DETERMINATIVE BACTERIOLOGY

Laboratory Manual

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PREFACE

A survey of textbooks in the field of bacteriology has shown a need for a laboratory manual dealing with the procedure for identifying unknown bacteria. Some texts consider a small group of organisms, such as the Enterobacteriaceae or the Streptococceae. The Manual of Microbiological Methods has excellent material for advanced students and research workers, but it is hardly suitable for classroom use without considerable elaboration by the instructor on how to carry out a systematic program for identification of an unknown bacterium.

This laboratory manual on the elements of determinative bacteriology has been compiled to meet the needs for a step-by-step procedure for identifying the common aerobic, microaerophilic and facultative anaerobic members of the Pseudomonadaceae, Spirillaceae and Eubacteriales.

The basic concept of this text is to lead the student through the various, progressive steps of isolation, purification, morphologic study, cultural study, and biochemical study of an organism. At all times the student is being trained to perfect his skill in routine techniques by repetition on many different organisms. He thereby gets a better picture of the "individuality" of organisms within certain "greater patterns" of shape and behavior.

It is not felt that this manual should cover every known genus of the Schizomycetes, hence, only the more common or representative aerobic groups are studied in detail. Likewise, since time is limited in course work the groups studied are those with the more rapid growth rates and without too fastidious nutritional requirements.

The manual was designed for a course having three two-hour laboratory sessions per week. The instructor may delete or add experiments to those presented herein to suit his own needs. To accomplish the majority of experiments as presented, it is presumed that the student will not have to use his laboratory class-time in washing glassware or preparing media.

During the course of the laboratory work, reference should be made to other available manuals of methods for pure culture study of bacteria, the 7th Edition of Bergey's Manual of Determinative Bacteriology and taxonomic monographs.

Of necessity many fundamental procedures learned in beginning courses have been repeated to aid the student in refreshing his memory and to give completeness to the manual. The author is grateful to several whose kind assistance was invaluable during the publication of this manual.

A text of this type invariably seems to have omissions and arrangements which are awkward for some users. Suggestions from users will be gratefully appreciated as an aid to improving the usefulness of future editions of the manual.

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Exercise I

ISOLATION TECHNIQUES FOR SECURING PURE CULTURES FROM NATURAL MATERIALS

The first step in making a determinative study of a bacterium from a natural material is to isolate the organism in pure culture upon a suitable medium. Many methods of isolation are available. The two following methods are satisfactory and have found wide favor.

First Day: Procedure A: The Streaked Plate Method

1. Melt a tube of sterile Nutrient Agar medium (or other suitable agar medium) at 100°C.

2. Cool the melted medium by placing in a water bath adjusted to 45°C. for 5 minutes.

3. Aseptically pour the cooled medium into a sterile Petri dish; tilt the dish to cause medium to cover the entire bottom of the dish.

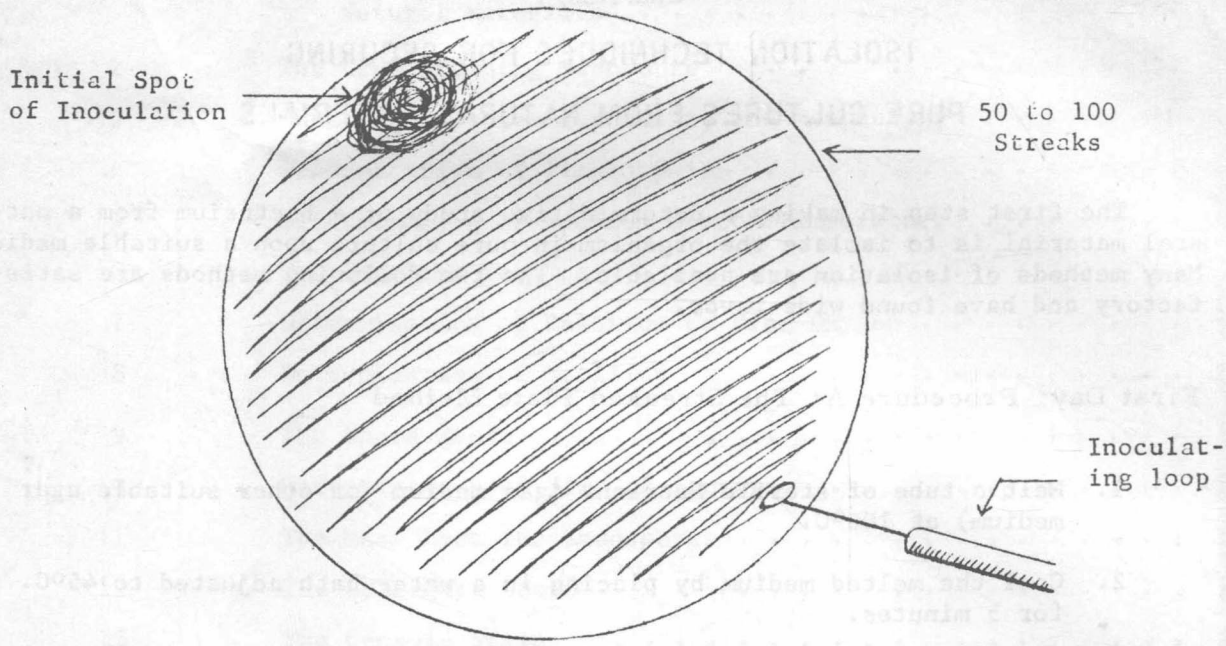
4. Allow medium to stand undisturbed until completely solidified, at least 15 minutes.

5. With a sterile inoculating loop (or special streaking needle) dip into the material to be cultured, then knock off the excess material from the loop against the inside of the container. This will leave the inoculating-loop wire coated with a microscopically thin film of material.

6. Rub the loop on the surface of the solid medium near one edge of the dish, spreading the material over an area about the size of a dime.

7. Starting at the spot of inoculation, streak back and forth across the surface of the medium, taking care not to cut into the surface of the agar. Keep the streaks as close together as possible (if they should overlap slightly it will not matter). Continue streaking back and forth until the entire surface of the medium has been covered with streaks as shown in the accompanying diagram. The success of this procedure depends on having from 50 to 150 streaks on the surface of the medium. (See page 2.)

8. Invert the inoculated plates and incubate at the temperature specified by the instructor for the particular organisms being sought.



Procedure B: The Loop Dilution (Poured Plate) Method

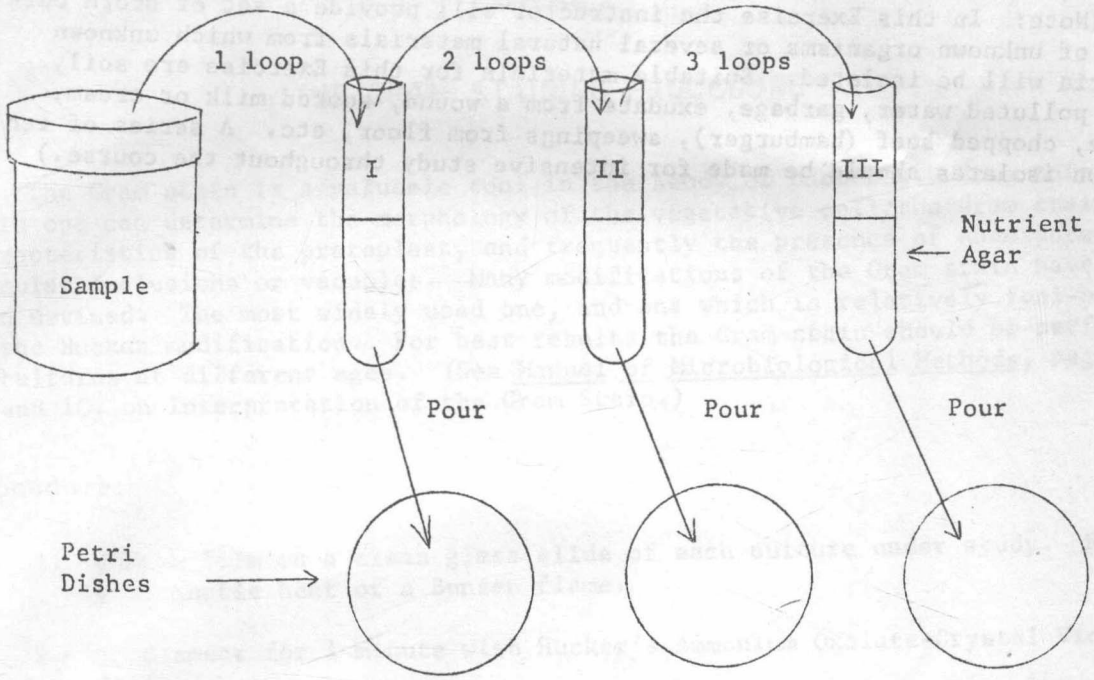
1. Obtain three tubes of liquid Nutrient Agar (or other suitable agar medium) and keep them in a water bath at a temperature of 45°C. to 48°C for 5 minutes. Label the tubes 1, 2, and 3. Label three sterile Petri dishes 1, 2, and 3.

2. With a sterile loop transfer one loopful of the material to be cultured to agar tube No. 1 and mix by stirring with the loop. Flame the loop. (Note: Return all tubes of medium to the water bath immediately after transfers are made. A few minutes at room temperature will cause agar media to solidify.)

3. Transfer two loopfuls of agar from tube No. 1 to tube No. 2 and mix. Flame the loop.

4. Transfer three loopfuls of agar from tube No. 2 to tube No. 3 and mix. Flame the loop.

5. Aseptically pour the agar in tube No. 1 into the dish No. 1, replace the cover immediately, and gently tilt the Petri dish until the agar has completely covered the bottom. In a similar manner pour the contents of tubes Nos. 2 and 3, into Petri dishes Nos. 2 and 3, respectively. (See page 3.)



6. Allow the agar to solidify in the plates and label the plates.

7. Invert the plates and incubate at the temperature specified by the instructor.

Second Day:

1. Observe the types of colonies developing on the plates from Procedure A or B.

2. Select ten colonies representative of the predominating organisms in the materials cultured, and encircle the colonies on the bottom of the dish with a wax crayon.

3. Describe the colonies and record the description on the Descriptive Chart in the space for Agar Colonies.

4. Make transfers from the selected colonies to Nutrient Agar slants or other appropriate medium; incubate at an appropriate temperature.

5. Make Gram stains of slides prepared from the original colonies selected and record morphologic features and Gram reaction of the organisms on the Descriptive Chart.

Discard any cultures which are not pure, and select new colonies from which to work.

The procedure for the Gram Stain is given in Exercise 2.

(Note: In this Exercise the instructor will provide a set of broth cultures of unknown organisms or several natural materials from which unknown bacteria will be isolated. Suitable materials for this Exercise are soil, dung, polluted water, garbage, exudate from a wound, soured milk or cream, cheese, chopped beef (hamburger), sweepings from floor, etc. A series of ten unknown isolates should be made for intensive study throughout the course.)

Exercise 2

THE GRAM STAINING PROCEDURE

The Gram stain is a valuable tool in the study of bacteria. From this stain one can determine the morphology of the vegetative cell, the Gram staining characteristics of the protoplast, and frequently the presence of endospores, granular inclusions or vacuoles. Many modifications of the Gram stain have been devised. The most widely used one, and one which is relatively fool-proof, is the Hucker modification. For best results the Gram stain should be performed on cultures at different ages. (See Manual of Microbiological Methods, pages 17 and 18, on Interpretation of the Gram Stain.)

Procedure:

1. Make a film on a clean glass slide of each culture under study. Fix with gentle heat of a Bunsen flame.
2. Flood smear for 1 minute with Hucker's Ammonium Oxalate-Crystal Violet Stain.
3. Wash thoroughly in water. (Tap water may be used here.)
4. Flood smear for 1 minute with Gram's Iodine Solution.
5. Wash in tap water and blot off excess water. Do not allow to become completely dry.
6. Decolorize by dripping 95% Ethyl Alcohol over the slide until streaks of color stop coming from the smear. (Usually about 30 seconds.)
7. Wash immediately in water. Drain off excess water from slide.
8. Counterstain 10 seconds in Safranin Counterstain solution.
9. Wash thoroughly in tap water, blot, dry, and examine under oil immersion objective.
10. Record results on the Descriptive Chart in the space provided for the Gram stain. Be sure to record the age of the culture at the time the smear was made!

Gram positive organisms are blue-violet, Gram negative organisms are red, and Gram variable organisms may show part blue-violet and part red, or be blue-violet at one age and red at another age. Interpretation must be made cautiously!

Exercise 3

CHECKING CULTURES FOR PURITY AND MORPHOLOGY

After isolating an organism from a material, it is then essential to determine whether the culture is pure. All work in determinative bacteriology presupposes that cultures are pure.

Procedure:

1. After 24 to 48 hours' incubation, observe the agar slant cultures prepared in Exercise 1 for growth.

2. Make a record of the appearance of each stroke culture on the Descriptive Chart in the space headed Agar Stroke.

3. Make Gram stains of all stroke cultures and check the smear for purity. Any culture which is obviously mixed as indicated by the presence of two or more morphologic types of bacteria or different Gram staining reactions should be repurified by making a heavy suspension of the growth in Nutrient Broth and reisolating according to Exercise 1. (Care must be taken to interpret the Gram reaction correctly since age, temperature of incubation, etc., may influence an organism so that part of the cells may be Gram positive and part may be Gram negative.)

4. Save all cultures proven to be pure as "Stock Cultures" and complete the part of the Descriptive Chart pertaining to Cell Morphology, as much as is applicable.

5. Record the Gram reaction and the age of the culture from which the smear was made.

6. Save the stained slides for future reference.

(Note: The following morphologic features should be especially noted: (a) Shape, (b) presence of branching, (c) trichome formation, (d) presence of endospores, conidia or other fruiting structures, (e) presence of sulfur granules or other granules, (f) vacuolation of cytoplasm, (g) irregular or bipolar staining, (h) presence of capsules or sheaths and (i) irregularly shaped cells. In all morphologic and/or cultural studies, note should be made of the age of cultures and medium employed for each test because these may be important factors in influencing the results of the tests. Any culture having morphologic features of a member of the order Actinomycetales should be discarded after consultation with the instructor.)

Exercise 4

DEMONSTRATION OF PLEOMORPHISM

Pleomorphism frequently occurs in cultures, especially with aging or transfer to different media. This phenomenon may give the student some consternation if he does not appreciate the extent to which an organism can change its morphology as a result of pleomorphism.

This Exercise is designed to impress the student with the variability in the morphology of an organism so that he will not be confused by similar observations in his future work.

Procedure:

1. Prepare a series of tubes containing 5 ml. Nutrient Agar medium and sterilize.

2. Divide the tubes into four lots.

3. To lot Number 1 add 1 ml. of sterile 10% Lithium Chloride solution, mix, and lay tubes in slanting position to solidify.

4. To lot Number 2 add 2 ml. of the LiCl solution and to lot Number 3 add 3 ml. of LiCl solution and treat as above.

5. Slant tubes from lot Number 4 without adding any LiCl.

6. When the media prepared above are solid inoculate one tube of each lot with a pure culture of *Escherichia coli*, and one tube of each lot with one of the bacilli or coccobacilli from the collection of "unknowns".

7. Incubate at optimum growth temperatures for the organisms used.

8. After 48 hours make Gram stains of the growth from each slant, and note the variability in the organisms with varying amounts of LiCl.

9. Re-examine the Gram stains prepared in Exercise 3 of the "unknown" cultures for evidence of pleomorphism.

Exercise 5

DETERMINATION OF OPTIMUM GROWTH TEMPERATURE

The temperature of incubation employed in the original isolation of a culture might not be the optimum for the organism. In order to be assured that the organism will be functioning efficiently in subsequent determinations, one should ascertain the optimum growth temperature rather early in the identification of the unknown organism.

Procedure: First Day.

1. Make stroke cultures on slants of a suitable agar medium, in quadruplicate, of each unknown organism in the collection isolated in Exercise 1.

2. Incubate one culture of each unknown at each of the following temperatures: 8°C., 20°C., 28°C., and 37°C. (If any thermophilic organisms are suspected, additional cultures should be added and incubated at temperatures of 55°C. and 65°C.)

Second Day.

3. All cultures should be observed at the end of 1, 2, 4, and 7 days' incubation for growth.

4. The lowest temperature of incubation giving the most abundant growth will be considered the optimum for the organism in question.

5. Record the optimum growth temperature on the Descriptive Chart.

(In all subsequent experiments the unknown organisms should be incubated at the optimum temperature, unless otherwise specified!)

Exercise 6

THE CATALASE TEST

The separation of genera within certain families of bacteria is dependent upon the demonstration of catalase production by the organism in question. Because the catalase test is simple to perform, it is advisable to perform it on all the unknown cultures in the collection.

Procedure:

1. Make one stroke culture on a slant of suitable nutrient medium of each unknown organism in the collection. (Extra cultures from Exercise 4 may be used for this Exercise.)

2. Incubate 48 hours at appropriate growth temperature.

3. Pour enough 3% Hydrogen Peroxide (ordinary U.S.P. strength H_2O_2) onto the growth to fill the tube about one inch full.

4. Note the presence or absence of bubbles of gas arising from the surface of the medium. This gas is the oxygen liberated when the hydrogen peroxide is decomposed by the enzyme catalase.

5. Record the liberation of gas bubbles as a positive catalase test on the Descriptive Chart.

Exercise 7

DETERMINATION OF RELATION TO FREE OXYGEN

Although most of the cultures selected from Exercise 1 will not be obligate anaerobes, information relative to the relation of the organisms to free oxygen may be helpful later in explaining certain behavior patterns of the isolated cultures.

Procedure: First Day.

1. Melt sufficient tubes of clear 0.1% Glucose Yeast Extract Agar for each culture in the collection of unknowns and steam at 100°C. for at least 20 minutes.

2. Cool the medium to 45°C. to 48°C. in a water bath.

3. With a sterile, straight, inoculating wire pick a small amount of culture from the surface of the "stock" culture of an "unknown" and inoculate the cooled medium by making a stab inoculation to the bottom of the tube. (Do not stir the medium!)

4. Chill the inoculated medium in cold water until solidification occurs.

5. Incubate at the optimum growth temperature for 48 hours.

Second Day.

6. Observe the cultures for the position and amount of growth in the medium.

7. Classify the organisms as:
   Obligate aerobes -- those which grow only on surface.
   Obligate anaerobes -- those which grow only in lower part of medium.
   Facultative -- those which grow both on surface and throughout depth of medium.
   Microaerophilic -- those which grow best in a layer about one-eighth inch below surface.

8. Record the results on the Descriptive Chart in the space provided for Relation to Free Oxygen.

(Note: In future experiments this procedure will be referred to as a "shake" culture even though the tube of liquid medium is <u>not</u> shaken or stirred!)

Exercise 8

DETERMINATION OF MOTILITY

Many species of bacteria possess flagella or other structures for locomotion. Because all species do not possess these structures, motility can be used as a distinguishing feature in determinative bacteriology. Often it is sufficient to determine the presence or absence of motility without consideration of the number or position of flagella. Presence or absence of motility should be determined for each organism in the "unknown" collection.

This Exercise describes three methods for determining motility.

Procedure A: The Simple Mount.

1. Clean a glass slide and coverglass thoroughly, and dry.
2. Put a small drop of water on the slide. With a sterile inoculating needle introduce a small amount of young (12 to 18 hour) culture sufficient to give very slight turbidity to the water.
3. Sterilize and cool the needle, and mix the bacterial suspension.
4. Carefully place the coverglass on the suspension.
5. Observe under the high dry objective. It will be necessary to reduce the illumination to the smallest amount needed to make the bacterial cells visible. Any true motility seen should be recorded in the space provided on the Descriptive Chart. Care must be taken to rule out movement caused by streaming as the preparation dries out. The absence of motility should not be recorded, but rather the determination should be repeated using one of the following techniques.

(The Simple Mount method of determining motility has the advantage that it is quick and will give accurate results for actively motile, large cells. By this screening test the time necessary in carrying out the more tedious and complex methods can be saved in the case of actively motile cultures.)

Procedure B: The Hanging Drop Technique.

1. Clean a coverglass thoroughly.
2. Put a small gob of vaseline on each corner of the coverglass.
3. Place a small loopful of water in the center of the coverglass, inoculate it lightly with a young (12 to 18 hour) culture, and mix to produce a homogeneous suspension.

4. Quickly invert the coverglass and gently place over the depression in a "hanging drop" slide. Do not press the coverglass down onto the slide. The vaseline will act as a buffer to help protect the coverglass from breakage.

5. Place a drop of immersion oil on the top of the mount and examine under the oil immersion objective. Note any vital motility, being careful to distinguish vital from Brownian movement. Record positive results on the Descriptive Chart.

(A negative result may occur if cultures are too old or for various other reasons. Consequently, all negative results should be checked culturally.)

Procedure C: Motility Determination in Semi-Solid Agar.

1. Using a straight inoculation wire, make a stab inoculation about 3/4th inch deep into a tube of Motility Test Agar Medium of each culture where doubtful or negative results were obtained on the microscopic motility determinations.

2. Incubate at the optimum temperature and observe after 8, 24, and 48 hours.

3. Motility is shown by the appearance of a diffuse zone of growth spreading from the line of inoculation. If the organism is very actively motile, the entire tube of medium may show the diffuse growth.

4. Record results of motility determination in Motility Test Agar Medium in the Descriptive Chart in the space headed "Motility in Semi-Solid Agar".

(All coverglasses and slides used in motility tests should be soaked in a disinfectant solution and boiled for at least 10 minutes before washing them!!)

Exercise 9

THE SPORE STAIN

(Snyder Modification of Dorner Method)

The family Bacillaceae is recognized by the presence of endospores in cells which have started to "age". Normally it is not difficult to find these endospores in Gram stains. The endospores will appear as unstained central to polar bodies of spherical to cylindric shape. Occasionally the number of endospores per smear may be very small, or the endospores can not be readily identified as such because of numerous unstained vacuoles in the cell.

To be certain of endospores, one should employ a procedure which will differentially stain the endospore. There are many methods which can be used. The Snyder modification of the Dorner spore stain is one of the best for student use. Cultures between 2 and 7 days old give best results, as a rule.

Procedure:

1. Prepare smear and fix by passing slide film side up through a Bunsen flame.
2. Cover smear with a piece of blotting paper, then saturate the blotting paper with Ziehl's Carbol Fuchsin stain.
3. Steam the preparation with gentle heat for 5 to 7 minutes. Be careful that the blotting paper does not dry out. Add more stain as necessary.
4. Quickly decolorize with 95% Ethyl Alcohol, then wash in water.
5. Apply a small drop of saturated Aqueous Nigrosin Solution and spread over the entire film. Tilt slide slightly to allow Nigrosin to be of different thickness over the slide area.
6. Dry quickly without washing.

Spores will stain red, vegetative cells will be colorless, and the background will be a purplish-black.

Record in the Descriptive Chart the size, shape, position, number of spores, and whether the sporangium is swollen. (See Exercise 10, Procedure B, for method of measuring spore size.)