Chapter 5
Use of a Plant Pathogen
to Examine Koch's Postulates*

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*Funds for the development of these materials were provided in part by the National Science Foundation in their program for undergraduate instructional improvement.
I. Introduction

This exercise allows students to examine Koch's postulates, the foundation of pathogenic microbiology. It does not require the expense or facilities involved with the use of test animals, nor does it involve any of the dangers associated with using animal pathogens. Students are able to: (1) reproduce Koch's postulates using a vegetable pathogen, *Erwinia carotovora*, and a carrot as a model host tissue; (2) distinguish between the ability to grow on a host and the ability to damage the host using a second organism, *Escherichia coli*. These two points highlight the distinction between normal flora and pathogens.

This exercise has been used for several years in a microbiology service course for agriculture, home economics and humanities majors. The students ranged from freshmen to seniors and from those with no previous biology or chemistry courses to those who had completed organic chemistry. This exercise is the first in an investigative project dealing with pathogens and, as such, extensive detail is provided for the students. This was done intentionally to acquaint students with the components of experimental design; succeeding experiments in the project require the students to be responsible for increasingly greater portions of the experimental design. The basic element of the exercise is quite straightforward, making it suitable for a variety of introductory-level laboratories in which Koch's postulates are to be examined. The exercise can be used in botany, general biology, microbiology, or plant pathology laboratories. The key modifications would fall into three categories: (1) modifying the student materials to remove the present detail; (2) changing the initial source of the pathogen by having students isolate their own pathogens from a mixed population on a rotted vegetable (obviously, a pure culture of *Erwinia* is not a "natural" situation); (3) expanding the section that deals with the characterization of the pathogen. The last modification could be a good place to introduce those biochemical tests used in clinical identification exercises in many courses. Preparation time and performance time depend, in part, on the students' training and available facilities. The only techniques students must know are basic aseptic technique and the Gram stain. Preparation for this exercise requires that the following be done:

1. Maintenance of the organism: The *Erwinia* culture must be transferred to a fresh nutrient agar slant or carrot slice every 4–6 weeks.
2. Preparation of infected carrot slices for students: This involves about one hour for a class of 20 students, one to two days before the exercise is begun.
3. Preparation of materials for the exercise: This will vary depending on the extent to which one provides materials for the students or has them prepare the materials. If the instructor does this, it will take approximately three hours, for media preparation and pouring plates.
The time involved for student performance can be apportioned in several ways. If no storage points are used, the exercise will require approximately one hour/day for four days running, assuming that the students have had prior experience in aseptic transfer and the preparation of Gram stains. If they have not, a simple laboratory exercise in aseptic technique and Gram stain should be done first. These techniques are described in Appendix II and Appendix III. The exercise need not be done on consecutive days. Generally, the time intervals (see student materials) may be increased to two days; if a longer interval is necessary the materials can be stored in a refrigerator or cold room between steps.

I will describe several follow-experiments that have been used with this exercise to highlight various aspects of pathogenic microbiology and how to use this material in an investigative format in Appendix I.

II. Student Materials

Upon first realizing that we exist in a world filled with bacteria, most people's immediate response is "Ugh!" There are microorganisms on every object with which we deal and in and on many locations on our bodies. You must realize that this is the natural circumstance and learn to differentiate between those organisms whose existence is either harmless or beneficial to us and those which produce disease, the pathogens.

A pathogen is something that generates suffering. The word comes from the Greek words "pathos," meaning suffering, and "genesis," meaning origin. Thus, a pathogenic or disease-causing organism is not simply one which will grow on a host, but one that damages the host in some way by its presence.

The first step in the study of a pathogen and the disease it causes is to isolate and identify the pathogenic organism. This can be quite difficult since a variety of organisms is often present during the diseased state and the pathogen must be distinguished from the other organisms. In bacterial rots, for example, as the primary pathogen rots the vegetable, the rotted tissue provides a food source for many microorganisms and many different types may be present.

One of the major achievements in the early history of microbiology was the development by Robert Koch of a set of criteria by which one can identify the organism responsible for a particular disease.

Koch's Postulates:
1. The presumptive pathogen must be present in all cases of the disease.
2. It must be isolated in pure culture from a diseased organism.
3. Inoculation of the isolated pathogen into a healthy organism should cause the same disease.
4. One must be able to re-isolate the pathogen from the newly infected organism.

This exercise deals with the study of pathogens and disease. Due to the difficulty one has in obtaining human subjects, we will confine our studies to the investigation of "sick" vegetables. We will investigate plant pathogens and use the destruction of vegetable tissue as a model disease system. In this exercise, you will (1) use Koch's postulates to show that a particular organism is a pathogen and (2) demonstrate that another organism which also grows on the vegetable is not necessarily a pathogen within the context of our definition.

**Koch's Postulates Exercise**

**Part I--Day 1**

1. Obtain or pour a nutrient agar (NA) plate.
2. Prepare carrot slices as follows. Cut 3 slices of carrot approximately \(\frac{1}{4}\)" thick. Using forceps, dip one slice into alcohol. Pass the slice through a flame. The carrot will catch on fire. Let the flame burn out and IMMEDIATELY place the carrot slice into a sterile plastic petri plate. Repeat for the other two slices, placing each in a separate plate. Add 5 ml sterile water to each plate.
3. From the rotting carrot provided, aseptically transfer one loopful of rotting material to the surface of a carrot slice in a plate. Label the plate #1. Examine the rot organism under the microscope using the Gram stain.
4. Using a sterile loop, mock-inoculate a carrot stick in a second plate. Why is this done? Label the plate #2.
5. Immediately after the mock inoculation, streak the loop onto an NA plate. Why? Label the plate NA #2.
6. Transfer a loopful of *E. coli* to the carrot slice in a third plate. Label the plate #3.
7. Incubate the three carrot plates and the NA plate at 30°C for 24 hours.

**Day 2**

8. Check the plates for (a) growth of microorganisms on each carrot slice (check by observing under the microscope using the Gram stain) and (b) apparent rot of slices. The centers of the slices often show the first signs of rot; they appear macerated and torn/shredded, lacking in firmness when compared to the tissue of a healthy carrot. The slices may even become liquefied, or nearly liquefied, due to the rot organism which attacks the material that makes the carrot tissue firm. You can test for firmness by poking the slice with a sterile loop.
Part 2: Can be done on Day 2
1. Obtain or pour 2 NA plates.
2. Obtain isolated colonies from plates #1 and #3 by streaking a small amount of material from the inoculated slices in each plate onto separate plates of NA. Use the dilution streak technique. Label the plate made from material in plate #1, NA #1, and the plate from the material in plate #3, NA #3.
3. Incubate the NA plates at 30°C for 24 hours.
4. The carrot plates #1 and #3 may be kept until you are sure there is growth on the NA plates. They may then be discarded, along with plate #2, by sealing with tape and placing in a special garbage pail.

Part 3: Day 3
1. 48 hours from the beginning of the experiment, prepare carrot plate #4 using the procedure in Part 1. Inoculate with a loopful of material from an isolated colony from plate NA #1. Be sure to use a rod-shaped organism.
2. Incubate carrot plate #4 at 30°C for 24-48 hours.
3. Examine the isolated colonies on NA #1 and NA #3 from Part 2 using Gram stains.

Part 4: Day 4
1. Obtain or pour an NA plate.
2. Check for rot in carrot plate #4. (Use criteria described in Part 1: #8)
3. Do a Gram stain on the material from liquid or carrot in plate #4.
4. Obtain isolated colonies from carrot plate #4 by streaking a small amount of material from the inoculated slice onto an NA plate. Label the plate #4 NA.
5. Incubate plate for 24 hr. at 30°C. Check an isolated colony with a Gram stain.

Discussion
1. Explain the way in which the results you obtained relate to Koch's postulates.
2. Does the Gram stain alone provide enough information to prove that the disease-causing organism you finally isolate is the same one with which you started? What else should be done?
3. What effects would contaminants have on your conclusion as to the cause of the rot?
4. Would you conclude that E. coli is or is not a pathogen in the context of this experiment?
III. Instructor's Materials

This exercise is technically simple, but does require care at several steps. For the exercise to be successful, you must use a pure culture of *Erwinia carotovora*, which must be maintained in an uncontaminated state, particularly on the carrot slices given to students. While students can reproduce Koch's postulates starting with a mixed culture (possibly a good idea for an advanced class), students in introductory courses have great difficulty when faced with contaminants.

A. Obtaining culture: The organisms used in this exercise are *Escherichia coli* (any strain) and *Erwinia carotovora* ATCC #25270. The *Erwinia* can be obtained from the American Type Culture Collection. The ATCC catalog lists other strains that cause soft rot, but this is the strain which we used. If you use this strain, you must order the culture several months ahead of time since it is a plant pathogen and you must receive clearance from your state agricultural commission to obtain the organism. Forms for this purpose will be sent to you by ATCC on their receipt of your order. The forms must be forwarded to the proper authority, who will notify ATCC. Clearance generally does not pose a problem in obtaining a culture. Because this is a plant pathogen, *all infected materials must be autoclaved before discarding.*

B. Setting up stock culture:

1. ATCC provides a lyophilized culture. Instructions for starting the culture are sent by ATCC. You will need nutrient broth (NB-Difco). A culture grown up in NB can be stored in the refrigerator (4°C) for several months.

2. After starting the culture in liquid, streak a loopful of culture onto nutrient agar (NA-Difco) to obtain isolated colonies (see Appendix 11) and incubate 24-48 hrs./30°C.

3. Check an isolated colony with a Gram stain (see Appendix 11) to make sure it contains only small, Gram-negative rods. Use this same colony to inoculate a carrot slice to check for rot. See below.

4. You should identify several colonies in this way and prepare a stock culture from each. This can be done in the following manner:
   a. Streak onto the surface of an NA slant, incubate 24-48 hrs./30°C and store in a refrigerator (4°C) for up to six months.
   b. Stab a tube of NA with the culture. Incubate 48 hrs./30°C. Tape the tube shut and store for 12-14 months in a refrigerator.

   Use one of these two methods for storage of permanent stocks.

   c. Inoculate a sterile carrot slice (see below, Section D) by touching a drop of culture to the surface of the carrot. Maintain at room
temperature. Transfer some of the rot material every 2–3 weeks to a fresh carrot slice. This is not the method of choice for long-term stock maintenance.

5. A simpler procedure for culture maintenance may be used, but there is a much higher risk of contamination. After starting a liquid culture of the *Erwinia*, inoculate 100 ml of NB with a drop or two of culture and incubate for 24–48 hrs./30°C. This culture may be used to inoculate the carrot slices and they can be stored for several months in the refrigerator. A loopful of this may be transferred to fresh NB for subsequent cultures.

C. Once a year, it is a good idea to key out the culture using *Bergey’s Manual* and the strain data provided by ATCC to make sure you are carrying the original strain.

D. Preparation of a carrot slice and inoculation.

1. Start with a healthy carrot. Wash carrot with alcohol using a cotton swab. Peel. Cut three slices of carrot approximately ¼” thick. Using forceps, dip one slice into alcohol. Pass the slice through a flame. The carrot will catch on fire. Let the flame burn out and IMMEDIATELY place the carrot slice into a sterile plastic petri plate. Repeat for the other two slices, placing each in a separate plate. Add 5 ml sterile water to each plate.

2. Transfer a loopful of *Erwinia* culture onto the surface of a carrot slice. Incubate inoculated carrot slices for 24–48 hrs./30°C. Check for rot simply by observing the slice and by touching it with a sterile loop. It is quite clear from the feel whether the carrot is rotting.

E. A deficiency in the experimental protocol is the use of the Gram stain as the only means of determining that the *Erwinia* remains the rot-producing organism and not a contaminant. The organism should actually be keyed out thoroughly in a more rigorous experiment. You may streak a line of cells from any sample to be tested, particularly NA1, NA3 and NA4, onto a plate of Simmon’s citrate agar. This will differentiate between *Erwinia* and *E. coli* and is a simple way to help confirm the identity of the *Erwinia*.

F. Time course of experiment for students

1. First lab: obtain infected carrot
   transfer infection to fresh carrot
   interval: 24–48 hours/30°C or plates can be stored at room temperature for up to a week

2. Second lab: streak to isolate
   interval: 24–48 hours/30°C. Plates must then be stored in the refrigerator if not used immediately. Can be stored one week.
3. Third lab: carrot slice
   interval: 24–48 hours/30°C or can be stored at room temperature for one week

4. Fourth lab: streak to isolate
   interval: 24–48 hours/30°C. Plates must then be stored in the refrigerator (up to one week) if not used immediately.

5. Fifth lab: check second streak

G. Materials and suppliers for Koch’s postulate exercise:
   1. Suppliers

   Difco Laboratories
   Detroit, Michigan 48232

   Scientific Products
   1750 Stoneridge Dr.
   Stone Mountain, GA 30083
   or
   Fisher Scientific Products
   P.O. Box 829
   Norcross, Georgia 30091

   American Type Culture Collection
   12301 Parklawn Drive
   Rockville, Maryland 20852

   2. Materials for 20 students

   80 sterile plastic petri plates
   (for carrot slices)
   60 glass petri plates (sterile)
   60 NA pours (25 ml/pour)
   (plates may be pre-poured for students)
   20 beakers of 10 ml 95% or absolute ethanol
   20 slices of *E. carotovora*-infected carrot in 20 separate petri plates

   20 scalpels or spatulas (to slice carrots)
   20 forceps
   20 bottles of 30 ml sterile water each
   60 ml stock culture of *E. coli* grown for 24–48 hrs. in NB 30°C incubator
   20 bunsen burners
   20 inoculating loops
   glass slides
20 Gram stain kits: dropper bottles of
(a) crystal violet;
(b) Lugol's iodine
(c) 95% alcohol
(d) safranin
50°C water bath for cooling
melted agar

References

APPENDIX I
Use of Related Materials in an Investigative Format

The exercise on Koch's postulates was developed as part of a course in which students participated in a multi-level investigative project. The investigative format is used to develop an understanding of what constitutes scientific investigation that transcends the piecemeal approach which stresses the learning of loosely related facts and the pursuit of cookbook experimentation. The factual and conceptual material is better understood and provides a basis for the integration of new information as it arises. The students are engaged in the same process as scientists, working toward a goal, as in actual scientific investigation.

In the course, students first learn basic laboratory technique and are then introduced to experimental design through a series of experiments. The first experiment in the series is presented with all details provided, from background information to ways in which results are interpreted. Subsequent experiments require that the student provide more and more of the design components. Finally, the students are given a catalog of techniques and assays and asked to develop a project in a particular area.

The Koch's postulates exercise was developed as the first experiment in a project that is intended to introduce the student to the area of pathogenic microbiology.

Once the identity of the pathogen has been established, a number of aspects of the disease may be studied. The means by which the organism damages the plant tissue can be examined. This is mediated by a set of enzymes which degrade the pectin found in the tissue. The activity of these enzymes may be measured as a function of colony growth or by measuring their presence in the growth medium. The vegetables
may be examined for the presence of other organisms that cause rot, or soil and other locations may be checked for this particular pathogen. Students can study the course of the disease itself, the spread of the organism or how it enters the vegetable. Students may also examine the effect of different storage conditions on the course of the disease or those factors involved in resistance to the disease or loss of virulence of the pathogen. The following are capsule forms of exercises we have used successfully as followups to the Koch’s postulates experiment.

I. Sick potato—live tissue
   A. Protocol exactly as given in student materials except for step 1 in part 1-day 1, where potato blocks are used instead of carrots. Prepare potato blocks as follows: scrub and rinse a potato. Soak potato for 10 min in a 7:3 ethanol: clorox mixture. Rinse with sterile water. Slice ends off potato with a sterile knife, then make 4 cuts to obtain rectangular block.
   B. This is an alternative to using carrot, but is not used routinely in large groups due to the difficulty in sterilizing the tissue. Can be made to work in small groups.

II. Sick potato—dead tissue
   A. Again protocol is the same as given except for tissue preparation. In this case, potatoes are peeled, cut into small blocks, placed in test tubes to which 5 ml water is added and autoclaved for 10 min.
   B. This is another alternative to using carrot but is not strictly Koch’s postulates since the tissue is not live. It is easy to set up a large lab and the students are generally not aware of the difference between live and dead tissue.

III. Assay
   Pectic substances occur in nature solely in the cell walls of plants, usually as the insoluble structural material, protopectin. A group of more soluble derivatives of this native substance can be extracted from plant tissues; these are the pectic acids and pectin. Pectic or polygalacturonic acid is usually considered to be a homopolysaccharide made up of unbalanced chains of \( \alpha-(1,4) \)-linked D-galacturonic acid residues; pectin itself is pectic acid with varying extents of methyl esterification (Starr and Chatterjee). Two basic types of pectin enzymes exist: pectin esterase which simply removes methoxyl residues from pectin, and a range of depolymerizing enzymes which can be distinguished under three headings: whether pectin or pectic acid is the preferred substrate; whether they act by transelimination or hydrolysis; and whether cleavage is random (endo-) or endwise (exo-) (Progress in Industrial Microbiology).

Pectin is available from several sources; Na polygalacturonate came from Sigma.

A. Growth on solid media
   1. PEC-SSA medium (Starr, Chatterjee, Starr, Buchanan). This is a gel of polypectate medium; if pectin digestion activity is present, colonies will sink into the agar. This medium is used to show pectin degrading activity with moderately vigorous digesters; for weak digesters, omit the agar from the recipe.

Place 100 ml distilled water and a magnetic stirring bar in a 1-liter Erlenmeyer flask on a heater with a magnetic stirrer. Add the following ingredients in order, while stirring and heating: 0.6 ml 10% aqueous CaCl\(_2\), 2H\(_2\)O; 1.0 ml of 0.1% aqueous bromothymol blue in 6.4 \( \times \) 10\(^{-4} \) N NaOH; 0.5 g Difco yeast extract; 3.0 g sodium polygalacturonate; and 0.3% Difco agar. Heat almost to boiling, watching carefully, as material has a tendency to foam. While solution is hot, adjust pH to 7.3 with 1 N NaOH. Autoclave and pour plates (PEC medium cannot be remelted).
2. PEC-YA medium (Starr, Chatterjee, Starr, Buchanan). Pectate digestion activity is shown by flooding a 24-hour plate with 2 N HCl. The areas around colonies that have digested pectate will show as clear against a hazier area of precipitated pectate. This is a good medium for spreading and isolating organisms. Replica plating can be done when isolating colonies. Theoretically, you can use pectin in this medium, but it has worked better for us with polypectate. Individual batches of polypectate should be tested before classroom use.

Place 100 ml distilled water and a magnet stirring bar in a 1-liter Erlenmeyer flask on a heater with a magnetic stirrer. Add the following ingredients in order, while stirring and heating: 1.0 g polygalacturonic acid; 1.0 g yeast extract; 1.0 ml of 1.0% aqueous BTB in 6.4 × 10⁻⁴ N NaOH; and 1.5 g agar. Heat as described for PEC-SSA, adjust pH to 7.3 and autoclave. Pour plates. To use this medium, streak or spot cultures on surface and allow to grow. Flood plates with 2 N HCl. Score by appearance of clear halos around and beneath colonies in otherwise turbid medium. See special instructions in Starr et al. (1974).

B. Enzyme activity in culture medium: viscosity

Viscosity measurements have found widespread use for determination of pectinolytic activity. This assay is based on the loss of viscosity of aqueous solutions of pectin or sodium pectate following decrease in chain length (Progress in Industrial Microbiology).

While there are quantitative assays using the Oswald viscometer and similar methods, we have found that a similar method using a narrow bore, long-tipped pipet gives sufficient accuracy in a classroom situation.

Protocol:
1. Prepare a 5% solution of pectin in water.
2. Place 1 ml pectin solution/tube. Prepare one tube for each solution to be tested and one control.
3. Add 1 ml solution to be tested to pectin. Add 1 ml water to control. Mix well.
4. Mix 0 time reading as follows: fill 1-ml pipet with pectin mixture. Time solution as it runs out of pipet to the 0.8-ml mark.
5. Take readings of run-out time at 5, 20, 15 min or as required.

IV. Isolation of rot-producing organisms: Crystal violet pectate medium (Cuppels and Kelman)

A. This method allows one to isolate Gram-negative rotters from field soil and distinguishes Erwinia from Pseudomonas. Using this medium you can quantify the amount of soft-rot organisms present in soil samples. We have had some problems with this medium; the pH determination is critical.

PROCEDURE FOR PREPARATION OF CRYSTAL VIOLET PECTATE MEDIUM FOR ISOLATION OF PECTOLYTIC BACTERIA (Cuppels and Kelman, 1974)
1. Preheat 5-cup Waring blender by rinsing with hot water.
2. Place 500 ml boiling water (distilled) in the blender.
3. Using a rheostat to control the speed of the blender, start the blender at low speed and add:
   a. 1.0 ml 0.75% (w/r) aqueous crystal violet solution (final concentration = 1.5 ppm crystal violet)
   b. 4.5 ml 1 N NaOH (8 g NaOH/200 ml)
c. 3 ml 10% CaCl₂ • 2H₂O (Use fresh solution. Do not store stock solution for more than 2 weeks)
d. 2.5 g Difco agar
e. 1 g NaNO₃
4. Blend at high speed for 15 sec.
5. Slowly add 15 g sodium polypectate while blending so that the sodium polypectate will not clump. Blend at high speed for 15 sec.
6. Place medium in a 2-liter flask. Add 0.5 ml 10% sodium lauryl sulfate (SDS-laboratory grade).
7. Cap with aluminum foil rather than a cotton plug. Autoclave for 25 min at 120°C and 15 lb pressure. Allow pressure to drop slowly to avoid bubbles.
8. Pour plates as soon as possible. Plates should be permitted to dry for 48 hr at room temperature before being used. No surface water should be present at time plates are used. Note: This is a very important aspect in the successful use of this medium.
9. (Optional) Add 0.9 ml 1% thallium nitrate-filter sterilized (final concentration = 0.0018%) to the hot medium (500 ml).
Addition of solution of thallium nitrate to give a final concentration of 0.0175 mg/ml in CVP medium will greatly enhance reduction of background populations, but also slightly reduce recovery of Erwinia. Medium containing thallium nitrate should be used immediately. There is an increase in toxicity to bacteria including Erwinia if this medium is stored.

V. Environmental factors affecting rot in vegetables
The key to examining these factors is a tissue system in which the amount of rot can be quantified. Once this is developed, one can examine the effect of environmental factors on the course of the disease. The tissue we use is potato. Its use yields relatively reproducible results.
A. Use a firm, healthy potato with no surface lesions. Swab the potato surface with alcohol. Using a sterile needle dipped into a liquid culture of the pathogen, inoculate four sites on the potato, making the inoculations as far from each other as possible. Use one potato for each environmental variable you wish to study. Incubate each potato in a beaker covered with foil. Environmental variables may include temperature, time, atmosphere (nitrogen, carbon dioxide, etc.), relative humidity, etc. At the time selected, remove the potato from the beaker and slice through the point of inoculation perpendicular to the surface. The rot will have spread perpendicularly to the initial stab, so you may measure the distance it has spread from the initial inoculation line. It is very easy to distinguish healthy tissue from rotted tissue. The average of four sites is used to increase the reproducibility of the data. One can then plot the spread of rot against the variable examined.

- Site of inoculation
\[ Cutting \ line \]
\[ Spread \ of \ rot \ from \ site \ of \ inoculation \ (cross-section) \]
B. A flamed dissecting needle will work for making the inoculations, although the precision of the inoculation can be increased by using a Hamilton syringe. In this case, you may inject a maximum of 5 microliters/site.

References for Appendix I
   A medium for the isolation of *Erwinia* from soil.
   A procedure to quantify the rotting ability of the organism in a vegetable. Allows one to measure effect of environment on rate and could be used to compare virulence of different strains *in situ*.
   Key reference detailing the chemistry of pectic compounds, the enzymology of pectin-degrading enzymes, and many assays of pectinolytic activity. Also deals with their occurrence in nature and industrial production and usage.
   Gives several assays of pectinase activity using solid medium.
   Details several methods of using solid growth medium to determine whether organisms have rotting (pectinolytic) ability.

APPENDIX II

I. Procedure for basic smear preparation and a simple stain
   A simple stain involves the use of a single dye and enables us to see the general shape and arrangement of cells.

Materials

- crystal violet
- safranin
- methylene blue
- various cultures will be available in the laboratory slides

Protocol
1. Pass a clean glass slide through a flame to clean off lint, etc. Place slide on table, flamed side up.
2. Hold an inoculating loop using the thumb and index finger.
3. Insert the loop in the upper area of the flame at a 60° angle until the entire wire portion is heated red-hot to kill any organisms on the loop.
4. Hold the culture tube in the palm of the left hand with the sides of the tube resting against the insides of the fingers. (If you are left-handed, reverse the directions.)
Hold the tube in place with the thumb. Remove the tube cap with the little finger of the hand holding the loop.

5. Pass the lip of the tube through the flame once to kill any organisms that might be present.

6. Insert the loop into the tube, jiggle it and withdraw a loopful of broth.

7. Flame the lip of the tube, bring the cap to the tube to close, and replace the tube in the rack.

8. Place the material on the loop onto the slide and spread the drop to cover an area approximately that of a dime. Reflake the loop before putting it down on the lab bench.

The smear is made by spreading a small amount of cellular material from a liquid culture on the surface of a clean glass slide. (The directions for preparing a smear of a culture on an agar plate are given following step 14.) The resulting film is air-dried and "fixed" to the slide by passing it through the flame of a bunsen burner. Fixing not only kills the cells but also coagulates the cellular proteins, thus fastening or "fixing" the organisms to the slide. The material is then ready for staining. Fixed smears are exposed to a stain for a specific amount of time, then washed, blotted dry and examined under the microscope.

9. Allow the smear to air dry. Do not apply heat before the smear is dry since this may cause boiling, which will result in broken cells. You should be able to see the smear, but it should be thin and fairly transparent. Circle the smear area with a wax pencil.

10. When the smear is dry, fix it as described above. Do not overheat. The slide should be uncomfortably hot but not intolerable when touched to your wrist.

11. Apply about 5-6 drops of dye to the slide. Stain for 45-60 seconds if using crystal violet or safranin. Methylene blue requires 2-3 minutes for good staining.

12. Pour off the stain and rinse gently with slowly running water. Do not allow the water to drop directly onto the smear.

13. Dry the slide by blotting (do not rub) between pages of bibulous paper.

14. Examine the slide under the oil immersion lens of your microscope.

NOTE: The same procedure may be followed for staining bacterial material taken from agar with the following exception: Place a drop of tap water on the slide, then follow steps 1-5. To remove cells, merely touch the loop to a colony. This should remove sufficient cells. Emulsify the cell material in the drop of water and continue with step 7. Continue as described.

II. The Gram stain—a differential stain

A second type of stain widely used in microbiology is the differential stain. As the name implies, it takes advantage of a differential response to the stain procedure. This differential response may be between different cells or between different portions of the same cell. Differential stains may involve the use of more than one dye and, sometimes, a specific de-staining step between the dye applications.

The most widely used differential stain is the Gram stain. It is used to divide nearly all bacteria into two major groups: Gram-positive and Gram-negative. Gram-positive organisms (such as Staphylococcus and Bacillus) retain the initial violet stain; Gram-negative organisms (such as Escherichia and Pseudomonas) lose the primary stain when decolorized and are then restained by the red counterstain. The Gram
reaction, besides being a constant characteristic (although there are some exceptions) of a cell and of great value in identification, correlates well with a variety of cell properties. (See chart.)

<table>
<thead>
<tr>
<th>Gram-Positive</th>
<th>Gram-Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>More susceptible to penicillin,</td>
<td>Less susceptible to penicillin,</td>
</tr>
<tr>
<td>sulfonamides and basic dyes</td>
<td>sulfonamides (except G-diplococci)</td>
</tr>
<tr>
<td>Less susceptible to streptomycin</td>
<td>More susceptible to streptomycin</td>
</tr>
<tr>
<td>Lysozyme degrades cell wall</td>
<td>Lysozyme digests cell wall only with addition of</td>
</tr>
<tr>
<td></td>
<td>EDTA or alkaline pH</td>
</tr>
<tr>
<td>Relatively thick cell walls, few acids,</td>
<td>Cell walls slightly thinner, but more</td>
</tr>
<tr>
<td>only 1–4% lipid</td>
<td>complex, consisting of several layers;</td>
</tr>
<tr>
<td></td>
<td>most of the amino acids found in proteins,</td>
</tr>
<tr>
<td></td>
<td>22% lipid</td>
</tr>
<tr>
<td>Nutrient requirements often complex</td>
<td>Nutrient requirements usually relatively simple</td>
</tr>
</tbody>
</table>

The basis for the differential response is one of rate rather than an absolute characteristic of bacteria. Thus, the procedure must be performed with great care. Too long a decolorization will result in Gram-positive organisms appearing to be Gram-negative. Also, many Gram-positive bacteria become Gram-variable as the culture ages, so only young cultures should be used in determining the Gram reaction.

Although the mechanism of the Gram reaction is still unknown, it seems to be related to differences in the cell wall structure. The thicker, Gram-positive walls appear to be impermeable to the dye-iodine complex, while the dye-iodine complex washes out quickly in Gram-negative cells exposed to the lipid solvents acetone and alcohol. While we do not generally deal with them in lab, you should be aware that there are two other Gram reactions: the Gram-variables (Neisseria), which may be either Gram-positive or Gram-negative, and the non-reactors which do not stain or stain very poorly (Spirochaetes, Mycobacteria).

The sequence of steps in the Gram stain is shown below:

<table>
<thead>
<tr>
<th>Primary stain (crystal violet)</th>
<th>Mordant (iodine)</th>
<th>Decolorization (acetone: alcohol)</th>
<th>Counterstain (safranin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram Positive</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gram Negative</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Materials
(Solution I) 1% aqueous crystal violet
(Solution II) 2% aqueous iodine
(Solution III) 95% ethanol:acetone (7:3)
(Solution IV) 2.5% safranin in 95% ethanol and water (1:9)
slides
various cultures will be available in the laboratory

Protocol
1. Prepare a smear of 12-18-hour culture. (See directions in Simple Stain.)
2. Fix the smear.
3. Stain I: Cover the smear with Solution I. Stain for 1-2 minutes. Pour off stain and rinse gently with a small amount of tap water.
4. Cover with Solution II for 2 minutes. Wash briefly with tap water.
5. Decolorize with Solution III by dripping it over the tilted slide until the wash is colorless. This is the most critical step and proper timing is important. It should not take more than 15-20 seconds.
6. Counterstain: Cover with Solution IV for one minute.
7. Rinse with tap water and blot carefully on bibulous paper.
8. View under oil immersion lens. Gram-positive bacteria are purple; Gram-negative bacteria are pink.

APPENDIX III
Aseptic Technique

Organisms must often be transferred from one container to another to begin growth in fresh medium or in a different medium, when changing growth conditions, for isolation, or for enumeration. This must be done without contaminating the test organism or having it contaminate anything. Since microorganisms are found everywhere, the test organism can be contaminated by another organism from the air, glassware, or people, thus spoiling the test results. Also, while we do not routinely work with disease-producing organisms, all microbes are potentially harmful under certain conditions, so we must control their spread. The methods of transfer all employ specific steps to minimize the possibility of contamination or spread. This general approach, termed aseptic technique, is not a particular method, but is a concept that covers almost every manipulation performed in the laboratory.

In this section we will describe the techniques for transfers used in growing microorganisms. In the section on stains you were told one method for sampling that involved aseptic technique. In the section on media you will learn how to perform transfers for isolation and enumeration.

There are two common tools to transfer organisms: the inoculating loop and the pipet. The inoculating loop we use is a thin metal wire formed into a loop at one end and held in a metal holder.

Pipets are basically calibrated glass tubes, tapered at one end, which are used like a straw for transferring a specific amount of liquid. If you have not previously used a pipet, the TAs will give a demonstration and a TV tape on pipetting is available for viewing.
Koch's Postulate

Transfers are made of organisms growing in either solid or liquid media to either solid or liquid media. They may be from one tube to another tube (tube-to-tube), from a tube to a plate or from a plate to a plate.

The directions given below are for a right-handed person. If you are left-handed, merely reverse the directions.

**Transfers Using a Loop**

1. Tube-to-tube transfer
   a. Loosen the tube caps, but do not remove them.
   b. Hold both tubes in the palm of the left hand, with the sides of the tubes resting against the insides of the fingers and using the thumb to hold them in place. The tube containing material to be transferred should be nearest the little finger.
   c. Hold the loop between the thumb and index finger of the right hand. Insert the loop into the upper portion of the flame at about a 60° angle. Flame the loop until it is red-hot, remove from the flame and let it cool. Briefly count to ten. The loop is flamed to kill any organisms that are on it. It is then cooled so it does not fry organisms in the culture.
   d. Using the ring and little fingers of the right hand, remove the cap from the tube containing the culture and flame the lip of the tube, passing it through the flame only once. The tube is flamed to kill organisms around the lip, but it is flamed only briefly so the tube does not get too hot.
   e. Insert the cooled loop without touching the sides of the tube and just touch the loop to the culture material. (With liquid medium, just immerse the loop in the top portion of the liquid after vortexing the tube to ensure distribution of the organism.) This is usually sufficient to obtain the proper number of cells for transfer.
   f. Withdraw the loop, flame the lip of the tube and replace the cap by bringing the cap to the tube. The culture tube is capped before continuing with the manipulation to protect the original culture. The cap is brought to the tube, rather than vice versa, to minimize the possibility of contamination by organisms in the air.
   g. Remove the cap of a fresh tube, flame the lip, insert the loop to the bottom of the slanted area of solid medium and draw it up across the surface to disperse the organisms on the fresh medium. (If transferring the liquid medium, insert the end of the loop into the liquid and gently jiggle the loop.) Withdraw the loop, flame the lip of the tube and replace the cap. Flame the loop.

Flame the loop before putting it down on the lab bench to kill any organisms remaining on it.

h. Tighten the caps on the two tubes and replace them in a rack.

i. Summary:
2. Stab/Stab and streak. These two methods are variations of the tube-to-tube transfer. In this procedure, the organism is removed from the first tube as described above. The fresh tube is inoculated by stabbing the inoculating loop through the medium to the bottom of the tube. Draw the loop straight out. For a stab-and-streak, stab to the bottom of the tube, then draw the loop back up across the slant surface. Complete the steps as described above.

3. Tube-to-plate
   a. In this method of transfer, material is removed from the culture tube as described.
   b. Using the left hand, lift the lid of the plate to approximately a 45° angle.
   c. Insert the loop to touch the surface of the agar at the furthest point from the open side and zig-zag the loop across the surface.

   In streaking the loop on an agar surface, you must be careful not to break into the agar with the loop.

   d. Withdraw the loop, lower the lid and flame the loop.
   e. Summary:
Transfers Using a Pipet

When bacteria are in a liquid medium and must be transferred from one container to another, pipetting may be the method used, particularly when a specified quantity of material is to be transferred. This method is not used when dealing with a dangerous
or potentially dangerous organism, as the possibility of ingesting some of the liquid while pipetting is too great.

Sterile pipets are kept in the lab in round or square metal cans labeled with the size pipet they contain. Bring the can to where it will be used. Do not carry sterile pipets across the room or allow them to touch anything or they will no longer be sterile. To remove sterile pipets from cans, lay the can on its side with the edge against the edge of the bench. Use the right hand to pull out a pipet from the top of the stack in the can, being careful not to touch the tip of the pipet to the ends of other pipets or to the lip of the can as these areas are usually not sterile. When using a sterile pipet, pass it through a flame only once to kill any surface bacteria before using it in sterile liquid. Do not overheat as this can be injurious to both bacteria and chemical solutions.

1. Tube-to-tube transfer
   a. Loosen the tube caps, but do not remove them.
   b. Hold both tubes in the left hand (see procedure for tube-to-tube transfers with a loop).
   c. Remove a sterile pipet from a pipet can using the method described above.
      If the pipet does touch something, discard it and take a fresh pipet. Do not put an unsterile pipet back in the can.
   d. Hold the pipet between the thumb and middle finger of the right hand, with the index finger resting loosely on top of the pipet. Flame the pipet by passing it once through the flame.
      An overheated pipet will kill many or all of the bacteria you are transferring. This can cause serious error when you are counting a specific volume of cells and is a factor to be considered if no growth appears after such a transfer.
   e. Using the little finger and palm of the right hand, remove the cap from the tube containing the culture. Flame the lip of the tube.
   f. Insert the pipet into the tube and withdraw culture to above the zero line on the pipet. Rest the tip of the pipet against the inside wall of the tube and let culture run out until the liquid is at the zero mark.
   g. Withdraw the pipet, flame the tube lip and bring the cap to the tube to close.
   h. Uncap the fresh tube, flame the lip, touch the tip of the pipet to the inside of the tube and let the required amount of inoculum run in.
   i. Withdraw the pipet, flame the tube lip and replace the cap.
   j. Immediately place the pipet, tip down, in a dirty pipet bucket. Never put a dirty pipet on the lab bench. Tighten the tube caps and replace the tubes in a rack.
   k. Summary:

![Diagram of steps](image-url)
2. Tube-to-plate transfers are similarly performed. As in transfers with a loop, the plate lid is lifted to a $45^\circ$ angle. Inoculate the plate by touching the tip of the pipet to the center of the agar and letting the required amount of liquid run out. Remove the pipet, lower the plate lid and immediately place the pipet, tip down, in a dirty pipet bucket.