add 0.3 g of MgSO₄•7H₂O, you have actually added only about half of the required amount of MgSO₄. How can you remedy this? Use the correct chemical or compensate for the extra mass of water. For example, the molecular weight of MgSO₄ is 120.4 g/mol. That of 7 H₂O is 7 x 18 g/mol = 126 g/mol. If the recipe calls for 0.3 g of MgSO₄ and you only have MgSO₄•7H₂O available, you must add:

$$0.3 \text{ g} \times \frac{\text{Mr (MgSO}_4\cdot\text{7H}_2\text{O)}}{\text{Mr (MgSO}_4)} = 0.3 \text{ g} \times \frac{246.4 \text{ g/mol}}{120.4 \text{ g/mol}} = 0.62 \text{ g}$$

When pure chemicals are compounded with water or some other substance, the molecular weight listed on the bottle will actually be the *formula weight*, which takes into account its hydration state.

***RULE 8** - Agar solutions at low pH hydrolyze in the autoclave. If you are attempting to make a medium at a low pH (<5.5), either do not autoclave it (this might be okay if the medium contains enough selective agents) or reduce its pH with a sterile acid solution *after autoclaving and cooling to 50°C*. Alternatively, autoclave the agar separately from the low pH medium and mix the components right before pouring plates.

***RULE 9** - Check the pH of all media unless instructed otherwise. For mineral salts media, the first thing to check if your organism does not grow is the pH of the medium. For reasons known only to chemistry, certain mineral salts media experience wild pH shifts when autoclaved. If this happens with any mineral medium you prepare, you may have to adjust the pH (aseptically, of course) after autoclaving.

RULE 10** - If agar is called for, ***always add agar as the last step in media preparation (the addition of agar will not change the pH or other chemical properties of the medium).

***RULE 11** – One last rule for media prep: If you make up a medium for the *first time*, follow any directions given **exactly**. If the medium works, be sure to note what it looks like to help you the next time. For example, what color is it? Does it have a slight turbidity that disappears with cooling? If a medium doesn't work, always check the pH of any remaining sterile media before setting up another enrichment with the same medium. Occasionally, the components of the medium will react in such a way that the pH is changed significantly. This is particularly a problem when precipitates form. Unless a major ingredient (carbon or nitrogen source) has been left out, media that are dismal failures (and that do not have water-related problems) usually have pH problems.

E. Autoclaving. You will be shown the operation of the autoclave, but in general, autoclaving will be done by the media preparation personnel in Room 115. All media to be autoclaved should be placed in the labeled plastic tubs in the front of the classroom before the end of the hour. They will be autoclaved when everyone's media are ready. If plates are to be poured, autoclaving will be done early during the period, so that you can pour your plates before you leave. Some tips for

proper autoclaving follow:

1. Do not fill any container more than 3/4 full of liquid (liquids expand when heated).
2. Never tightly close a vessel closure (bottle cap, *etc.*), because pressure that builds up in the autoclave can burst a tube or bottle, if they are tightly clamped. This is generally not a problem with small screw-capped tubes, but it is best to not tighten caps in general.
3. Remember that autoclaved liquid media generally take one or more hours (depending on the vessel size) to reach room temperature. Don't grab a hot tube or bottle! The standard autoclave time/temperature setting is 15-20 min at 121°C (15 psi pressure). However, if an autoclave is full, or if your container of medium is large, the material within it will not reach 121°C as fast as the saturated steam inside the autoclave (which is what the thermometer actually measures). Therefore, large volumes of media (> 250 ml) should be heated for a proportionally *longer* period of time than small volumes of media. Empty glassware (pipettes, empty tubes, *etc.*) requires 40–60 minutes to sterilize by autoclaving. Following autoclaving, liquids are returned to atmospheric pressure *slowly*, a procedure called **SLOW EXHAUST**. Since during autoclaving the temperature of the liquid rises well above its boiling point at atmospheric pressure, the return to atmospheric pressure must be accompanied by a slow return to temperature below 100°C. Never rush the slow exhaust of an autoclave. If you do, you'll risk being burned with superheated liquids as they boil over at reduced pressures, or at the very least you'll lose a good portion of the medium you worked so hard to prepare.

F. Filter Sterilization: Heat labile materials must be sterilized by filtration. The technique of filter sterilization will be demonstrated. The filter apparatus consists of a filter support and an O-ring. The filter is placed in the apparatus (using forceps). The assembled apparatus is wrapped in aluminum foil and is autoclaved for 10 minutes only. To filter sterilize a solution, a syringe is attached to the inlet and the liquid to be sterilized slowly pushed through the filter into a sterile tube. If liquid flows through the filter without pressure on the syringe plunger, the filter is broken and sterilization has not been achieved. Before attaching a syringe, it is best to gently tighten the filter assembly to ensure that the gasket is seated properly. A sterile filter apparatus should be handled using aseptic technique. **Never touch the outlet.**

III. ENRICHMENT AND ISOLATION

1. **Enrichment.** Rarely does a natural environment contain only a single type of microorganism. In most cases, a wide variety of organisms are present, and it is the task of the microbiologist to devise methods and procedures that will permit the isolation and culture of the organism(s) of interest. The most common approach to this goal is the **enrichment culture method**. In the enrichment culture method, a medium or set of incubation conditions are used which are **selective** for the desired organisms and which are inhibitory or counter-selective for undesired organisms. It is not a straightforward task to design appropriate enrichment methods, but over the ~100 years since Beijerinck first clearly stated the enrichment culture method, a large body of knowledge has accumulated on how to enrich for particular organisms or groups of organisms (for example, see Reference 1 below). Some of the methods used are rather obvious. For instance, if the only energy source available to organisms is light, then only phototrophic organisms should develop in the enrichment. If oxygen is excluded, only organisms capable of anaerobic growth will develop. Some enrichment procedures are less obvious and have been developed through trial and error. Throughout the first part of MICR 481 you will be using enrichment procedures selective for various groups of bacteria; in each case your instructors will explain the rationale behind these procedures in pre-lab lectures. Be sure you understand the theory behind an enrichment, and if you don't, just ask.

For incubation purposes, three different temperatures will commonly be used. Incubations referred to as 25°C will be considered **room temperature** and can be set up in your lab drawers. Incubations at 32°C will be in the small laboratory incubator and 37°C incubations will be in the walk-in incubator across from Room 119.

Excellent reference material on enrichment cultures can be found in:

Balows A, Trüper HG, Dworkin M, Harder W and Schleifer K.-H. (eds.) 1992.

The Prokaryotes. 2nd edition. Springer-Verlag, Berlin. [available in Morris Library]

Brock Biology of Microorganisms, 14th ed., Pearson, San Francisco, CA

Bergey's Manual of Systematic Bacteriology. [available in Morris Library]

2. **Inoculum.** Successful enrichment requires that an appropriate inoculum source be used. The old saying that "Everything is everywhere, the environment selects" is not always true, and thus it should be understood that a given handful of soil or mud, or a sample of pond or lake water will not necessarily contain the organisms sought. Thus, we will begin each exercise by discussing the appropriate *habitat* from which to obtain our inoculum. Use only a **small** amount of inoculum for a particular enrichment procedure. Adding too much inoculum can change the chemical or physical properties of the enrichment and work against your isolating the desired organisms.
3. **Crude Enrichment Cultures.** After inoculation of the appropriate medium and incubation for an appropriate period of time (all prescribed in the exercises to follow), the desired organisms may have developed in the enrichment culture to a reasonable cell density. But a crude enrichment culture is rarely if ever pure, being mixed with one or more other organisms that are also able to grow under the conditions used. However, in a successful enrichment culture, the sought-after organism should predominate over other types, and can often be identified under the microscope or through chemical changes which have been brought about in the medium. The next task, and often the most difficult, is to obtain a *pure* culture of the desired organism. Generally, this can be done by streaking on an appropriate agar medium, permitting single colony isolation, or diluting the culture in agar shake tubes.
4. **Streak Plate Method.** There are many variants on this method, but we suggest you adapt and stick to the method described below. Plates used for streaking should be free of excess surface moisture. Ideally, plates should be poured 1–2 days ahead of time and stored at room temperature to permit drying of the agar surface. Inspect the plates you are provided or that you have prepared yourself for excess moisture and for contamination. Discard any contaminated plates.

To streak a plate using a colony from another plate, the following procedure should be used. Make sure that the loop is clean of grit to avoid cutting into the agar. Flame the loop until it is *red hot*, allow it to cool and pick up a loopful of sterile water. Touch the water-filled loop carefully to the surface of the colony. This will transfer some of the cells from the colony to the loop, and will allow for better colony separation on your new plate. It is not satisfactory in most cases to streak directly from a colony on agar, as the loop picks up so many cells that it may be difficult to obtain well-isolated colonies on the streak plate. However, with organisms that form hard or compact colonies, insufficient cells may be obtained using the water-loop method, and direct streaking from the colony may be necessary. If a fluid suspension is to be used to streak a plate, insert the sterile loop into the bacterial suspension and pick up a loopful of material. Pick up the plate to be streaked and hold it vertically, then deposit the inoculum at the periphery of the plate. Use a three-sector streaking pattern or with experience, you may be able to streak a four-sector streaking pattern. Get as many streaks as possible within each of the sectors, but be careful not to overlap streaks. After the first sector is streaked, *flame the loop*, allow it to cool, then streak the second sector, removing inoculum from the tail end of the first streak as illustrated. Flame again and streak the third (or

fourth) sector. The most common source of errors in this technique are not flaming the loop before streaking each sector or using a loop that is still too hot.

Once well-isolated colonies have been obtained, pick a typical colony and streak again. The loop must be COOL before it touches the inoculum.

5. **Examination of your Primary Enrichment.** In a primary enrichment culture designed to isolate a specific group from some natural source, you should first make a careful gross examination of the culture **after carefully** removing it from the incubator (never shake or otherwise disturb an enrichment until you have had a chance to look at the growth vessel first). What kind of observations might you make? Has growth occurred? Is growth extensive or fairly minimal? In what form has growth occurred (even turbidity, surface pellicle, bottom pellet)? Are there obvious signs of gas formation? If a solid substance was initially present in the medium (e.g., CaCO_3 , cellulose, meat), has it disappeared or shows signs of decomposition? Was any marked color produced in the culture? Is there any marked odor? Odor may be misleading, but is often a useful characteristic. On differential media, color changes are especially useful in detecting an organism of interest.

The examination of colonies from enrichment cultures is the first important step in characterizing an organism. Start with a gross examination, supplemented by observation with the aid of a dissecting microscope at low power. What are the features of the predominant colonies? How many colony types are present? What are their characteristics? Have any changes in the medium occurred? Pigment formation? Odor? Proceed to prepare **wet mounts** from two or three colonies of the predominant type, as well as from any other colonies that appear of interest. Note principal morphological characters; be sure to look carefully for possible morphological heterogeneity, since colonies on first plates are often mixed ones. Make Gram stains, again checking for possible heterogeneity with respect to the Gram reaction. You may in some cases wish to perform a catalase test (placing a drop of dilute H_2O_2 on a small portion of the colony and observing for O_2 evolution), since the presence or absence of catalase is an important differential character in certain bacterial groups. **Be sure to mark with an identifying number the location of each colony examined** (make the mark with your marking pen on the back of the Petri dish). Once your observations are completed, you can prepare a second plate from the colony or colonies of interest. **Never purify a colony that you have not previously examined, even if it appears identical with the one actually examined**; you cannot be certain without microscopic examination, that it is not a mixed colony or even a totally different organism from that originally examined. Emulsify a portion of the colony in sterile water and use this suspension to streak a second plate.

There is a good chance that colonies in a first plate streaked from an enrichment culture may be contaminated. This risk of contamination is greatly reduced, when a second plate is prepared from a single, morphologically homogeneous, well-isolated colony from the first plate. Nevertheless, the second plate should be examined with care before it is used to prepare a pure culture slant; if there is obviously a mixture of colony types present, a third plate should be prepared. Never prepare a stock culture from a plate that is not *uniform* with respect to colony appearance (an occasional colony derived from an air contaminant, recognizable by the fact that it occurs singly on a plate and is usually well away from streak lines, can be disregarded as far as this rule goes). Also, always check the microscopic appearance of the cells from the *colony* from which you intend to make a slant. Is the culture homogeneous? Does the morphology correspond to that recorded for the parent colony on the previous plate? The considerations outlined above also apply, of course, to the isolation of anaerobes by the shake culture and other methods. A good notebook will help a lot in jogging your memory. **All cultures should be carefully labeled**, with your name or initials, the

group under study, any additional identifying marks and the date. **Clear** labeling with this information is essential, since it will frequently be necessary for the instructors to examine your work in progress between laboratory periods in order to determine the progress of enrichments.

6. Microscopic examinations

- A. Living preparations:** Initial microscopic examination should be done on live preparations using wet mounts. With bright-field microscopy, however, you must carefully adjust the illumination such that you keep glare to a minimum. Otherwise you will end up seeing very little. Ask your instructor for help in properly adjusting the light on your microscope, if you have problems adjusting it yourself. Initial observations should be performed at 400x on a wet mount, prepared by placing a drop of the culture on a slide and covering with a cover slip. Always try to cover the drop in such a way that a few air bubbles are trapped in the liquid (drop the slip on). One of the characters that you will want to determine is motility, and strictly aerobic microorganisms in a dense wet mount can rapidly become non-motile, when conditions become anoxic. If there are air-bubbles in the preparation, they provide an oxygen reservoir, and strict aerobes will consequently remain motile in the immediately surrounding liquid. In fact, relations of a motile organism to oxygen can often be inferred simply from such a microscopic examination. Use a phase-contrast microscope here to better observe motile cultures. A careful examination of the wet mount will reveal the morphological homogeneity or heterogeneity of the microbial population and the size, shape and motility of the predominant forms. In some cases, other significant taxonomic features can be recognized, such as spores or sulfur inclusions. You may then wish to proceed to the examination of a fixed and stained preparation. However, staining should not be used as a substitute for wet mount observations. Gross morphological features are better determined on living cells.
- B. Fixed and Stained Preparations:** Microscopic examination of stained preparations is of paramount importance. Either Gram-stained or simple stained preparations can be used. For simple staining, flood your smear with either methylene blue or crystal violet. Are cells separate, or are they arranged in groups (chains, packets, clumps, pairs)? Can you see any structures inside the cells, such as granules? For Gram stains, is the organism gram-positive or gram-negative? Always repeat your Gram stains, once you are sure you have a pure culture. Microscopic examination of the first enrichment cultures and well-isolated colonies obtained from streak plates should be done. Always record your observations as you make them, as you will probably need to refer back to them at subsequent periods. For staining procedures, see also section V.

7. Other Characteristics

- A. Colony Characteristics.** The appearance of colonies is quite useful to note, especially as purification proceeds. Note especially the size, shape, color and overall appearance of colonies. Be certain that colonies observed have grown *well isolated* from other colonies. Be sure to note the culture medium, temperature of incubation and any other incubation conditions used, since colony characteristics can often vary markedly with cultural conditions. When you pick a colony and streak it onto a subsequent plate, make sure the colonies are similar to those seen on the original plate.

A large number of terms have been used to describe the appearance of bacterial colonies on agar plates: *smooth, irregular, wrinkled, rough filamentous, flat, raised, mucoid, glistening, dull etc.* It is sometimes useful to examine colonies from streak plates under a dissecting microscope, since this permits not only a closer examination of the morphology of the colony, but permits visualization of colonies at earlier stages of growth. In some cases, it may be

desirable to pick very tiny colonies at an early stage of growth to avoid overgrowth of a minor member of the population, and this can best be done under the dissecting microscope. Once a pure culture is available, a variety of diagnostic tests can be used to better identify the organism. However, diagnostic tests should **never** be performed on impure cultures, since the results will usually be meaningless.

- B. Criteria of Purity.** For most purposes, a culture can be adjudged pure, if only a single type of cell is seen microscopically, and if only a single type of colony is obtained when the culture is streaked on an appropriate culture medium. For research purposes, it is often necessary to make further tests, such as streaking out on a variety of selective culture media, some of which do not support the growth of the desired organisms, but which will support the growth of suspected contaminants. In general, the most difficulty is experienced in obtaining pure cultures of phototrophic and chemolithotrophic microorganisms. Although these organisms are grown primarily in inorganic media, which might at first seem rather selective, in actuality these organisms often excrete organic materials in small amounts, that will support the growth of small numbers of chemoorganotrophic contaminants. Such contaminants are often carried along from transfer to transfer with the desired organism; these can usually be recognized by inoculating a rich organic medium that does not support growth of the desired organism, but will support growth of the contaminant.
- C. Biochemical characterization of the isolate.** For several commonly applied biochemical tests, please see section IV of the manual. Further tests are explained in your lab manual from MICR 301.
- D. Stock Cultures.** Once a pure culture is obtained, stock cultures should be prepared, so that the organism can be maintained in a viable state during the time it is being studied. From the final single colony isolate obtained, a culture on an agar slant is made, and after this has grown up, additional slants can be prepared or the original slant stored. It should be emphasized that cultures can lose viability if incubated too long, so that stock cultures should be removed from the incubator as soon as visible growth has been obtained. For the purposes of the present course, stock cultures can be reasonably well preserved by storage in your media area at room temperature or in the refrigerator. Some organisms (e.g., lactic acid bacteria) keep better in stab cultures than on slants. We will make stock cultures of some of the isolates obtained.

IV. BIOCHEMICAL TESTS USEFUL FOR IDENTIFYING BACTERIA

A. LITMUS MILK REACTIONS

Litmus milk medium is inoculated and incubated for at least 2 days. If no reactions are evident, incubation should be continued. The observed changes in litmus milk are to mostly due to bacterial action on the protein (casein) and/or the carbohydrate (lactose).

Neutral litmus milk (pH about 6.8) is lavender in color and becomes *pink to red* with acid production and *blue* with development of alkaline conditions. The following are some of the reactions that can be detected:

1. **ACID PRODUCTION.** Pink color. The lactose is fermented with the production of acid.
2. **CURD FORMATION.** Casein is coagulated, usually contracting and squeezing out the whey. This is due to one of the following:
 - a. acid from lactose fermentation which drops the pH to the isoelectric point of casein (pH 4.8–5.0).
 - b. "sweet-curdling" by rennin-like enzymes (alkaline conditions). The milk stays a lavender in color.
3. **REDUCTION.** White color. Certain organisms can reduce litmus to a colorless leuco base. When this occurs, the ability of the litmus to indicate pH is lost; the medium turns partially or totally white. There is often an area of unreduced litmus at the top of the medium.
4. **GAS PRODUCTION.** Production of hydrogen and carbon dioxide (often only the latter) from non-homofermentative lactose fermentation. Gas can be detected by frothing of the milk.
5. **PROTEOLYSIS.** Proteolytic activity is shown by a clearing of the medium; a slight amber color may be seen. Frequently proteolysis begins at the top of the medium.
 - a. There may be an associated development of alkalinity due to reamination of amino acids.
 - b. Curd formed under acid conditions may be solubilized (peptonized). Often the curd is digested down one side of the tube.
6. **ALKALINE REACTIONS.** Blue or purple color. Result of one of the following:
 - a. Deamination of amino acids formed after proteolysis of the casein (see 5a).
 - b. Utilization of citrate from the milk with the release of calcium and magnesium ions.
7. **ROPINESS OR SLIME.** Certain organisms can produce a polysaccharide slime which can be detected by its adherence to an inoculating loop. Immerse a sterile cooled loop into the milk and observe for a stringy, slimy mucus.
8. **PRODUCTION OF HYDROGEN SULFIDE.** Black color. Cysteine from hydrolysis of proteins is reduced, resulting in H₂S and alanine; the H₂S reacts with the reduced iron added to the litmus milk to form ferrous sulfide, a black precipitate.

B. GLUCOSE O/F MEDIUM

This semisolid medium contains a relatively high concentration of glucose and a small amount of peptone to permit the detection of an acidic reaction with a minimum of interference from the deamination of amino acids (which produces ammonia, an alkaline product). The medium is inoculated by stabbing duplicate tubes with a layer of sterile mineral oil added to one of the tubes to create anoxic conditions. Strictly aerobic organisms that do not ferment, but instead only oxidize sugars (e.g., *Pseudomonas* species), will grow and produce a slight acidic reaction (yellow color) at the top of the medium without the mineral oil. Facultatively aerobic bacteria (e.g. enterics) will grow in both tubes, and the acid produced from fermentation will impart a yellow color to the entire medium in each tube. Occasionally a strictly aerobic organism is encountered which will not produce any acid; in that case an alkaline reaction (blue color) is usually seen at the top of the "open" tube.

C. CATALASE TEST

The enzyme catalase, present in many aerobes, catalyzes the formation of O₂ from hydrogen peroxide (H₂O₂). The activity of this enzyme can be detected by adding a small amount of H₂O₂ to a culture and observing for the formation of gas bubbles, indicative of the release of O₂. The culture should be grown on an agar slant or plate which has been heavily inoculated, so that dense cell mass is obtained. Dropwise, add 3% hydrogen peroxide over the mass. Observe for the **immediate** appearance of bubbles.

An alternative procedure can be used to test for catalase. Place a drop of 30% H₂O₂ on a clean microscope slide. With a loop, pick up the cell mass from a single colony and emulsify it in the H₂O₂. Immediate bubbling indicates the presence of catalase. Care must be taken if the organism has been grown on blood agar, since blood cells contain catalase. As a control, touch a loop to a portion of the uninoculated culture medium and carry out the same microscope slide test.

D. OXIDASE TEST

The detection of oxidase activity is used as a differential test mainly for the aerobic and facultatively anaerobic groups of Gram-negative bacteria. Activity is correlated with the occurrence of a cytochrome of the c type.

The oxidase test itself is a test for the ability to oxidize aromatic amines to colored products. The substrate is almost immediately converted to a colored oxidation product by oxidase-positive bacteria, whereas oxidase-negative bacteria do not produce a color change. The color change should be rapid.

Procedure I: Apply a 1% aqueous solution of para-amino-dimethylaniline to a plate showing well-isolated colonies. Note any colonies that first turn pink and then brown-black. This is a positive oxidase test reaction.

Procedure II: To approximately 5 ml of broth culture or a nutrient agar slant culture add several drops of 1% para-aminodimethylalanine. Shake vigorously and allow to stand. Development of a blue color *within one minute* indicates a positive oxidase test.

E. NITRATE REDUCTION (NITRITE AND N₂ FORMATION)

Certain bacteria can utilize compounds other than molecular oxygen as terminal electron acceptors during oxidative metabolism (anaerobic respiration). Some species employ nitrate (NO₃⁻) as the terminal electron acceptor producing nitrite (NO₂⁻) as final product. Certain species may further reduce nitrite to molecular nitrogen (N₂).

A broth medium containing a small amount of potassium nitrate and an inverted Durham tube is inoculated with the test bacterium. After incubation, the test for nitrite is performed by adding two or three drops of sulfanilamide reagent and an equal number of drops of N (1-naphthyl)-ethylenediamine reagent to the culture. If nitrite is present, a pink to red color develops immediately. The presence of gaseous nitrogen (N₂) can be detected by examining for gas in the Durham tube inserted in the original culture.

Reagents:

Nitrite A solution: Sulfanilamide reagent: Add 5 g of sulfanilamide to 300 ml of H₂O containing 50 ml concentrated HCl. Carefully adjust the volume to 500 ml with distilled H₂O.

Nitrite B solution: N-(1-naphthyl)-ethylenediamine reagent: 0.1% aqueous solution of N-(1-naphthyl)-ethylenediamine dihydrochloride

Negative tests should be confirmed by adding a small amount of zinc dust to the tube. This will reduce nitrate to nitrite and a red color will develop. If a red color does NOT develop in a negative test after adding zinc dust, this indicates that all the nitrate has been reduced, probably to N₂.

F. TESTS FOR EXTRACELLULAR ENZYMES (EXOENZYMES):

Exoenzymes are released by bacteria into the culture medium and are responsible for the conversion of polymers, such as proteins, starches or fats, into monomers which can be transported into the cell. Exoenzymes are *hydrolytic*, in that they utilize water to split complex organic compounds into smaller subunits.



To detect the presence of extracellular enzymes, a plate of nutrient medium containing a complex organic compound is inoculated with a single streak of the test bacterium. After incubation, suitable reagents are added to the plate to detect the disappearance of the polymer, usually signalled by a clearing in an area surrounding growth of the bacterium. An alternative procedure is to note the appearance of certain byproducts of the enzymatic cleavage.

Examples:

1. Starch hydrolysis

Amylase: After incubation the plate is flooded with *Grams' iodine* which will react with starch to form a blue color. A clear zone indicates starch hydrolysis.

2. Protein hydrolysis

a. **Caseinase:** After incubation, the test plate is flooded with 1% HCl. This will precipitate any remaining casein, leaving a clear zone around the bacterial growth if the enzyme caseinase has been produced.

b. **Gelatinase:** After incubation the test plate is flooded with HgCl₂/HCl (caution:**toxic**) solution (15 g HgCl₂ + 20 ml concentrated HCl in 100 ml H₂O). *Unhydrolyzed* gelatin will form a precipitate; *hydrolyzed* gelatin will remain clear.

V. STAINING METHODS

1. GRAM STAIN

- a. Prepare a thin bacterial smear on a clean glass slide, allow it to dry, and heat fix as shown by your instructor. (When preparing smears from colonies make sure that the smear is not too thick).
- b. Immerse the smear for 1 min. in crystal violet.
- c. Wash the film in a gentle stream of tap water for 2 sec.
- d. Immerse the smear in Gram's iodine for 1 min.
- e. Wash the smear in a gentle stream of tap water for 2 sec. and then gently blot the film dry with a paper towel.
- f. While holding the slide **at an angle**, add 95% alcohol, drop by drop, for about 10 sec.
- g. Wash as above.
- h. Immerse the smear in safranin to counter-stain for 1–2 min.
- i. Observe microscopically using the bright-field stop on your condensor (phase-contrast optics will not always give you a good reading of the color of your Gram stain). Also, diaphragm adjustments may be necessary to get a correct color reading.

2. ENDOSPORE STAIN

- a. Prepare an air-dried heat fixed smear in the usual way. Cut a piece of filter paper to fit over the slide.
- b. Saturate the filter paper with the stain Malachite Green.
- c. Steam the slide for 5 min. by placing it over a small beaker of gently boiling water. Replace any evaporated stain with fresh stain.
- d. Cool, wash as above, and counter-stain with safranin for 1 min. Wash, blot dry and examine.

Endospores are bright bluish-green and vegetative cells are pink to brownish red. Use either the brightfield (condensor stop 0) or the dark-field stop on your phase-contrast microscope to see color in the spores the best. Diaphragm adjustments may be necessary to obtain a bluish-green color.