EXPERIMENT 1

Effectiveness of Hand Washing

Unlike the sterile human skin found *in utero*, adult human skin is colonized by about one trillion (10^{12}) bacteria, which constitute the normal residential and transient flora of the skin. The necessity for surgical washing of hands was introduced in the mid-nineteenth century, in Vienna, by Ignatz Semmelweis. Semmelweis showed that hand washing prior to delivery decreased the incidence of puerperal fever (child birth fever) resulting in maternal mortality. Routine surgical scrubbing by surgeons is an essential practice for all surgical procedures in modern medicine.

Although the skin is never completely sterilized, the residential and transient flora can be significantly reduced by prolonged hand washing with soap and hot water.

Materials

Media

	Per Lab Group	Per Class
Nutrient agar plates	4	

Equipment

	Per Lab Group	Per Class
Liquid antibacterial soap	1	
Sterile cotton swabs	8	
Sterile saline tubes	2	
Bunsen burner	1	
Glass marking pencil	1	
Surgical hand brush	1	
Stop watch	1	
Quebec colony counter	1	

Procedural Points to Emphasize

Since this is the first laboratory experiment for beginning students, the following points should be thoroughly explained and/or demonstrated by the lab instructor.

1. The role of the student washer and student assistant.
2. Since students have not yet learned aseptic techniques, the instructor should demonstrate to the class the method used to inoculate sterile agar plates with sterile cotton swabs.

3. The proper technique for opening and closing of sterile agar Petri dishes.
4. Streaking the agar surface of the plates without gouging the surface.
5. Proper opening and closing of saline tubes after passing the lips of the tube through the Bunsen burner flame.
6. Dividing agar plates into halves using the glass marking pencil.
7. Proper method for incubating inoculated agar plates.
8. Use of the Quebec colony counter.

Tip

This being the first laboratory session for students, the instructor should circulate through the laboratory and assist students who are having problems with dexterity and manipulation of equipment.

Additional Reading

- Katz J. D. (2004). Hand washing and hand disinfection: More than your mother taught you. *Anesthesiology Clinics of North America*, 22(3):457–71.

Answers to Review Questions

1. The oil layer, dead cells, and organisms trapped in hair follicles prevent the removal of all microorganisms from the skin with water alone. Soap helps to remove the oil and soap plus vigorous scrubbing will maximize the removal of most bacteria from the skin.
2. The flora of the skin (transient and residential) is usually nonpathogenic and may benefit the host by preventing transient pathogens from colonizing the skin surface. This is done by competition for nutrients, secretion of chemical substances, and by stimulation of the body's immune system. On the other hand, the residential flora is capable of causing skin diseases when they are able to enter the blood, especially in immunosuppressed people.
3. The residential and transient microorganisms of the skin respond differently to hand washing. Transient flora are susceptible to antiseptics and are easily removed with hand washing. Residential organisms are more difficult to detach from the skin because of a layer of oil and entrapment in the hair follicles and dead skin cells that obstruct their removal by simple hand washing and require vigorous hand scrubbing with soap and water.
4. Surgical gloves play a significant role in preventing cross-contamination of both the surgeon and patient. Surgeons wear gloves because it is impossible to remove all of the organisms from the skin even with the most vigorous hand washing. However, surgical gloves are not a substitute for hand washing. Tiny holes in the surgeons' gloves are not uncommon and can occur during the handling of instruments, from pieces of cut bone or bone fragments, and during extended surgical procedures. Tears in gloves may also occur if fingernails, natural or artificial, are too long.

EXPERIMENT 2

Culture Transfer Techniques

Aseptic technique forms the basis for the successful manipulation of organisms in the microbiological laboratory. The development of proper aseptic transfer methods can be acquired only through the repetitive performance of this task until the steps involved become second nature to the student. To accomplish this end, it is advisable to allow students to practice this technique using cultures and sterile media in various forms, e.g., agar slants, agar deeps, and broths. The necessary manual dexterity required for the handling of culture tubes and closures while flaming inoculating instruments will be acquired through repetition.

Materials

Cultures

- 24-hour nutrient broth culture of *S. marcescens*
- 24-hour nutrient agar slant culture of *S. marcescens*

Media

	Per Lab Group	Per Class
Nutrient broth tube	1	
Nutrient agar slant tube	1	
Nutrient agar deep tube	1	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop/needle	1	
Glassware marking pencil	1	

Procedural Points to Emphasize

1. Beginning students in microbiology have difficulty appreciating the diminutive size of microorganisms. Thus, they have the tendency to procure excessive amounts of inoculum for transfer. It should be stressed that the inoculating instrument needs only to touch the growth, not to be dragged over the agar, to obtain a sufficient number of cells for the transfer. When broth cultures are used, the organisms must be suspended by vigorous tapping of the bottom of the tube. A single loopful will suffice for use as the inoculum.
2. It should be stressed that the transfer procedure should be performed as rapidly as possible. However, to ensure that viable cells are obtained from the stock culture, the hot loop or needle must be cooled by tapping it against the inner surface of the culture tube before securing the inoculum.
3. The students should be reminded that the entire inoculating wire must be flamed until it turns red.

Tip

Considering students are novices and lack the necessary manual dexterity at this point, it is wise for the instructor to circulate through the laboratory and assist students who are unable to manipulate the uncapping and recapping of culture tubes while holding the transfer instrument.

Additional Reading

- Lypson, M. L., Hamstra, S. J., Ross, P. T., Gruppen, L. D., & Colletti, L. M. (2010). An assessment tool for aseptic technique in resident physicians: A journey towards validation in the real world of limited supervision. *The Journal of Graduate Medical Education*, 2(1):85–9.

Answers to Review Questions

1. a. The inoculating instrument is flamed prior to inoculation to prevent contamination of the stock cultures. Flaming after inoculation prevents contamination of the laboratory table when the instrument is returned to the table.

   b. The test tube closures are held in the manner prescribed to maintain their sterility. Once removed, they must be kept between the fingers of the hand and never placed on the laboratory tabletop.

   c. Insertion of a hot needle directly into or onto the culture medium must not be done, as this will kill the cells.

   d. Flaming the neck of the test tube is a precaution intended to kill any organisms that might be present on the neck of the tube or the inner surface of the closure if the aseptic procedure has been compromised.

2. The purposes of the subculturing procedure are intended to establish a routine method for the transfer from one medium to another for the preparation and maintenance of stock cultures and to provide media for the performance of microbiological test procedures.

3. A straight inoculating needle is used to inoculate an agar deep tube in order to maintain the redox potential of the medium.

4. The absence of pigmentation on some *S. marcescens* colonies is not necessarily indicative of contamination. This organism is capable of producing variants that may not produce any pigment. Thus, some colonies are red, while others are colorless. Also, the rate of pigment production may vary within one culture, producing a mixture of pigmented and nonpigmented colonies.

5. To determine the presence of contamination in the *S. marcescens* culture, make Gram-stained preparations of both a colony suspected of contamination and a pigmented colony. Streak-plate preparations of both colonies may also be helpful for a comparison of cultural characteristics.

EXPERIMENT 3

Techniques for Isolation of Pure Cultures

The purposes of this experiment are to instruct students in the preparation of pure cultures from a mixed microbial population and to compare the cultural characteristics of the resultant agar plate and agar slant cultures. Toward this end, students are first introduced to two methods that are used to separate microorganisms, namely the streak-plate and spread-plate techniques. The ensuing transfer of isolated colonies onto agar slants will also enhance the students' ability to use aseptic techniques (Figures 3.1, 3.2, and 3.3).

Materials

Cultures

PART A

24- to 48-hour nutrient broth cultures of:

- 1:3 *S. marcescens/M. luteus* mixture
- 1:10 *E. coli/M. luteus* mixture
- Environmental culture obtained by students

PART B

24- to 48-hour streak-plate and/or spread-plate cultures of:

- 1:3 *S. marcescens/M. luteus* mixture
- 1:10 *E. coli/M. luteus* mixture
- Environmental culture from Part A

Media

PART A	Per Lab Group	Per Class
Trypticase® soy agar plates	3	

PART B	Per Lab Group	Per Class
Trypticase soy agar slants	4	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop/needle	1	
500-ml beaker of 95% ethyl alcohol	1	
Turntable	1	
L-shaped bent glass rod	1	
1-ml tube of sterile water	1	
Cotton swabs	as needed	
Test tube rack	1	

Procedural Points to Emphasize

1. Students should be made aware that the streak-plate technique is the most frequently used procedure for the separation of organisms from a mixed culture, whereas spread-plate preparations are used preferentially for the quantitation of cell populations.
2. Students should be apprised of the following when performing the streak-plate procedure:

   a. Petri dish covers should never be completely removed; this will avoid exposing the medium and the cover to exogenous contamination. The cover should be raised and held at the smallest angle that is sufficient for the introduction of the inoculating wire, and it should be done only for as long as it takes to inoculate each designated area of the plate.

   b. It is essential that the inoculating instrument be flamed and cooled prior to the inoculation of each area of the plate.

   c. Once the inoculum is obtained from the previously streaked area, the loop or needle should not be passed over that area again during the streaking process.
3. As this is the first time students are performing plate inoculations, they should be reminded of the fact that agar plate cultures are always incubated in an inverted position.
4. Spread-Plate Technique: Using a "lazy-Susan" Petri dish turntable (Figure 3.3 or Figure 55.2) and a sterile bent glass rod, a drop of mixed culture is placed on the surface of the agar and is spread by spinning the turntable and moving the glass rod back and forth over the agar surface. In this way, the culture is distributed evenly and should produce distinct discrete colonies.

Optional Procedural Additions or Modifications

Because of the time constraints in the laboratory, an expanded examination of cultural characteristics, as presented in Experiment 4, is frequently omitted. In order to gain an awareness of differences in cultural characteristics, it is suggested that students observe their culture preparations from Experiment 3 to note these variations.

Additional Readings

- Glasson, J. H., Guthrie, L. H., Nielsen, D. J., & Bethell, F. A. (2008). Evaluation of an automated instrument for inoculating and spreading samples onto agar plates. *Journal of Clinical Microbiology*, 46(4):1281–4.
- Gröbner, S., Beck, J., Schaller, M., Autenrieth, I. B., & Schulte, B. (2012). Characterization of an *Enterococcus faecium* small-colony variant isolated from blood culture. *International Journal of Medical Microbiology*, 302(1):40–4.

Answers to Review Questions

1. A pure culture can be obtained from a mixed culture only by first performing a streak-plate or spread-plate inoculation for the separation of the organisms into discrete colonies.
2. If Quadrant 4 of a streak-plate inoculation contains more growth than Quadrant 3, either the inoculating wire was repeatedly dragged through Quadrant 3 or, more likely, it entered Quadrant 1 during its inoculation.
3. The inoculating needle is the instrument of choice to isolate individual discrete colonies because it is thin enough to touch the center of the colony.

   The center of the colony is the best area for isolation and transfer to an agar slant as a subculture. An inoculating loop is too imprecise and therefore unsatisfactory.
4. The purity of a chosen colony may be determined by the following:

   a. Subculturing the isolate in a broth medium or on an agar slant medium

   b. Gram staining the subculture following incubation to verify its purity

EXPERIMENT 4

Cultural Characteristics of Microorganisms

Cultural characteristics are determined genetically for each particular organism. As such, these characteristics remain constant and are reproducible. This property of colonial constancy is important because it allows the microbiologist to use these macroscopic growth patterns as an aid in the identification of various microbial species. A standard descriptive vocabulary has been developed to describe the cultural and colonial appearance of microorganisms grown in artificial culture media. This vocabulary is used in a source such as *Bergey's Manual of Systematic Bacteriology*.

Materials

Cultures

24-hour nutrient broth cultures of:

- *P. aeruginosa*
- *B. cereus*
- *M. luteus*
- *E. coli*

72- to 96-hour Trypticase soy broth culture of *M. smegmatis*.

Media

	Per Lab Group	Per Class
Nutrient agar plates	5	
Nutrient agar slants	5	
Nutrient gelatin tubes	5	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop	1	
Inoculating needle	1	
Glassware marking pencil	1	
Crushed ice	as needed	

Organisms are prepared in bulk (inoculated into 500 ml of broth) and then dispensed in 10-ml aliquots in sterile 16 × 100-mm test tubes.

Procedural Points to Emphasize

1. A single cell dividing by binary fission on agar divides thousands of times, producing a single round colony. Its appearance is determined by fundamental characteristics, such as pigment production, type of cell wall, presence or absence of a capsule, and motility.
2. These characteristics are under genetic control; however, the cell's macroscopic expression may be tempered by environmental conditions, such as temperature, nutrients, and pH.
3. Because of environmental conditions, growth patterns may not always coincide exactly with those illustrated in the figures in the manual.

Tip

- Gelatin cultures: For the rapid resolidification of liquefied gelatin, cultures should be refrigerated for about 30 minutes. This process can be expedited by placing liquefied tubes in a beaker of crushed ice for a few minutes to determine if gelatin remains liquefied.

Additional Reading

- Sutula, J., Coulthwaite, L., & Verran, J. (2012). Culture media for differential isolation of *Lactobacillus casei* Shirota from oral samples. *Journal of Microbiological Methods*, [Epub ahead of print] PubMed PMID: 22484087.

EXPERIMENT 5

Microscopic Examination of Stained Cell Preparations

The compound microscope is an indispensable tool in the study of microbiology. Instructors may find that some of their students' past experience with microscopy has been limited to use of the low- and high-power objectives, which provide only sufficient magnification for viewing eukaryotic cells. However, the visualization of microorganisms, particularly prokaryotes, requires that the students become adept in the use of the oil-immersion objective.

Materials

Slides

Stained slides of selected microorganisms, prepared by the instructor, may be substituted for the commercial slide preparations. Following their use, the immersion oil can be removed from the slides with the application of xylol and gentle blotting with lens paper.

Cultures

- *S. aureus*
- *B. subtilis*
- *A. itersonii*
- *S. cerevisiae*
- Human blood smear

Equipment

	Per Lab Group	Per Class
Compound microscope	1	
Lens paper	as needed	
Immersion oil	as needed	
Xylol	as needed	

Procedural Points to Emphasize

1. Students should be made aware of the fact that the microscope is an expensive piece of equipment, and therefore, proper care is required at all times. To prevent damage to the microscope, it is important to emphasize the proper means of transporting it to and from the laboratory bench. Also, to maintain the instrument in proper working condition, students must check the objective lenses for the presence of residual oil at the start and end of

each laboratory session. Oil is removed with lens paper; the lenses are then cleaned with Windex® and wiped with dry lens paper. Xylol is never to be used by students for the removal of oil from the lens system of the microscope.

2. In addition to the instructions in the manual for the proper use of the oil-immersion objective, the following are some suggestions that may be helpful to facilitate student use of this objective.

   a. Following the addition of the immersion oil to the slide or its coverslip, rotate the nosepiece to the oil objective in the direction that does not bring the high-power objective into contact with the immersion oil.

   b. Viewing specimens under oil immersion requires more light than under the lower-power objectives. To ensure proper light transmission through the specimen, the condenser must be fully raised to the fixed platform, and the iris diaphragm must be adjusted. Students should also be made aware of the fact that differences in specimen density will require the readjustment of the iris diaphragm with each slide preparation.

   c. Because microscopes are parfocal, when focusing under the oil-immersion objective, the coarse adjustment is never used. The fine-adjustment knob is turned slowly in both directions until the specimen is in sharp focus.

Additional Reading

- Santos, M. J., Cavaleiro, F., Campos, P., Sousa, A., Teixeira, F., & Martins, M. (2010). Impact of amoeba and scuticociliatidia infections on the aquaculture European sea bass (Dicentrarchus labrax L.) in Portugal. *Veterinary Parasitology*, 171(1–2):15–21.

Answers to Review Questions

1. The body tube of a microscope is never lowered while looking through the ocular lens to ensure that the objective lens and the slide are not damaged by the forceful contact between the two.

2. a. The iris diaphragm adjusts the amount of light coming through the specimen.

   b. The coarse adjustment is used to bring the specimen into view.

   c. The fine adjustment brings the specimen into sharp focus.

   d. The condenser directs the light from the light source into the lens system.

   e. The mechanical stage controls the position of the specimen over the central opening in the stage.

3. a. Inability to bring the specimen into sharp focus may be caused by an insufficient or an excessive amount of oil on the slide or failure to position the fine adjustment at the midpoint of its range prior to focusing with the coarse-adjustment knob. Repeat the procedure for focusing under oil immersion with special attention to the instructions above.

   b. Insufficient light may be corrected by raising the Abbé condenser completely and adjusting the iris diaphragm.

   c. Accumulation of dust particles and debris on the ocular lens or the prepared slide is a frequent cause of the appearance of artifacts in the microscopic field. Clean both with lens paper and Windex.

EXPERIMENT 6

Microscopic Examination of Living Microorganisms Using a Hanging-Drop Preparation or a Wet Mount

Visualization of the single-celled bacteria in the unstained state is a challenging experience for beginning students of microbiology. To lower the frustration level of students, the instructor should apprise them of the fact that differentiating living bacteria from microscopic debris is an arduous task.

Materials

Cultures

24-hour nutrient broth cultures of:

- *P. aeruginosa*
- *S. aureus*
- *B. cereus*
- *P. vulgaris*
- Hay infusion* or pond water (optional)

*See Appendix 3 for preparation of hay infusion broth.

Equipment

	Per Lab Group	Per Class
Compound microscope	1	
Bunsen burner	1	
Inoculating loop	1	
Depression slides	4–6	
Glass microscope slides	4–6	
Coverslips	4–6	
Petroleum jelly	as needed	
Cotton swabs	as needed	
Eyedropper	1	

Procedural Points to Emphasize

1. It may be helpful to the students for the instructor to prepare a demonstration to clarify the distinction between Brownian movement and bacterial motility.
2. As this may be the first time that students are required to transfer microorganisms from sterile cultures, it is important to stress the principles of aseptic transfer techniques. Review the steps as outlined in Experiment 2.
3. This is a good opportunity to instruct the students in the proper procedures for the disposal of contaminated materials and equipment. The immersion of the hanging-drop slides into a container of disinfectant is recommended.

Additional Reading

- Minion, J., Pai, M., Ramsay, A., Menzies, D., & Greenaway, C. (2011). Comparison of LED and conventional fluorescence microscopy for detection of acid fast bacilli in a low-incidence setting. *PLoS One*, 6(7):e22495.

Answers to Review Questions

1. Bacteria are more difficult to observe in an unstained state because of their small size, the movement of cells caused by Brownian movement or motility, and their refractive index, which is similar to that of water.
2. Living microbial preparations are done to detect physiologic processes, such as motility and binary fission, and to observe their natural size and shape. Stained smears, on the other hand, distort the size, shape, and arrangement and allow you to view only dead organisms; thus, it is not possible to see motility.
3. True motility is a directional movement, while with Brownian movement, the microorganisms vibrate at a constant rate without progressing in any particular direction.
4. True motility and uniformity in the shape of the particles can be used as criteria for differentiation of living organisms from debris. The distinction between viable and nonviable particles may not always be accurate, particularly when viewing the smaller life forms. Similarities between refractive indices, size, shape, and their movement may preclude distinction between the particles.

EXPERIMENT 7

The Microscopic Measurement of Microorganisms

The microorganisms used in this experiment have been selected to illustrate the size variations that exist in the microbial population. The approximate size of the protozoans is 1.0 mm, which is about 100 times larger than red blood cells. The eukaryotic yeast cells are slightly smaller than red blood cells, measuring about 90 µm. The prokaryotic bacterial cells are almost one-tenth the size of the erythrocytes. The above spectrum of cell types may be viewed with the light microscope. Viruses, subcellular particles, are measured in nanometers and can be seen only by means of an electron microscope.

Materials

Slides

One of each prepared slide per lab group:

- Yeast cells
- Protozoa
- Bacterial cocci
- Bacterial bacilli

Equipment

	Per Lab Group	Per Class
Ocular micrometer	1	
Stage micrometer	1	
Compound microscope	1	
Immersion oil and lens paper	as needed	

Procedural Point to Emphasize

The instructor should indicate that the critical step in this procedure is the alignment of the stage and ocular micrometers so that the lines on both coincide. A demonstration and individual assistance may be required.

Additional Reading

- Cheadle, M. A., Dame, J. B., & Greiner, E. C. (2001). Sporocyst size of isolates of Sarcocystis shed by the Virginia opossum (Didelphis virginiana). *Veterinary Parasitology*, 95(2–4): 305–11.

Answers to Review Questions

1. The same calibration factor cannot be used to determine the size of an organism under all objectives. The stage micrometer is calibrated only for use with the oil-immersion objective.

2. If a stage micrometer division contains 12 ocular divisions, the distance between two lines on the ocular micrometer is 0.833 µm.

   Calculation:
   0.01/12 = 0.000833 mm or 0.833 µm

3. The size of a bacterium that measures 3 µm × 1.5 µm is 1.18×10^{-4} inch by 5.9×10^{-5} inch.

   Calculation:
   1 µm = 1/25,400 inch; therefore 1µm = 3.9×10^{-5}

   length: $3\ \mu m \times 3.9 \times 10^{-5} = 1.17 \times 10^{-4}$ inch

   width: $1.5\ \mu m \times 3.9 \times 10^{-5} = 5.9 \times 10^{-5}$ inch

4. a. The measurements of *Bacillus subtilis* in the stained and unstained state would not be the same.

   b. Heat fixation causes dehydration of the cells, and their measurements will be less than that of their native state.

Preparation of Bacterial Smears
Simple Staining

These initial staining exercises are designed to instruct students in the proper technique for the preparation of a bacterial smear, which is the prerequisite for all staining procedures. In addition, the microscopic observation of the stained smears is intended to familiarize students with cellular morphology—the size, shape, and arrangement of bacteria. The performance of these experiments will also reinforce the use of the oil-immersion lens.

Materials

Both broth and agar slant cultures of the selected organisms should be available in order for students to gain experience in performing aseptic transfers and bacterial smear preparations from both types of cultures.

Preparation of Bacterial Smears

Cultures

24-hour cultures of:
- Nutrient agar slant of *B. cereus*
- Nutrient agar broth of *S. aureus*

Equipment

	Per Lab Group	Per Class
Glass microscope slides	7	
Bunsen burner	1	
Inoculating loop	1	
Inoculating needle	1	
Glassware marking pencil	1	

Simple Staining

Cultures

24-hour cultures of:
- Nutrient agar slant of *B. cereus*
- Nutrient agar slant of *E. coli*
- Nutrient broth of *S. aureus*

Reagents

- Methylene blue
- Crystal violet
- Carbol fuchsin

Equipment

	Per Lab Group	Per Class
Microscope	1	
Glass microscope slides	3	
Bibulous paper	as needed	
Staining tray/rack	1	
Bunsen burner	1	
Inoculating needle	1	
Glassware marking pencil	1	

Procedural Points to Emphasize

1. As the students are still novices in aseptic transfer techniques, a review of this procedure is recommended.
2. Students should be cautioned to clean slides well. A dirty or greasy slide will produce a poor smear preparation because grease may prevent the smear from adhering to the glass. Likewise, grease may cause the suspension to coalesce and not spread evenly on the slide. Dust particles on a slide might easily be mistaken for microorganisms.
3. Students tend to use too much inoculum when preparing their bacterial smears from agar slant cultures. It should be stressed that a sufficient number of organisms will be obtained by touching the surface of the culture with a loop or needle without digging into the agar. It should also be mentioned that broth cultures should be gently mixed to suspend the microorganisms that may have settled in the bottom of the tube prior to the transfer of the loopful of inoculum to the slide.
4. When heat fixing a smear, students should be instructed to pass the slide through the outer portion of the flame to prevent overheating the smear. Excess heat can distort the morphology through plasmolysis of the cell wall.
5. In performing the staining procedure, students are frequently afraid to sufficiently wash their slides following the application of each staining reagent. It should be emphasized that if the smear is heat fixed properly prior to the start of the staining procedure, then this fear is unfounded. Furthermore, they should be instructed to wash both sides of the slide under running water to remove all residual stain, as it may interfere with the microscopic recognition of the microorganisms.

Tips

- Agar cultures are used in this experiment to help the student prepare smears of the correct thickness. A good smear should allow the student to read newsprint through the smear. Broth cultures, on the other hand, allow the student to view the morphological characteristics on the smear because the cells are widely separated and do not clump on the smear.
- These slides could be saved and used in Experiment 9. Finished slides can be wrapped in paper toweling and secured with a rubber band.

Additional Readings

- Weinstein, R. A., Bauer, F. W., Hoffman, R. D., Tyler, P. G., Anderson, R. L., & Stamm, W. E. (1975). Factitious meningitis. Diagnostic error due to nonviable bacteria in commercial lumbar puncture trays. *Journal of the American Medical Association*, 233(8):878–9.
- Youssef, D., Shams, W., Ganote, C. E., & Al-Abbadi, M. A. (2011). Negative image of blastomyces on diff-quik stain. *Acta Cytologica*, 55(4):377–81.

Answers to Review Questions

Preparation of Bacterial Smears

1. Thick smears do not allow sufficient light to pass through the preparation for good visualization of the organisms. Also, dense smears contain tightly packed and superimposed cells that do not lend themselves to accurate determination of cell shape and arrangement.
2. Air-drying prevents the cells from shrinkage and distortion, thereby protecting their size and shape, and allows for the visualization of the natural cellular morphology.
3. Excessive heating may distort the morphology, causing plasmolysis of the cell wall. On the other hand, an improperly heat-fixed smear could wash off the slide.
4. The presence of grease from fingers or any other exogenous source may interfere with the adherence of the culture to the slide and will result in the production of an unsatisfactory smear. The presence of dirt or dust on the glass surface will produce artifacts in the stained smear and serve as a source of confusion for the student viewing the organisms in the stained smear.

Simple Staining

1. Basic dyes are used preferentially for bacterial staining because the chromogen is cationic and has an affinity for the negatively charged DNA. Also, the bacterial cell surface generally has a negative charge, which attracts the basic stain.
2. Simple staining procedures cannot be used for purposes other than the determination of cell morphology. The structural bacterial components are too small to be viewed with a simple light microscope.
3. Failure to heat fix the *E. coli* smear would result in the loss of the smear during the staining process. Heat is required to cause coagulation of bacterial proteins, which then adhere to the glass slide. The sparse number of remaining cells would not be readily discernible.
4. The coffee-discolored laboratory coat is not permanently stained, and the color will wash out. The reason for this is that the coffee is not a stain. It is only a chromogen and lacks the auxochrome component. Therefore, ionization cannot occur, and there will be no binding to the cloth fibers.

EXPERIMENT 10

Negative Staining

Negative staining is presented as an alternative technique to the hanging-drop procedure for the observation of living cells. Because the smears are not heat fixed and the stain used does not penetrate into the cells, the organisms remain viable.

Materials

Cultures

24-hour nutrient agar slant cultures of:

- *M. luteus*
- *B. cereus*
- *A. itersonii*

Reagent

- Nigrosin stain

Equipment

	Per Lab Group	Per Class
Microscope	1	
Glass microscpe slides	6	
Lens paper	as needed	
Staining tray/rack	1	
Bunsen burner	1	
Inoculating loop	1	

Procedural Points to Emphasize

1. As bacterial smear preparations for negative staining differ to some extent from conventional staining procedures, students should be reminded not to heat fix the smear. Also it may be advisable to demonstrate the technique for spreading the smear with the aid of a second glass slide.
2. As the bacteria are not killed during the negative-staining procedure, students should be instructed in the importance of discarding the slides into a beaker containing disinfectant following their microscopic examination.

Optional Procedural Additions or Modifications

The experimental procedure may be modified to include the staining of an organism by both simple and negative staining to allow students to compare the observed results.

Tips

- The instructor should emphasize that these organisms are not heat fixed and thus are viable. Students should be given the option to use disposable gloves.
- Some labs reuse slides for negative staining. Only new and clean slides should be used in this experiment.

Additional Reading

- Baradkar, V., Mathur, M., De, A., Kumar, S., & Rathi, M. (2009). Prevalence and clinical presentation of Cryptococcal meningitis

among HIV seropositive patients. *Indian Journal of Sexually Transmitted Diseases*, 30(1):19–22.

Answers to Review Questions

1. Methylene blue as a basic, cationic dye cannot be used in negative staining. An acidic stain, such as nigrosin, is required so that it does not bind to the negatively charged cell surface.
2. Negative staining allows the visualization of living microbial cells that have not undergone distortion by heat fixation.
3. The nigrosin is an anionic acidic stain and does not have an affinity for the negatively charged cell surfaces. As such, the dye colors the background, and the cells remain unstained.

EXPERIMENT 11

Gram Stain

The Gram stain is one of the first procedures to be performed for the identification of microorganisms. As such, it is the "workhorse" for microbiologists in both academic and health-related fields. In the classroom setting, it serves as the prototype for a variety of other differential staining procedures.

Materials

Cultures

18- to 24-hour nutrient agar slant cultures of:

- *E. coli*
- *B. cereus*
- *S. aureus*

Reagents

- Crystal violet
- Gram's iodine
- 95% ethyl alcohol
- Safranin

Equipment

	Per Lab Group	Per Class
Microscope	1	
Glass Microscope slides	4	
Staining tray	1	
Lens paper	as needed	
Bibulous paper as needed	as needed	
Bunsen burner	1	
Inoculating loop/needle	1	

Procedural Points to Emphasize

1. As the Gram stain is the most frequently performed differential staining technique, the instructor should explain the functions of the chemicals used in differential staining as well as the chemical basis of this procedure.
2. The most critical step in all differential staining procedures, including the Gram stain, is the decolorization process. Students should be cautioned that the density of the smear will be a major factor in determining the amount of decolorizing agent necessary for proper decolorization of the smear. Thus, the method used by the instructor should be explained and demonstrated. The authors have found that this step can be best achieved by the application of 95% ethyl alcohol in a dropwise fashion with intermittent washing. When the water bubble clinging to the edge of the slide is almost clear, decolorization is complete.
3. It should again be stressed that thorough washing of the slides under running water between the applications of all staining reagents is essential to remove excess chemicals.
4. Caution students to *blot* with bibulous paper and not to *rub* the bibulous paper.

Tips

- Fresh cultures, 18–24 hours old, are necessary for optimum Gram staining reactions. Older cultures tend to produce gram-variable results.
- Aqueous crystal violet should be fresh and filtered before use.
- Washing of stained smears should be done carefully. Overwashing should be avoided so as not to overdecolorize the preparation.
- Clothespins may be used as slide holders if desired.
- Considering this is the first time students are performing a differential stain, the instructor may wish to demonstrate the method for the class.

Additional Reading

- Uehara, Y., Yagoshi, M., Tanimichi, Y., Yamada, H., Shimoguchi, K., Yamamoto, S., Yanai, M., & Kumasaka, K. (2009). Impact of reporting gram stain results from blood culture bottles on the selection of antimicrobial agents. *American Journal of Clinical Pathology*, 132(1):18–25.

Answers to Review Questions

1. Simple staining uses a single dye and stains all cells and their cytological components the same color. Thus, these procedures can be used only to determine cell morphology. Differential staining utilizes two stains of contrasting colors that allow for the separation of bacteria into groups, e.g., Gram stain, or for the visualization of cellular structures, e.g., flagella.
2. a. The primary stain is the first stain used and imparts color to all cells.

   b. The mordant is a chemical that acts as an intensifier in the Gram staining procedure. It forms a complex with the crystal violet, which cannot be easily removed from gram-positive cells with the decolorizing agent.

   c. The decolorizing reagent functions to remove the primary stain only from some cell types or cell structures, thus allowing for their differentiation, on the basis of color, following the application of the counterstain.

   d. The counterstain is the second, contrasting-color stain that is applied. This stain will be absorbed only by decolorized cells.
3. Considering bacteria cannot be separated on the basis of differences in cell morphology, differential staining, using dyes of contrasting colors, allows for the microscopic separation of organisms into groups based on a difference in color.
4. Decolorization is the most crucial step. The basis of the Gram stain is the ease with which the primary stain can be removed by the decolorizing agent. Therefore, over-decolorization will remove the primary stain from gram-positive organisms, causing many cells to appear to be gram negative. Insufficient decolorization fails to remove the primary stain from organisms that are gram negative, thereby resulting in a gram-positive reaction.
5. With increasing age of a culture, the ability of organisms to absorb the stain becomes variable because of changes in cell wall structure. Thus, a uniformly colored preparation is not possible and results in a gram-variable reaction with the *B. cereus* cells ranging in color from intense blue to pink. This phenomenon of gram variability is noted more frequently with gram-positive organisms. Included among these are members of the genus *Bacillus*.

EXPERIMENT 12

Acid-Fast Stain

The acid-fast stain is a highly specialized diagnostic staining procedure that is used to identify members of the genus *Mycobacterium*. Its application in the clinical setting is for the diagnosis of tuberculosis and leprosy.

Materials

Cultures

- 72- to 96-hour Trypticase soy broth (TSB) culture of *M. smegmatis*
- 18- to 24-hour TSB culture of *S. aureus*

Reagents

- Carbol fuchsin
- Acid-alcohol (3% HCl plus 95% ethyl alcohol)
- Methylene blue

Equipment

	Per Lab Group	Per Class
Microscope	1	
Glass microscope slides	3	
Staining tray	1	
Hot plate	1	
Lens paper	as needed	
Bibulous paper	as needed	
Bunsen burner	1	
Inoculating loop	1	
250-ml beaker	1	

Procedural Points to Emphasize

1. Considering that mycobacteria have a tendency to clump, students should be instructed to vigorously spread the inoculum on the slide to separate the organisms.
2. When preparing the mixed-culture smear, students should be cautioned to use a more concentrated sample of *M. smegmatis* than *S. aureus*.
3. In order to obtain a satisfactory acid-fast reaction using the heat method, the following points should be stressed:

   a. The carbol fuchsin–covered smear must be heated for the required period of time.

   b. The carbol fuchsin must be maintained at a steaming rather than a boiling temperature to prevent rapid evaporation of the stain.

   c. Additional applications of carbol fuchsin will be required during the heating process even though the slide is maintained at a steaming temperature.

   d. Following the application of heat, the slide preparations must be allowed to cool prior to their vigorous washing with water to prevent breakage of the slides.

Tips

- The steps for decolorization should be reviewed so as not to overdecolorize the smear.
- Clothespins may be used as slide holders.
- Students should be reminded to blot the stained smear with bibulous paper but not to rub the bibulous paper over the wet slide.
- Three- to 4-day cultures of *M. smegmatis* are required to maximize the bacteria's growth. Specialized media, such as the Lowenstein-Jensen medium, may be used to culture *Mycobacterium* sp. If a broth medium is used, the addition of 0.4- to 1.0-percent Tween 80 per liter of medium will reduce the tendency of the mycobacteria to clump.
- If the heatless modification of the Ziehl-Neelsen method is used, add 2 drops of Triton X per 100 ml of carbol fuchsin.

Additional Reading

- Wilmer, A., Bryce, E., & Grant J. (2011). The role of the third acid-fast bacillus smear in tuberculosis screening for infection control purposes: A controversial topic revisited. *Canadian Journal of Infectious Diseases and Medical Microbiology*, 22(1):e1–3.

Answers to Review Questions

1. The application of heat or a surface-active agent is essential to soften the waxy cell wall components to facilitate the penetration of the primary stain into the cells.
2. Acid-alcohol is used preferentially over 95% ethyl alcohol to ensure that the primary stain is removed from the non–acid-fast organisms.
3. The acid-fast staining procedure is used for the diagnosis of leprosy and tuberculosis, both of which are caused by members of the genus *Mycobacterium*.
4. Application of heat or a surface-active agent is not required during the application of the counterstain. The acid-fast organisms, because of the waxy nature of their cell walls, are not decolorized, and the red stain remains trapped inside the cells. The non–acid-fast organisms lack the lipoidal cell wall components. Therefore, the primary stain is easily removed during decolorization, and the colorless cells are readily stained by the counterstain.
5. The presence of acid-fast bacilli in the gastric washing suggests that the tubercle bacilli, released from the lungs, were swallowed by the child rather than eliminated by coughing. This evidence is suggestive of a tuberculosis infection.

EXPERIMENT 13

Differential Staining for Visualization of Bacterial Cell Structures

These differential staining procedures are used to demonstrate anatomical structures that may be present in bacteria, namely the endospore and the capsule. The procedures, although of academic interest, are not frequently performed.

Materials

Cultures

PART A: Spore Stain

48- to 72-hour nutrient agar slant culture of
B. cereus

48- to 72-hour thioglycollate broth culture of
C. butyricum

PART B: Capsule Stain

48-hour skim milk cultures of:

- *A. viscolactis*
- *L. mesenteroides*
- *E. aerogenes*

Reagents

PART A: Spore Stain

- Malachite green
- Safranin

PART B: Capsule Stain

- 1% crystal violet
- 20% copper sulfate ($CuSO_4 \bullet 5\ H_2O$)

Equipment

	Per Lab Group	Per Class
Microscope	1	
Glass microscope slides	8	
Staining tray	1	
Bibulous paper	as needed	
Lens paper	as needed	
Hot plate	1	
Bunsen burner	1	
Inoculating loop	1	

Procedural Points to Emphasize

Spore Stain

1. Reemphasize the precautions outlined in Experiment 8 for the application of dyes with heat. As in the acid-fast staining procedure, the absorption of the primary stain requires the application of sufficient heat.
2. Be sure to tell students not to allow malachite green to evaporate from the smear during heating.
3. Be careful not to wash more than 35–45 seconds with tap water or the malachite green stain will overdecolorize. Overdecolorization is a common mistake made by students.

Capsule Stain

1. Caution students to avoid vigorous spreading with the loop or needle during smear preparation because of the fragile nature of the capsular material. Also, remind students that water is not used in this procedure for washing.
2. Remind the students not to heat fix the capsule smears.

Optional Procedural Additions or Modifications

Projected slides, commercially prepared slides, or colored transparencies can be used to acquaint students with these cytological structures.

Additional Readings

- Jöbstl, M., Heuberger, S., Indra, A., Nepf, R., Köfer, J., & Wagner, M. (2010). *Clostridium difficile* in raw products of animal origin. *International Journal of Food Microbiology*, 138(1–2):172–5.
- Martin, M., Turco, J. H., Zegans, M. E., Facklam, R. R., Sodha, S., Elliott, J. A., …Whitney, C. G. (2003). An outbreak of conjunctivitis due to atypical *Streptococcus pneumoniae*. *New England Journal of Medicine*, 348(12):1112–21.

Answers to Review Questions

1. Because of the impervious nature of the protein spore coats, the stain-covered smear is heated to ensure penetration of the stain into the spore.
2. The function of water is to remove excess primary stain from the spore. The vegetative cells lack an affinity for this stain; thus it is removed by water, rendering the vegetative cells colorless.
3. a. Acid-alcohol would not decolorize the stained spore, and the final observations would be the same as with the use of water.

   b. If safranin is applied with heat, both the endospore and the vegetative cell will accept the stain and appear red in color. Tap water will not remove the stain, and therefore, malachite green would not be accepted. Both the endospore and the vegetative cell will be red.

   c. Failure to apply heat with the primary stain will not allow the stain to penetrate into the endospore. The vegetative cell will be red, and the endospore will be colorless and refractile.
4. The capsule is a viscous structure with a polysaccharide/protein composition that is found outside of the cell wall of some microorganisms. It is of medical significance as its presence renders the cell resistant to the phagocytic activities of WBCs, thereby increasing the virulence of the organism.
5. The capsule is nonionic and as such will not bind with the cationic primary stain, crystal violet. In this method, copper sulfate is used rather than water to wash out excess stain from the cell. During this process, the copper sulfate is absorbed into the capsule, giving it a light blue color in contrast to the deep purple color of the cell.

EXPERIMENT 14

Nutritional Requirements: Media for the Routine Cultivation of Bacteria

The purpose of this experiment is twofold. First, it will evaluate synthetic (chemically defined) media, complex (chemically undefined) media, and enriched media for their ability to support microbial growth. Second, students will ascertain the degree of fastidiousness of selected microorganisms.

Materials

Cultures

Saline suspensions of 24-hour Trypticase soy broth cultures, with adjusted absorbances (A) to A = 0.05 at 600 nm:

- *E. coli*
- *A. faecalis*
- *S. mitis*

Media

Three test tubes (13 × 100 ml) of each:

- Inorganic synthetic broth
- Glucose salts broth
- Nutrient broth
- Yeast extract broth

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
1-ml serological pipettes	3	
Mechanical pipetting device	1	
Glassware marking pencil	1	
Test tube rack	1	
Spectrophotometer	1	

Procedural Points to Emphasize

1. As this is the first time students will be using a spectrophotometer, they should be given a complete explanation and a demonstration of its use. Ancillary information should include the following reminders:

   a. The organisms in each culture must be resuspended. However, the cultures must be allowed to stabilize until the bubbling subsides prior to the determination of the A readings. Otherwise, erroneous readings will be obtained.

   b. The outside of all culture tubes must be wiped with lens paper to remove finger marks before their insertion into the test tube well.

   c. All culture tubes must be inserted into the test tube well in the same position. The etched marking on the test tube may be used as a guide.

   d. The test tube well cover must be closed prior to obtaining A readings.

2. Students should be reminded that pipetting by mouth is prohibited.

Optional Procedural Additions or Modifications

If a spectrophotometer is not available, observation of the turbidity of the cultures may be made visually and recorded on a scale of 0 through 4+.

Additional Reading

- Lindqvist, R. (2006). Estimation of *Staphylococcus aureus* growth parameters from turbidity data: characterization of strain variation and comparison of methods. *Applied and Environmental Microbiology*, 72(7):4862–70.

Answers to Review Questions

1. Absorbance is directly proportional to the amount of microbial growth, whereas percent T is inversely proportional to the number of cells present.

2. Uninoculated media tubes, representative of the media in which the cultures have been grown, are used as blanks. As the different media exhibit variations in color, the blanks must be used to standardize the spectrophotometer to 100% T prior to obtaining the A readings of the cultures.

3. Artificial media are used for the routine cultivation of microorganisms as the peptones and beef extract are sufficient to provide the nutritional growth requirements for most microorganisms. Thus, knowledge of the specific nutritional needs of the organism is not needed.

4. Heterotrophic organisms require the use of organic carbon sources and, in some cases, organic nitrogen sources and vitamin supplements. These organisms would not grow in an inorganic medium.

5. a. If the organism showed minimal growth in a basic artificial medium, yeast extract could be added as a supplement, as it contains all the B vitamins.

   b. To determine the specific vitamin needs of the organism, a vitamin assay is required. In performing the assay, the control would contain all the vitamins. Each of the remaining assay culture media would contain all the vitamins present in the control culture with the deletion of one different vitamin from each test tube. Culture tubes lacking growth in the absence of a particular vitamin would indicate that this vitamin is an essential growth factor.

EXPERIMENT 15

Use of Differential, Selective, and Enriched Media

The purpose of this experiment is to demonstrate the functions of special-purpose media used for the isolation and the identification of specific groups of microorganisms. In the clinical laboratory, these media are frequently used to facilitate the rapid detection and isolation of possible pathogens from mixed microbial populations in biological specimens.

Materials

Cultures

24- to 48-hour Trypticase soy broth cultures of:

- *E. aerogenes*
- *E. coli*
- *Streptococcus* var. Lancefield Group E
- *S. mitis*
- *E. faecalis*
- *S. aureus*
- *S. epidermidis*
- *S. typhimurium*

Media

	Per Lab Group	Per Class
Mannitol salt agar plate	1	
Eosin-methylene blue agar plate	1	
Phenylethyl alcohol agar plate	1	
MacConkey agar plate	1	
Blood agar plate	1	
Crystal violet agar plate	1	
7.5% sodium chloride agar plate	1	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop	1	
Glassware marking pencil	1	

Procedural Points to Emphasize

1. Students should be reminded of the necessary precautions to prevent exogenous contamination when performing multiple inoculations on a single plate.
2. Students should be cautioned to confine the line of inoculation of each organism well within its designated section of the plate.

Tip

- Although blood agar is not truly classified as a differential or selective medium, it can be used as such in the separation and classification of the streptococci on the basis of their hemolytic patterns (alpha, beta, and gamma) on blood agar. It is a good opportunity for students to become familiar with this, considering they will see it again in Experiment 64.

Additional Reading

- Craven, R. R., Weber, C. J., Jennemann, R. A., & Dunne, W. M. Jr. (2010). Evaluation of a chromogenic agar for detection of group B streptococcus in pregnant women. *Journal of Clinical Microbiology*, 48(9):3370–1.

Answers to Review Questions

1. a. Crystal violet in MacConkey agar medium is an inhibitor to suppress the growth of gram-positive organisms.

   b. Blood serves to enrich an agar medium to support the growth of fastidious organisms and to differentiate microorganisms, particularly streptococcal species, on the basis of their hemolytic activities.

   c. The eosin and methylene blue in the EMB agar medium is used to identify *E. coli*. The large amount of acid produced by these organisms causes the dyes to precipitate out onto the surface of the colonies, thereby producing a green coloration to the growth. Methylene blue also partially inhibits growth of gram-positive organisms.

   d. High salt concentration in the mannitol salt agar medium is used to inhibit the growth of organisms other than halophiles.

   e. Lactose is a major microbial carbon source. In MacConkey agar medium, it serves to differentiate between lactose fermenters and nonfermenters on the basis of their ability to produce acid.

   f. Phenylethyl alcohol in the phenylethyl alcohol agar medium partially inhibits growth of gram-negative organisms; thus, the number and size of gram-negative colonies is markedly reduced.

2. Gram-positive organisms are very sensitive to the basic dye crystal violet. The exact mechanism of action by which crystal violet acts is still unclear. However, it may be that the dye has an affinity for the nucleic acids and may interfere with reproduction in gram-positive organisms, therefore inhibiting their growth and "selecting" gram-negative bacteria. When incorporated into a medium, 7.5% sodium chloride agar produces an osmotic environment not conducive for the growth of most organisms other than those classified as halophilic (salt loving). This medium is excellent for the selection and differentiation of different species of staphylococci, which are halophilic organisms.
3. A boil is usually the result of a staphylococcal or a streptococcal infection. The exudate should first be cultured in a broth medium, followed by streak-plate inoculations on blood and mannitol salt agar plates for the isolation of discrete colonies. If the etiological agent of the boil is *Staphylococcus aureus*, a yellow halo will be present surrounding some of the colonies on the mannitol salt agar plate, and beta-hemolysis will be evident on the blood agar plates. If the causative agent is a pathogenic streptococcus, evidence of beta-hemolysis will be present on the blood agar plate; however, none of the colonial growth on the mannitol salt agar plate will exhibit a yellow halo.

Physical Factors: Temperature

Physical Factors: pH of the Extracellular Environment

Physical Factors: Atmospheric Oxygen Requirements

These experiments are designed to demonstrate the microbial diversity as it relates to the specific environmental requirements essential to support microbial growth. This diversity is dependent upon the enzymatic capabilities of specific microorganisms.

Materials

Temperature

Cultures

24- to 48-hour nutrient broth cultures of:
- *E. coli*
- *B. stearothermophilus*
- *P. savastanoi*
- *S. marcescens*

24-hour Sabouraud broth culture of:
- *S. cerevisiae*

Media

	Per Lab Group	Per Class
Trypticase soy agar plates	4	
Sabouraud broth tubes with Durham tubes	4	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop	1	
4°C refrigerator	1	
37°C incubator	1	
60°C incubator	1	
Pasteur pipette	1	
Test tube rack	1	
Glassware marking pencil	1	

pH of the Extracellular Environment

Cultures

Saline suspension of 24-hour nutrient broth cultures with A = 0.05 at 600 nm:
- *A. faecalis*
- *E. coli*

Saline suspension of 24-hour Sabouraud broth with A = 0.05 at 600 nm:
- *S. cerevisiae*

Media

Trypticase soy broth tubes at each of the following pH designations*:

	Per Lab Group	Per Class
pH 3	3	
pH 6	3	
pH 7	3	
pH 9	3	

*The pH of the broth is adjusted with 1 N NaOH or 1 N HCl.

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
1-ml pipettes	3	
Mechanical pipetting device	1	
Test tube rack	1	
Glassware marking pencil	1	
Inoculating loop	1	
Spectrophotometer*	1	

* Bausch and Lomb, Spectronic 20

Atmospheric Oxygen

Cultures

24- to 48-hour nutrient broth cultures of:
- *S. aureus*
- *C. xerosis*
- *E. faecalis*

48- to 72-hour Sabouraud broth cultures of:
- *S. cerevisiae*
- *A. niger*

48-hour thioglycollate broth culture of:
- *C. sporogenes*

Media

	Per Lab Group	Per Class
Brain heart infusion agar deep tubes	6	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Waterbath	1	
Iced waterbath	1	
Thermometer	1	
Pasteur pipettes	6	
Test tube rack	1	
Glassware marking pencil	1	

Procedural Points to Emphasize

Temperature

Students should be made aware of the presence of a Durham tube in each of the carbohydrate broth medium tubes and cautioned not to accidentally introduce air into this gas collection vial.

Atmospheric Oxygen

For the preparation of the shake-tube inoculations, students should be reminded to:

1. Cool the test tubes of molten agar to 40°C prior to their inoculation.
2. Rapidly rotate the test tubes between the palms of the hands for the even distribution of the organisms throughout the medium.
3. Rapidly solidify the inoculated cultures by placing them into an ice-water bath.

Optional Procedural Additions or Modifications

Atmospheric Oxygen

Stab culture preparations in nutrient agar deep tubes may be substituted for the molten agar shake-tube procedure.

Additional Readings

- Konishi, T., Yamashiro, T., Koide, M., & Nishizono, A. (2006). Influence of temperature on growth of Legionella pneumophila biofilm determined by precise temperature gradient incubator. *Journal of Bioscience and Bioengineering*, 101(6):478–84.
- Stingl, K., Uhlemann, E. M., Schmid, R., Altendorf, K., & Bakker, E. P. (2002). Energetics of *Helicobacter pylori* and its implications for the mechanism of urease-dependent acid tolerance at pH 1. *Journal of Bacteriology*, 184(11):3053–60.
- Das, D., & Bishayi, B. (2009). Staphylococcal catalase protects intracellularly survived bacteria by destroying H_2O_2 produced by the murine peritoneal macrophages. *Microbial Pathogenesis*, 47(2):57–67.

Answers to Review Questions

Temperature

1.

Environment	Type of Organism	Optimum Temperature
Ocean bottom near shore	Psychrophile	15°C
Ocean bottom near hot vent	Extreme thermophile	>100°C
Hot sulfur spring	Extreme thermophile	80–100°C
Center of compost pile	Mesophile	37°C
High mountain lake	Psychrophile	15°C
Center of an abscess	Mesophile	37°C
Antarctic ice	Extreme psychrophile	5°C

2. Enzymes will be denatured above the maximum growth temperature. Below the minimum growth temperature, enzymes are inactivated but not destroyed.
3. To determine whether an organism is a psychrophile or a mesophile, cultures should be incubated at 15°C and 37°C. If growth occurs at 37°C but not at 15°C, it is a mesophile. However, if growth occurs only at 15°C, the organism is a psychrophile.
4. It is not possible for obligate thermophiles to induce infections in warm-blooded animals because they require temperatures that exceed body temperature for growth. Theoretically, facultative thermophiles can grow at 37°C. However, their optimum growth temperature is 45°C, well above body temperature; therefore, it is unlikely that they would cause infection.

pH of the Extracellular Environment

1. If the pH decreases, the salt of the weak base will accept excess H^+, forming a weak acid. If the pH increases, the weak acid will donate H^+, thereby forming a weak base.
2. Microbial metabolic activities will generate shifts in pH within the culture. For example, if carbohydrates are primarily utilized, the accumulation of acidic end products will lower the pH of the medium. If protein compounds are metabolized, alkaline end products are released into the environment, thereby raising the pH.
3. Proteins and amino acids, because of their amphoteric nature, serve as natural buffers, as they can either donate or accept H^+.
4. Differences in pH requirements among microorganisms are dependent upon the susceptibility of their individual enzyme systems to denaturing at various pH levels.
5. Not all organisms grow optimally at a neutral pH, as their enzyme systems have already adapted for existence in their natural environment. For example, many fungi grow best in an acidic environment, while many species of soil bacteria prefer an alkaline pH.
6. In the chemically defined *E. coli* culture, which lacks a buffer, growth was terminated due to a radical shift in pH. In the nutrient broth medium, which contains natural buffers, there was no pH shift, and growth was sustained in the presence of adequate nutrients.

Atmospheric Oxygen

1. The inoculated molten agar cultures require rapid solidification to prevent the diffusion of atmospheric oxygen into the depth of the cultures and to maintain the even distribution of the organisms throughout the medium.
2. Facultative anaerobes possess the most extensive bioenergetic enzyme systems because of their dual capability to respire aerobically and anaerobically.
3. Aerobes cannot grow in the absence of atmospheric oxygen, as these organisms can utilize this molecule only as the final electron acceptor in aerobic respiration.
4. Microaerophiles have specific atmospheric oxygen needs, requiring a reduced oxygen tension that exists in a narrow zone beneath the surface of the agar preparation.
5. Facultative anaerobes, which grow throughout the entire test tube culture, are capable of anaerobic and aerobic respiration. However, they preferentially use the aerobic pathway, which is more efficient, and thus exhibit more growth toward the surface of the culture.
6. *Clostridium sporogenes* is a strict anaerobe and therefore grows in the depth of the agar culture. This organism generates copious amounts of gas that fractures or elevates the medium from the bottom of the test tube.
7. *Streptococci* are aerotolerant organisms (facultative anaerobes) that have the enzymatic capacity to survive in the presence or absence of oxygen. These organisms do not use oxygen in their metabolism, and use other molecules as a final electron acceptor. They are homofermentative and are able to obtain their energy from fermenting sugars (glucose) to lactic acid via the hexose monophosphate pathway.

EXPERIMENT 19

Techniques for the Cultivation of Anaerobic Microorganisms

The purpose of this experiment is to introduce students to the use of specialized techniques for the cultivation of anaerobic microorganisms. In clinical medicine, the use of anaerobic culture procedures has gained significance over the years. Currently, pseudomembranous enterocolitis may be caused by the anaerobe *Clostridium difficile* following certain types of antibiotic therapy.

Materials

Cultures

24- to 48-hour nutrient broth cultures of:

- *B. cereus*
- *E. coli*
- *M. luteus*

48-hour thioglycollate broth culture of:

- *C. sporogenes*

Media

	Per Lab Group	Per Class
Screw-cap tubes of thioglycollate medium	4	
Nutrient agar plates	4	

Equipment

	Per Lab Group	Per Class
GasPak® anaerobic system	1	
Test tube rack	1	
Inoculating loop/needle	1	
Glassware marking pencil	1	
Bunsen burner	1	

Procedural Points to Emphasize

1. Students should be provided with a thorough demonstration in the use of the GasPak system. Furthermore, they should be cautioned to strictly adhere to the steps as outlined under the procedure section of this exercise to ensure the development of anaerobiosis.
2. Students should be instructed to:

   a. Handle the test tubes of thioglycollate carefully, with minimal agitation, to prevent the introduction of oxygen into the medium.

   b. Introduce the inoculum into the bottom of the test tube.

3. Be sure that the caps of screw-cap tubes are fully tightened when tubes are incubated.
4. Slightly loosen screw-cap tubes incubated in the GasPak about a half of a turn to neutralize the oxygen inside the tubes by the generated hydrogen gas.
5. Be sure the lid of the jar is on correctly and sealed properly.

Optional Procedural Additions or Modifications

To reduce laboratory expenses, have students perform the GasPak procedure in groups of four students. Two groups may share a single GasPak.

Tips

- The GasPak jar not only provides excellent anaerobic conditions, but it also provides increased concentrations of CO_2 that can be used for enrichment alone.
- If time and equipment are available, some other methods (Figure 19.2) may be tried by the class or perhaps as a demonstration by the instructor.
- Fluid thioglycollate, dispensed in screw-cap culture tubes, must be freshly prepared to show a pink coloration in the upper third of the medium.

Additional Reading

- Donelli, G., Vuotto, C., Cardines, R., & Mastrantonio, P. (2012). Biofilm-growing intestinal anaerobic bacteria. *FEMS Immunology and Medical Microbiology*, 65(2):318–25, doi: 10.1111/j.1574–695X.2012.00962.x.

Answers to Review Questions

1. These media contain meat products that reduce the redox potential of the medium, thus establishing conditions that favor anaerobiosis.
2. In the GasPak system, the gas generator component serves to replace the oxygen in the vessel with hydrogen gas and carbon dioxide. The indicator strip is used to indicate the development of anaerobic conditions in the system.
3. Heroin is usually diluted with a redox reducing agent such as quinine. Upon injection, if a spore-laden needle misses the vein, leakage of the heroin into the tissues establishes a focal point for an infection such as tetanus by the spore-forming *Clostridium*.
4. The infections of concern are gas gangrene and tetanus, whose etiological agents are spore forms that are present in the upper layers of the soil. The deep puncture wound caused by the tine would result in conditions favorable for spore germination. The absolute anaerobic condition is generated by the elimination of oxygen from the deep wound by aerobic and facultatively anaerobic contaminants. In addition, the breakdown of dead tissues provides the essential nutritional source for rapid germination.
5. A drain is inserted into a deep puncture wound to ensure that healing occurs from the depth of the wound outwards. Also, the drain provides an outlet for the passage of dead tissue debris, a contributory factor in spore germination, and it serves as an inlet for oxygenation of the infected area.

EXPERIMENT 20

Serial Dilution–Agar Plate Procedure to Quantitate Viable Cells

The purpose of this experiment is to allow students to count the number of viable cells in a culture. As part of this procedure, students will be introduced to two new methodologies, namely the pour-plate preparation and pipetting techniques that are essential for the serial dilution of cultures.

Materials

Culture

- 24- to 48-hour nutrient broth culture of *E. coli*

Media

	Per Lab Group	Per Class
20-ml nutrient agar deep tubes	6	
9-ml sterile water blanks	7	

Equipment

	Per Lab Group	Per Class
Hot plate	1	
Waterbath	1	
Thermometer	1	
Test tube rack	1	
Bunsen burner	1	
Mechanical pipetting device	1	
Sterile Petri dishes	6	
Quebec® colony counter and manual hand counter	1	
Disinfectant solution	as needed	
500-ml beaker	1	
Glassware marking pencil	1	

Optional Equipment

For the optional modifications or additions listed below:

- Spread-plate preparation: turntable, bent glass rod, and 95% ethyl alcohol
- Direct microscopic count: Petroff-Hausser chamber

Procedural Points to Emphasize

1. As this is the students' first encounter with the use of pipettes and mechanical pipetting devices, complete instructions and a demonstration should be given as to their proper use.
2. As numerous dilution tubes and Petri dishes are used in this exercise, students should be cautioned to carefully label and organize all the glassware before proceeding with the experimental procedure.
3. Students should be reminded to deposit all contaminated pipettes into a disinfectant-containing receptacle. These are never placed on the bench top.
4. The pour-plate method should be demonstrated with emphasis on the gentle swirling of the molten agar so as to prevent the agar from sloshing onto the cover of the plate.
5. Students should be cautioned to cool the molten agar to approximately 40°C prior to pouring it into the Petri dish.
6. The significance of TNTC and TFTC, in terms of the validity of the experimental data, should be adequately explained.

Optional Procedural Additions or Modifications

1. The spread-plate technique (Experiment 3) may be substituted for the pour-plate preparation. The advantage of this is twofold: First, this procedure can be more rapidly performed, and second, only surface colonies will be present for counting.
2. The performance of a direct microscopic count with the Petroff-Hausser chamber may be added to illustrate the difference in the results obtained when performing a viable cell count and a total cell count.

Additional Reading

- Sanderson, M. W., Sreerama, S., & Nagaraja, T. G. (2007). Sensitivity of direct plating for detection of high levels of E. coli O157:H7 in bovine fecal samples. *Current Microbiology*, 55(2):158–61.

Answers to Review Questions

1. Methods other than the serial dilution–agar plate method count both viable and dead cells.
2. Dilution refers to varying the concentration of a substance. The dilution factor is expressed mathematically as the reciprocal of the dilution.
3. The advantage of the serial dilution–agar plating procedure is that the cell count represents only viable cells. The disadvantage of this method is that it requires an incubation period that precludes the ability to obtain immediate results.
4. There are 5.6×10^8 cells per ml of culture.
5. The number 305 is recorded as TNTC. The number 15 is recorded as TFTC.
6. A variety of parameters can be used to measure cell growth chemically: for example, by an increase in protein and DNA concentrations or by determination of dry cell mass. Metabolic parameters can also be used: for example, the measurement of oxygen uptake by aerobic cells or the amount of carbon dioxide used by anaerobic organisms.
7. Dilution plates showing spreading colonial growth should be eliminated from the experimental data because they may be obscuring the presence of small colonial forms, and they represent the growth of more than a single colony.

EXPERIMENT 21

The Bacterial Growth Curve

The primary purpose of this experiment is to help students to visualize the exponential growth dynamics of microorganisms in culture. Toward this end, students will use two parameters to measure growth. The indirect method employs spectrophotometric absorbance (A) readings, while the direct method uses the serial dilution–agar plate method for the determination of viable cell counts. The graphic representation of the growth curve, using data from both the indirect and direct methods, will illustrate the major growth phases of the culture and will allow for the determination, by extrapolation, of the generation time (T1/2) for the culture.

Materials

Cultures

- 5- to 10-hour (log phase) brain heart infusion broth culture of *E. coli* with an A of 0.08–0.1 at 600 nm

Media

	Per Lab Group	Per Class
100-ml brain heart infusion in 250-ml Erlenmeyer flask	1	
99-ml sterile water blanks	18	
100-ml bottles of nutrient agar	4	

Equipment

	Per Lab Group	Per Class
37°C waterbath shaker incubator	1	
Spectrophotometer*	1	
13- × 100 mm test tubes (cuvettes)	1	
Quebec colony counter	1	
Sterile Petri dishes	24	
1-ml sterile pipettes	18	
10-ml sterile pipettes	6	
Mechanical pipetting device	1	
Glassware marking pencil	1	
1000-ml beaker	1	
Bunsen burner	1	

*Bausch and Lomb, Spectronic 20

Procedural Points to Emphasize

1. The procedure for harvesting aliquots for A determination and serial dilution for cell counts requires strict adherence to the prescribed time schedule, as outlined in the procedure section of the exercise. To facilitate the efficient performance of these procedures within the designated time frames, a review of the use of the spectrophotometer and the serial dilution–pour-plate technique will be helpful.
2. A demonstration and an explanation of the proper way to plot a graph using semilog paper and determination of the generation time by extrapolation from the plotted data should be presented.

Optional Procedural Additions or Modifications

To reduce the number of experimental manipulations, it is suggested that the spread-plate preparation (Experiment 3) be substituted for the pour-plate procedure.

Tips

- The inoculum must be composed of cells in midlog phase of growth (10–12 hours of incubation). These cultures may be placed into an ice-water bath to maintain this phase of growth for a short period of time.
- Because the Spectronic 20 uses a tungsten light source, rather than ultraviolet, new carefully checked and unscratched 10-$\times$100-ml tubes may be used rather than expensive quartz cuvettes.

Additional Reading

- Peleg, M., & Corradini, M. G. (2011). Microbial growth curves: what the models tell us and what they cannot. *Critical Reviews in Food Science and Nutrition*, 51(10):917–45.

Answers to Review Questions

1. In multicellular organisms, the term *growth* generally implies an increase in cell mass, whereas in bacteria, it implies an increase in cell number.
2. a. Variations in generation time among different species of organisms have a genetic basis that governs the enzymatic utilization of substrates.

   b. Variations in generation time within a single species are dependent upon differences in the environmental and nutritional conditions.
3. a. The growth curve of the organisms in an abscess would not mimic a laboratory growth curve because the organisms in the abscess are maintained in the stationary phase within the walled-off infected area where the host's defenses are ineffective.

   b. Antibiotics would not be effective unless the abscess was incised and allowed to drain. It is only under these conditions that the antibiotic could penetrate the infected area and act on the exponentially growing cells.
4. Generation time is a useful parameter to indicate the most suitable medium to support the growth of a specific organism. The medium in which the culture exhibited the shortest generation time would support the most abundant growth.

EXPERIMENT 22

Extracellular Enzymatic Activities of Microorganisms

The identification of microorganisms in both the academic and clinical settings is dependent upon the detection of specific enzymes that produce biochemical transformations of substrates exogenously or endogenously. In this experiment, extracellular enzymatic activities will be studied. These exoenzymes hydrolyze environmental macromolecules into their basic building blocks for their transport into the cell. Within the cell, these serve as metabolites for endoenzymatic activities. The presence of a specific exoenzyme is determined by the absence of its macromolecular substrate in the environment (medium) following the growth of the culture.

Materials

Cultures

Short Version: 24- to 48-hour TSB cultures of:

- *E. coli*
- *B. cereus*
- *P. aeruginosa*
- *S. aureus*

Long Version: 24- to 48-hour brain heart infusion broth cultures of:

- *A. faecalis*
- *B. cereus*
- *C. xerosis*
- *E. aerogenes*
- *E. coli*
- *K. pneumoniae*
- *L. lactis*
- *M. luteus*
- *P. vulgaris*
- *P. aeruginosa*
- *S. typhimurium*
- *S. dysenteriae*
- *S. aureus*

Media

Short Version	Per Lab Group	Per Class
Starch agar plate	2	
Tributyrin agar plate	2	
Milk agar plate	2	
Nutrient gelatin deep tubes	4	

Long Version	Per Lab Group	Per Class
Starch agar plate	4	
Tributyrin agar plate	4	
Milk agar plate	4	
Nutrient gelatin deep tubes	13	

Reagent

- Gram's iodine solution

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop and needle	1	
Glassware marking pencil	1	
Test tube rack	1	
Refrigerator 4°C	1	

Procedural Point to Emphasize

Gelatin Hydrolysis

It is essential that the instructor impress upon the student the fact that the gelatin cultures must be refrigerated following their incubation and prior to their observation.

Optional Procedural Additions or Modifications

1. At this point in the semester, aseptic techniques should be routine for the students. Consequently, to conserve laboratory time and media, it is suggested that students work in groups of four for the performance of this experiment as well as all the exercises in Part 5 of the manual, entitled "Biochemical Activities of Microorganisms."
2. Long and short versions are presented in all the experiments in this section of the manual. The long version provides the students with a comparative overview of the enzyme-dependent microbial activities on a variety of substrates. Most of the test procedures are basic; therefore, several experiments can be readily performed during a single laboratory session by groups of four students.

Tips

- The preparation of tributyrin agar requires that the tributyrin be incorporated into the nutrient agar at 90°C and emulsified in a blender.
- Soluble starch is added to nutrient agar to make a final starch concentration of 1%. The use of dehydrated starch agar is less satisfactory.
- Milk agar is best prepared by using double-strength nutrient agar autoclaved for 15 minutes at STP, and skim milk autoclaved for 10 minutes at 12 PSI. After cooling to 45–50°C, equal parts of each are mixed together.

Additional Reading

- Juarez, Z. E., & Stinson, M. W. (1999). An extracellular protease of *Streptococcus gordonii* hydrolyzes type IV collagen and collagen analogues. *Infection and Immunity*, 67(1):271–8.

Answers to Review Questions

1. All biochemical reactions that occur in living cells are regulated by enzymes. Without the action of enzymes many of these reactions would not take place at perceptible rates. All aspects of cellular metabolism are catalyzed by enzymes. This includes the digestion of large nutrient molecules such as proteins, carbohydrates, and lipids that are broken down into smaller molecules; the production and transformation of energy; and the synthesis of larger macromolecules essential for the viability of the cell.
2. Although milk is a sterile body fluid, microorganisms gain entry during the milking process. Many of these microorganisms contain enzymes that degrade milk carbohydrates, proteins, and lipids with the production of acid end products. Organisms such as *Lactobacillus* and *Streptococcus spp.* ferment lactose to lactic acid and acetic acid, turning milk sour. They may produce enough acid to curdle the milk protein, forming a curd.
3. Large, complex, highly branched polysaccharides, such as starch and cellulose, are macromolecules that cannot pass through the cell membrane and therefore must be degraded outside the cell by exoenzymes excreted by the cell. Once degraded, these smaller molecules, such as disaccharides like lactose and sucrose, may pass through the cell membrane and be further degraded into lactose and galactose by endoenzymes of the cell.

EXPERIMENT 23

Carbohydrate Fermentation

Carbohydrates serve as the major intracellular substrates for the production of cellular energy. Thus, one group of endoenzymes, the carbohydrases, is responsible for sustaining the bioenergetic activities in most microbes. The purpose of this experiment is twofold. First, it helps students ascertain the ability of a microorganism to utilize carbohydrates as a principal energy source. Second, it enables them to determine which specific carbohydrates can be metabolized by a microorganism; this is dependent upon the presence of complementary intracellular carbohydrases.

Experimentally, carbohydrate utilization can be used as a parameter for the identification of microorganisms. This is accomplished by the action of a specific carbohydrase on the carbohydrate substrate with the production of a metabolic waste product that is then detectable in the medium.

Materials

Cultures

Short Version: 24- to 48-hour TSB cultures of:

- *E. coli*
- *A. faecalis*
- *S. typhimurium*
- *S. aureus*

Long Version: 24- to 48-hour TSB cultures of:

- *A. faecalis*
- *B. cereus*
- *C. xerosis*
- *E. aerogenes*
- *E. coli*
- *K. pneumoniae*
- *L. lactis*
- *M. luteus*
- *P. vulgaris*
- *P. aeruginosa*
- *S. typhimurium*
- *S. dysenteriae*
- *S. aureus*

Media

Short Version	Per Lab Group	Per Class
Phenol red lactose broth	5	
Phenol red dextrose broth	5	
Phenol red sucrose broth	5	

Long Version	Per Lab Group	Per Class
Phenol red lactose broth	14	
Phenol red dextrose broth	14	
Phenol red sucrose broth	14	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop	1	
Glassware marking pencil	1	

Procedural Points to Emphasize

1. A brief discussion of the fermentative processes with an emphasis on end-product formation would be helpful to the students.
2. An explanation of the role of the pH indicator and the Durham tube in the carbohydrate broth medium for end-product detection should be presented.
3. Students should be cautioned that the presence of an acid in the medium is only discernible by the development of a canary yellow coloration throughout the culture medium.
4. The Durham gas vials must be submerged beneath the broth medium.
5. In large experiments (long version) requiring multiple inoculations, students may sometimes forget to inoculate a tube; in this case, the absence of growth should not be considered a reflection of the organism's fermentative capabilities.
6. Students need to know that, in some cases, they may obtain variable results, and this can be expected because of strain variations, incubation times, size of inoculum, or color reversion caused by refrigeration following incubation.
7. All carbohydrate media must be labeled immediately upon their preparation.
8. Students should set the experimental carbohydrate media in a definite order in the test tube rack and label them carefully to prevent any mix-up, especially considering that all phenol red broths look alike.
9. Students should be reminded to flame the loop before and after each carbohydrate broth is inoculated.

Tips

- Prior to autoclaving the carbohydrates, an inverted Durham tube should be placed into each of the carbohydrate tubes. Do not attempt to get the medium into the inverted tube; usually this tube floats on top of the medium. However, upon autoclaving, the medium will be forced up into the inverted Durham tube.
- The presence of a gas bubble in the Durham tube must be definite. Occasionally tiny bubbles in the gas vials may represent the loss of oxygen as the temperature changes in the medium from the refrigerator to the incubator.

Additional Reading

- Zoetendal, E. G., Raes, J., van den Bogert, B., Arumugam, M., Booijink, C. C., Troost, F. J., …Kleerebezem, M. (2012). The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *The ISME Journal*, 6(7):1415–26, doi: 10.1038/ismej.2011.212. [Epub ahead of print]

Answers to Review Questions

1. Cellular respiration is a biooxidative process that occurs aerobically, with molecular oxygen serving as the final electron acceptor, or anaerobically, with an inorganic ion acting as a final electron acceptor. Fermentation is a biooxidation that utilizes an organic compound as the final electron acceptor.
2. Microorganisms differ in their use of pyruvic acid. Some organisms use the pyruvic acid as a final electron acceptor, resulting in the formation of acids, alcohols, and solvents. Other organisms use this compound as a stepping stone into the Krebs cycle for further ATP production.
3. The strict anaerobes utilize the Embden-Meyerhof glycolytic pathway to pyruvic acid with limited ATP production. The pyruvic acid is then further metabolized through fermentative pathways.
4. *Pseudomonas* species hydrolyze proteins to amino acids that then enter the Krebs cycle for generation of ATP.
5. *Clostridium perfringens* is a saccharolytic organism and can utilize carbohydrates anaerobically; therefore, there is no evolution of carbon dioxide. However, the proteolytic enzymes of its exotoxin degrade proteins with the evolution of hydrogen gas, which destroys body tissues with the further release of carbohydrates for the continued generation of ATP.

EXPERIMENT 24

Triple Sugar–Iron Agar Test

The purpose of this experiment is to illustrate a rapid diagnostic procedure for the separation and presumptive identification of the enteric bacilli on the basis of differences in their carbohydrate fermentative patterns.

Materials

Cultures

Short Version: 24-hour TSB cultures of:

- *E. coli*
- *S. typhimurium*
- *S. dysenteriae*
- *P. aeruginosa*
- *P. vulgaris*
- *A. faecalis*

Long Version: 24-hour TSB cultures of:

- *A. faecalis*
- *B. cereus*
- *C. xerosis*
- *E. aerogenes*
- *E. coli*
- *K. pneumoniae*
- *L. lactis*
- *M. luteus*
- *P. vulgaris*
- *P. aeruginosa*
- *S. typhimurium*
- *S. dysenteriae*
- *S. aureus*

Media

Short Version	Per Lab Group	Per Class
Triple sugar-iron agar slants	7	

Long Version	Per Lab Group	Per Class
Triple sugar-iron agar slants	14	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating needle	1	
Test tube rack	1	
Glassware marking pencil	1	

Procedural Points to Emphasize

1. The stab-and-streak method of inoculation should be demonstrated to the students.
2. It is essential to adhere to the designated incubation period of 18–24 hours to prevent the complete degradation of the carbohydrate substrates, thereby inhibiting the catabolism of proteins.
3. Students should be reminded not to fail to inoculate the butt of the TSI agar. Failure to do so will produce invalid results.

4. Students should be told that they cannot use an inoculating loop for this experiment. The loop will split the butt, giving the medium the false appearance of gas production.
5. The butt may be stabbed first and then the slant streaked, or the reverse order may be used, depending on the instructor's preference.

Tip

- A prolific H_2S producer may produce so much black precipitate (ferrous sulfide) that acidity produced in the butt is completely masked. If H_2S is produced, an acid condition does exist even though it is not discernible, and the sample should be reported as positive for acid.

Additional Reading

- Ma, M., Amano, T., Enokimoto, M., Yano, T., Moe, K. K., & Misawa, N. (2007). Influence of pH of TSI medium on the detection of hydrogen sulfide production by *Campylobacter hyointestinalis*. *Letters in Applied Microbiology*, 44(5):544–9.

Answers to Review Questions

1. The TSI test is designed for the rapid separation and presumptive identification of enteric organisms.
2. The lower concentration of glucose in the medium allows for detection of the utilization of this substrate only.
3. The purpose of the phenol red pH indicator is to detect carbohydrate fermentation that is indicated by a color change in the medium from orange-red to yellow, which is caused by the presence of acidic end products.
4. The thiosulfate serves as a substrate for hydrogen sulfide production.
5. The limited length of the incubation period is important to prevent the breakdown of proteins in the medium, which would result in the formation of end products that would obscure the observation of the results.

IMViC Test
Hydrogen Sulfide Test
Urease Test

The purpose of this battery of biochemical tests is to separate and identify the members of the family *Enterobacteriaceae*. Once again, the basis of these tests is predicated on the availability of specific endoenzymes whose presence is determined by the presence or absence of their metabolic end products in the medium.

This particular group of tests has clinical as well as environmental significance. From the medical perspective, members of this family of microorganisms include pathogens and organisms that constitute the normal intestinal flora. From an environmental aspect, *E. coli* is the index marker for fecal pollution of water and food sources.

Materials

IMViC Test

Cultures

PART A: Indole
Short Version: 24- to 48-hour TSB cultures of:
- *E. coli*
- *P. vulgaris*
- *E. aerogenes*

PART B: Methyl Red
Short Version: 24- to 48-hour TSB cultures* of:
- *E. coli*
- *E. aerogenes*
- *K. pneumoniae*

*Aliquots of these experimental cultures must be saved for the Voges-Proskauer test.

PART C: Voges-Proskauer Test
Short Version: 24- to 48-hour TSB cultures* of:

- *E. coli*
- *E. aerogenes*
- *K. pneumoniae*

*All aliquots of these experimental cultures must be set aside from the methyl red test.

PART D: Citrate Utilization Test
Short Version: 24- to 48-hour TSB cultures of:
- *E. coli*
- *E. aerogenes*
- *K. pneumoniae*

Media

PART A: Short Version	Per Lab Group	Per Class
SIM agar deep tubes	4	

PART A: Long Version	Per Lab Group	Per Class
SIM agar deep tubes	14	

PART B: Short Version	Per Lab Group	Per Class
MR-VP broth	4	

PART B: Long Version	Per Lab Group	Per Class
MR-VP broth	14	

PART D: Short Version	Per Lab Group	Per Class
Simmons citrate agar slants	4	

Media (*continued*)

PART D: Long Version	Per Lab Group	Per Class
Simmons citrate agar slants	14	

Reagents

PART A: Kovac's reagent
PART B: Methyl red indicator
PART C: Barritt's reagents A (alpha-naphthol) and B (potassium hydroxide)

Equipment

PART A,C,D	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating needle/loop	1	
Test tube rack	1	
Glassware marking pencil	1	

PART B	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop	1	
Test tube rack	1	
Glassware marking pencil	1	
Sterile pipettes	as needed (3 minimum)	
Sterile 13- × 100-ml test tubes	3	

Hydrogen Sulfide Test

Cultures

Short Version: 24- to 48-hour TSB cultures of:
- *E. aerogenes*
- *S. dysenteriae*
- *P. vulgaris*
- *S. typhimurium*

Media

Short Version	Per Lab Group	Per Class
SIM agar deep tubes	5	

Long Version	Per Lab Group	Per Class
SIM agar deep tubes	14	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating needle	1	
Test tube rack	1	
Glassware marking pencil	1	

Urease Test

Cultures

Short Version: 24- to 48-hour TSB cultures of:
- *E. coli*
- *P. vulgaris*
- *S. typhimurium*
- *K. pneumoniae*

Media

Short Version	Per Lab Group	Per Class
Urea broth tubes	5	

Long Version	Per Lab Group	Per Class
Urea broth tubes	14	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop	1	
Test tube rack	1	
Glassware marking pencil	1	

IMViC Test, Hydrogen Sulfide Test, Urease Test

Cultures

Long Version: 24- to 48-hour TSB cultures of:

- *A. faecalis*
- *B. cereus*
- *C. xerosis*
- *E. aerogenes*
- *E. coli*
- *K. pneumoniae*
- *L. lactis*
- *M. luteus*
- *P. vulgaris*
- *P. aeruginosa*
- *S. typhimurium*
- *S. dysenteriae*
- *S. aureus*

Procedural Points to Emphasize

Students should be cautioned to be careful to add the correct test reagents to the appropriate cultures. In addition, the following points should be stressed for the performance of the biochemical tests.

1. **Indole and Hydrogen Sulfide Tests** These are performed and read in the same medium, SIM agar.
2. **Methyl Red Test** No attempt should be made to record the methyl red results prior to 48 hours of incubation. They should be checked, however, after 24 hours. If the methyl red tests are performed too early, false-positive results may occur because the organisms have not had sufficient time to completely metabolize the acidic products that have accumulated following the fermentation of the glucose.
3. **Hydrogen Sulfide Test** Students should be made aware that H_2S production is most prominent with motile organisms and that they will produce a black color throughout the medium. Nonmotile organisms may exhibit blackening along the line of growth.
4. **Methyl Red and Voges-Proskauer Tests** The MR-VP broth cultures must be separated into two aliquots prior to the addition of the test reagents. For the V-P test, the addition of Barritt's test reagents requires the introduction of solution A first, followed by solution B. A reversal of the order may give a weak-positive or false-negative result. Also, a 10- to 20-minute reaction time is required prior to the observation of the cultures.
5. **Citrate Test** A positive reaction is ascertained by the presence of growth on the slant surface and not by the color change of the medium that accompanies the microbial growth.
6. **Citrate Test** It is a good idea to streak the citrate medium first when inoculating a number of biochemical tests from the same culture. Any carryover of glucose or other nutrient onto the citrate medium may produce a false-positive result.

Tips

IMViC Test

- Kovac's reagent should be fresh; however, it can be kept in the refrigerator (4–10°C). Standing at room temperature for any length of time may cause color changes and decrease sensitivity. Store the reagent in an amber bottle with a glass stopper.
- Motile cultures grown in SIM media display a diffuse growth away from the line ofinoculation.

Urease Test

- *Proteus* spp. give strong positive results when the pH reaches 8 or higher.
- Variations in the pH of positive reactions can occur because of differences in the size of the inoculum.

Additional Readings

- Lee, J. I., Kim, S. J., & Park, C. G. (2011). *Shigella flexneri* infection in a newly acquired rhesus macaque (*Macaca mulatta*). *Laboratory Animal Research*, 27(4):343–6.
- Stoffels, L., Krehenbrink, M., Berks, B. C., & Unden, G. (2012). Thiosulfate reduction in *Salmonella enterica* is driven by the proton motive force. *Journal of Bacteriology*, 194(2):475–85.
- Murphy, T. F., & Brauer, A. L. (2011). Expression of urease by *Haemophilus influenzae* during human respiratory tract infection and role in survival in an acid environment. *BMC Microbiology*, 11:183.

Answers to Review Questions

IMViC Test

1. The IMViC test is used for the identification of enteric organisms, which include both pathogens and nonpathogens.
2. Kovac's reagent acts to extract indole from the medium into the reagent layer. The indole then forms a cherry-red complex with *p*-dimethylaminobenzaldehyde.
3. Citrate utilization produces oxaloacetic acid and acetate, which are enzymatically converted to CO_2 and pyruvic acid. The CO_2 combines with sodium and water to form Na_2CO_3, an alkaline product.
4. Both *E. coli* and *E. aerogenes* produce acidic end products during early incubation. The low pH is maintained by *E. coli*, thereby producing a positive methyl red test. On the other hand, *E. aerogenes* converts the acids to acetylmethylcarbinol, a nonacidic end product that elevates the pH of the culture later in the incubation period.
5. Pyruvic acid is a utilizable intracellular metabolite and therefore is not excreted into the medium. Indole is a waste product and can be detected in the medium.
6. The rationale for the use of Simmons citrate is to identify organisms that are enzymatically capable of metabolizing citrate as the sole carbon source for energy production.

Hydrogen Sulfide Test

1. The substrates for hydrogen sulfide production include the amino acid cysteine and inorganic sulfur-containing compounds such as thiosulfates, sulfates, and sulfites.
2. The growth of a nonmotile organism would be restricted to the line of inoculation, whereas motility is recognized by growth that is not restricted to the line of inoculation.
3. The ferrous ammonium sulfate serves as an indicator by combining with the hydrogen sulfide gas to form a detectable, insoluble, black ferrous sulfide precipitate within the medium.
4. *E. aerogenes* lacks the desulfurase that is present in *P. vulgaris*.
5. Because *E. coli* is a member of the natural intestinal flora, the differentiation between *Salmonella* and *Shigella* can be made on the basis of hydrogen sulfide production. Only *Salmonella* species produce hydrogen sulfide.

Urease Test

1. Urease is a hydrolytic enzyme that degrades amide compounds such as urea with the formation of ammonia, which is alkaline.
2. Phenol red is incorporated into the urea broth for the detection of the alkaline end products with the resultant development of a deep pink color.
3. Urease activity is more rapid in *Proteus* species than in other urease-positive organisms. Thus, this reaction will be detected earlier in a *Proteus* culture than in other cultures.
4. The possible contaminant may be a *Proteus* or a *Salmonella* organism. Differentiation between the two genera may be accomplished by the urease test. Only *Proteus* organisms will produce a positive result.

EXPERIMENT 28

Litmus Milk Reactions

Milk, as it contains lactose and casein, serves as an excellent medium for the cultivation of microorganisms. Enzymatically, these substrates are acted upon differently by various organisms. In this experiment, students will have the opportunity to determine the substrate that is being metabolized and its concomitant reactions.

Materials

Cultures

Short Version: 24- to 48-hour TSB cultures of:

- *E. coli*
- *A. faecalis*
- *L. lactis*
- *P. aeruginosa*

Long Version: 24- to 48-hour TSB cultures of:

- *A. faecalis*
- *B. cereus*
- *C. xerosis*
- *E. aerogenes*
- *E. coli*
- *K. pneumoniae*
- *L. lactis*
- *M. luteus*
- *P. vulgaris*
- *P. aeruginosa*
- *S. typhimurium*
- *S. dysenteriae*
- *S. aureus*

Media

Short Version	Per Lab Group	Per Class
Tubes of litmus milk broth	5	

Long Version	Per Lab Group	Per Class
Tubes of litmus milk broth	14	

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop	1	
Test tube rack	1	
Glassware marking pencil	1	

Procedural Points to Emphasize

1. A 48-hour incubation period is necessary for the complete development of the biochemical reactions in the cultures.
2. The basic observable reactions should be explained, and the fact that more than one change may be noted in a single culture should be stressed.
3. For accurate observation and interpretation of the experimental results, the following precautions should be brought to the attention of the students:

   a. Cultures should be handled with minimal agitation.

b. The determination of coagulation or curd formation requires the gentle tilting of the culture tube following the notation of color changes in the medium.

c. The presence of minimal reduction in the very bottom of cultures frequently occurs following 48 hours of incubation. Its presence should be disregarded.

Tips

- Litmus milk broth should be autoclaved at 12 pounds/inch^2 for 15 minutes to maintain the chemical integrity of the medium. Overheating caramelizes the milk sugars.
- Litmus milk broth must have a pH of 6.8. To be sure of the pH, three control organisms with a broad spectrum of results should be used. These are as follows:

  Alcaligenes faecalis: This gives a blue alkaline reaction in milk broth.

  Clostridium welchii: The clostridia in milk broth give a stormy fermentation and an acid clot, which is torn apart by gas.

  Proteus vulgaris: This produces no change in the milk broth.
- Homogenized milk should never be used for the preparation of litmus milk broth.

Additional Reading

- Schierl, E. A., & Blazevic, D. J. (1981). Rapid identification of enterococci by reduction of litmus milk. *Journal of Clinical Microbiology*, 14(2):227–8.

Answers to Review Questions

1. An acid curd is a nonretractable, hard clot, whereas the rennet curd is a soft clot that retracts from the walls of the tube.
2. The reactions that occur as a result of the catabolism of proteins are proteolysis and an alkaline reaction. In proteolysis, the proteins are degraded to amino acids with the production of ammonia, an alkaline end product. This is evidenced by the appearance of a deep-purple band at the surface of the culture. The medium below shows a translucent brown color. In an alkaline reaction, the casein is partially degraded to shorter polypeptides with the release of some alkaline end products. As a result, the medium assumes a deeper blue color.
3. The litmus in the uninoculated medium is in the oxidized state and is purple in color. It becomes reduced when it gains hydrogen ions and the medium turns white starting at the bottom of the test tube.
4. A pink coloration indicates lactose fermentation, and a translucent, brownish color with a deep-purple band at the surface is indicative of proteolysis. Both reactions cannot coexist in one culture, as this would indicate the simultaneous degradation of both lactose and proteins.
5. Litmus milk can serve a differential purpose in that it separates lactose fermenters from nonfermenters.

EXPERIMENT 29

Nitrate Reduction Test

The purpose of this experiment is to identify organisms that are enzymatically capable of using nitrate as the final electron acceptor in the absence of molecular oxygen.

Materials

Cultures

Short Version: 24- to 48-hour TSB cultures of:

- *E. coli*
- *A. faecalis*
- *P. aeruginosa*

Long Version: 24- to 48-hour TSB cultures of:

- *A. faecalis*
- *B. cereus*
- *C. xerosis*
- *E. aerogenes*
- *E. coli*
- *K. pneumoniae*
- *L. lactis*
- *M. luteus*
- *P. vulgaris*
- *P. aeruginosa*
- *S. typhimurium*
- *S. dysenteriae*
- *S. aureus*

Media

Short Version	Per Lab Group	Per Class
Trypticase nitrate broth tubes	4	

Long Version	Per Lab Group	Per Class
Tryticase nitrate broth tubes	14	

Reagents

- Solution A (sulfanilic acid)
- Solution B (alpha-naphthylamine)
- Zinc powder

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop	1	
Test tube rack	1	
Glassware marking pencil	1	

Procedural Point to Emphasize

Students should be cautioned that the reaction from the addition of zinc powder to the cultures for the reduction of nitrate to nitrite is a delayed reaction; therefore, the development of the cherry color in the medium is not immediate.

Tips

- When performing a number of determinations, examine the tubes immediately. When doing the nitrate reduction test using alpha-naphthylamine, the color produced in a positive test can fade quickly.
- In some cases, a strong nitrate-positive reducing organism may exhibit a brown precipitate immediately after the addition of the reagents.

Additional Reading

- Khan, A., & Sarkar, D. (2012). Nitrate reduction pathways in mycobacteria and their implications during latency. *Microbiology*, 158(Pt 2):301–7.

Answers to Review Questions

1. The agar in the nitrate medium serves to lower the redox potential to favor the anaerobic requirement for nitrate reduction.
2. Solutions A and B are used to detect the presence of nitrite in the culture, which is evidenced by the immediate appearance of a red color in the medium.
3. If no color change occurs in the medium upon the addition of solutions A and B, either nitrate reduction has not occurred or it was reduced past the nitrite stage.
4. The development of a red color upon the addition of zinc indicates that nitrate is still present in the culture; the zinc, rather than the microorganisms, reduced it to nitrite.
5. A direct relationship exists between the organism's ability to reduce nitrate past the nitrite stage and its vigorous proteolytic ability.

EXPERIMENT 30

Catalase Test

The purpose of this experiment is to illustrate the enzymatic degradation of cytotoxic hydrogen peroxide, formed during aerobic respiration, as a result of catalase activity. This procedure is of clinical significance for the presumptive identification of members of the genus Streptococcus.

Materials

Cultures

Short Version: 24- to 48-hour TSB cultures of:

- *S. aureus*
- *M. luteus*
- *L. lactis*

Long Version: 24- to 48-hour TSB cultures of:

- *A. faecalis*
- *B. cereus*
- *C. xerosis*
- *E. aerogenes*
- *E. coli*
- *K. pneumoniae*
- *L. lactis*
- *M. luteus*
- *P. vulgaris*
- *P. aeruginosa*
- *S. typhimurium*
- *S. dysenteriae*
- *S. aureus*

Media

Short Version	Per Lab Group	Per Class
Trypticase soy agar slants	4	

Long Version	Per Lab Group	Per Class
Trypticase soy agar slants	14	

Reagent

- 3% hydrogen peroxide

Equipment

Tube method	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop	1	
Test tube rack	1	
Glassware marking pencil	1	

Slide Method	Per Lab Group		Per Class	
	Short Version	Long Version	Short Version	Long Version
Inoculating loop	1	1		
Bunsen burner	1	1		
Glassware marking pencil	1	1		
Petri dishes	4	14		
Glass microscope slide	4	14		

Procedural Points to Emphasize

Tube Method

1. Students should be directed to tilt the agar slant cultures while adding the hydrogen peroxide so that it makes contact with the bacterial growth along the entire slant surface.
2. When not in use, hydrogen peroxide should be kept under refrigeration at all times.
3. Although only a 3% hydrogen peroxide solution is used, if spilled on the skin, it should be neutralized with 70% ethyl alcohol rather than water.

Slide Method

1. Slides should be placed in Petri dishes.
2. Do not mix culture with hydrogen peroxide.
3. Cover Petri dishes to prevent aerosols.

Tips

- Hydrogen peroxide must be fairly fresh. Because it is unstable and breaks down when exposed to light, it should always be kept in a brown or amber bottle.
- Catalase cultures should be about 24–48 hours old for the best results. Older cultures may lose catalase and possibly result in a false-negative reaction.
- There is no specific concentration for hydrogen peroxide used in the catalase test, considering that the speed and intensity of the reaction is independent of the concentration. However, 3% seems to be the concentration used in most laboratories.

Additional Reading

- Das, D., & Bishayi, B. (2009). *Staphylococcal* catalase protects intracellularly survived bacteria by destroying H_2O_2 produced by the murine peritoneal macrophages. *Microbial Pathogenesis*, 47(2):57–67.

Answers to Review Questions

1. Hydrogen peroxide is formed as a result of aerobic respiration. This is a cytotoxic compound that requires the presence of specific enzymes for its degradation. Strict anaerobes lack these essential enzymes.
2. Catalase catabolizes hydrogen peroxide to water and molecular oxygen.
3. Catalase is an endoenzyme, as cellular respiration is an intracellular process.
4. The streptococci, which are catalase negative, can grow aerobically on blood agar, as the RBCs in the medium serve as a source of catalase to degrade the H_2O_2.

EXPERIMENT 31

Oxidase Test

The purpose of this experiment is to illustrate cytochrome oxidase activity, which is of value in the identification of medically important members of the genera *Neisseria* and *Pseudomonas*.

Materials

Cultures

Short Version: 24- to 48-hour TSB cultures of:

- *E. coli*
- *P. aeruginosa*
- *A. faecalis*

Long Version: 24- to 48-hour TSB cultures of:

- *A. faecalis*
- *B. cereus*
- *C. xerosis*
- *E. aerogenes*
- *E. coli*
- *K. pneumoniae*
- *L. lactis*
- *M. luteus*
- *P. vulgaris*
- *P. aeruginosa*
- *S. typhimurium*
- *S. dysenteriae*
- *S. aureus*

Media

Short Version	Per Lab Group	Per Class
Trypticase soy agar plate	1	

Long Version	Per Lab Group	Per Class
Trypticase soy agar plates	4	

Reagent

- *p*-aminodimethylaniline oxalate

Equipment

	Per Lab Group	Per Class
Bunsen burner	1	
Inoculating loop	1	
Petri dishes	1 (short vers.) 4 (long vers.)	
Glassware marking pencil	1	
Filter paper (for filter-paper method only)	1 (short vers.) 4 (long vers.)	

Procedural Point to Emphasize

This test procedure should not present any problems in its performance.

Optional Procedural Alterations or Additions

The instructor may have the students perform either the plate method or the filter paper method or opt to do both depending upon time constraints.

Tips

- If cultures are grown on a medium containing glucose, then the oxidase test should not be performed. Glucose fermentation will inhibit the oxidase enzyme and result in false-negative reactions.
- The *p*-aminodimethylaniline oxalate may be replaced with tetramethyl-p-phenylenediamine dihydrochloride, but it is not simply Kovac's reagent used in the indole test. This reagent should be labeled Kovac's oxidase reagent.

Additional Reading

- Slesak, G., Douangdala, P., Inthalad, S., Silisouk, J., Vongsouvath, M., Sengduangphachanh, A., … Newton, P. N. (2009). Fatal *Chromobacterium violaceum* septicaemia in northern Laos, a modified oxidase test and post-mortem forensic family G6PD analysis. *Annals of Clinical Microbiology and Antimicrobials*, 8:24.

Answers to Review Questions

1. Cytochrome oxidase catalyzes the oxidation of reduced cytochrome A_3 by molecular oxygen, resulting in the formation of water or hydrogen peroxide.
2. Strict aerobes are oxidase positive because molecular oxygen is the final electron acceptor in aerobic respiration.
3. The oxidase test is used for the differentiation of oxidase-positive members of the *Neisseria* and *Pseudomonas* from the oxidase-negative members of the family *Enterobacteriaceae*.
4. The reagent serves as an artificial electron donor that becomes oxidized to a purple-black compound in the presence of cytochrome oxidase and oxygen.
5. The swarming colonies of the gram-negative bacilli may be either a *Pseudomonas* or a *Proteus* species. The oxidase test can be used to differentiate between the oxidase-positive *Pseudomonas* and the oxidase-negative *Proteus*.

EXPERIMENT 32

Utilization of Amino Acids

The purpose of this experiment is to illustrate the ability of some microorganisms to enzymatically degrade amino acids via the process of decarboxylation and deamination reactions. They are clinically useful in the differentiation and identification of members of the gram-negative *Enterobacteriaceae*.

Materials

Cultures

PART A
Short Version: 24-hour nutrient broth cultures of:
- *P. vulgaris*
- *C. freundii*
- *E. coli*

PART B
Short Version: 24-hour nutrient broth cultures of:
- *E. coli*
- *P. vulgaris*

PARTS A and B
Long Version: 24- to 48-hour nutrient broth cultures of:
- *A. faecalis*
- *B. cereus*
- *C. xerosis*
- *E. aerogenes*
- *E. coli*
- *K. pneumoniae*
- *L. lactis*
- *M. luteus*
- *P. vulgaris*
- *P. aeruginosa*
- *S. typhimurium*
- *S. dysenteriae*
- *S. aureus*

Media

PART A: Short Version	Per Lab Group	Per Class
Tubes of Moeller's DB with L-lysine (labeled LD+)	3	
Tubes of Moeller's DB w/o L-lysine (labeled LD–)	3	

PART A: Long Version	Per Lab Group	Per Class
Tubes of Moeller's DB with L-lysine (labeled LD+)	13	
Tubes of Moeller's DB w/o L-lysine (labeled LD–)	13	

PART B: Short Version	Per Lab Group	Per Class
Phenylalanine agar slants	2	

PART B: Long Version	Per Lab Group	Per Class
Phenylalanine agar slants	13	

Reagents

PART A: N/A
PART B: 10–12% ferric chloride solution

Equipment

PARTS A and B	Per Lab Group	Per Class
Bunsen burner	1	
Glassware marking pencil	1	
Inoculating loop/needle	1	
Sterile Pasteur pipettes	2	
Rubber pipette bulbs	2	
Test tube rack	1	
Sterile mineral oil	as needed	

Procedural Points to Emphasize

1. Decarboxylase reactions occur under anaerobic conditions. Anaerobiosis is established by sealing the tubes with sterile mineral oil. Using a sterile Pasteur pipette, overlay the slanted surface of the culture with the oil. It is important to stress that the pipette should not touch the walls or sides of the tube.
2. Deaminase reactions must be read immediately following the addition of the ferric chloride solution.
3. Students must label all decarboxylase tubes prior to inoculation. The accidental mix-up of tubes will invalidate results for bacterialidentification.
4. Following incubation (18–24 hours), the decarboxylase control tubes must remain yellow, indicating that only glucose has been fermented. A positive control tube (purple color) invalidates the decarboxylase test, and interpretation of the results is not possible.
5. A positive phenylalanine test must be interpreted immediately after the addition of the ferric chloride reagent because the green color fades rapidly.
6. Rolling the ferric chloride reagent over the slant produces a more intense color and more rapid reaction.

Tips

PART A

- Falkow lysine decarboxylase medium may be substituted for Moeller's decarboxylase medium. The latter medium requires a low pH and anaerobic conditions. Falkow medium simply depends on the change in the pH indicator (bromcresol purple). The composition of Falkow lysine decarboxylase medium is found in Appendix 1.

PART B

- The 10–12% ferric chloride solution must be refrigerated in a dark brown or amber bottle. Its stability should be checked weekly, using positive cultures.

Additional Readings

- Curiel, J. A., Ruiz-Capillas, C., de Las Rivas, B., Carrascosa, A. V., Jiménez-Colmenero, F., & Muñoz, R. (2011). Production of biogenic amines by lactic acid bacteria and enterobacteria isolated from fresh pork sausages packaged in different atmospheres and kept under refrigeration. *Meat Science*, 88(3):368–73.
- Clayton, T. A. (2012). Metabolic differences underlying two distinct rat urinary phenotypes, a suggested role for gut microbial metabolism of phenylalanine and a possible connection to autism. *FEBS Letters*, 586(7):956–61.

Answers to Review Questions

1. Only glucose was fermented, and therefore, cadaverine was not produced.
2. The results must be read quickly because the ferric chloride is not too stable and the color reaction fades rapidly.
3. Ferric chloride acts as a chelating agent (binding agent) and chelates phenylpyruvic acid to form a green color.
4. Oxygen is consumed in the medium by microorganisms during the growth phase and rises (alkalinity) as carbon dioxide is produced. Because the pH can be controlled, it is now possible to add a pH indicator to the medium.

5. a. This is a disorder that affects the way the body metabolizes certain amino acids found in proteins, mainly leucine, isoleucine, and valine. These amino acids accumulate in the bloodstream and interfere with brain function. There is an autosomal genetic basis for this disease. It occurs in newborns, accompanied by lethargy, convulsions, and, if not treated, death within the first few days of life. The sweet burnt sugar (maple syrup) smell in the urine is responsible for its name.

b. The disease is treated through strict dietary restriction of branched amino acids throughout the lifetime of the affected individual.

EXPERIMENT 33

Genus Identification of Unknown Bacterial Cultures

The purpose of this experiment is to provide the students with an opportunity to bring together all of the techniques and accumulated information that they have mastered at this point in their laboratory aspect of the course. This basic exercise for the genus identification of an unknown culture is designed to put this assimilated knowledge to practical use.

Materials

Cultures

- One unknown pure culture per student

Number-coded 24- to 48-hour Trypticase soy agar slant cultures of:

- *A. faecalis*
- *B. cereus*
- *C. xerosis*
- *E. aerogenes*
- *E. coli*
- *K. pneumoniae*
- *L. lactis*
- *M. luteus*
- *P. vulgaris*
- *P. aeruginosa*
- *S. typhimurium*
- *S. dysenteriae*
- *S. aureus*

Media

	Per Student	Per Class
Trypticase soy agar slants	2	
Phenol red lactose broth	1	

Media (*continued*)

	Per Student	Per Class
Phenol red sucrose broth	1	
Phenol red dextrose broth	1	
SIM agar deep tube	1	
MR-VP broth	1	
Trypticase nitrate broth	1	
Simmons citrate agar slant	1	
Urea broth	1	
Litmus milk	1	
Trypticase soy agar plate	1	
Nutrient gelatin deep tube	1	
Starch agar plate	1	
Tributyrin agar plate	1	

Reagents

- Crystal violet stain
- Gram's iodine
- 95% ethyl alcohol
- Safranin stain
- Methyl red
- 3% hydrogen peroxide
- Barritt's reagent
- Solution A (sulfanilic acid)
- Solution B (alpha-naphthylamine)
- Zinc powder
- Kovac's reagent
- *p*-aminodimethylaniline oxalate

Equipment

	Per Student	Per Class
Bunsen burner	1	
Inoculating loop	1	
Inoculating needle	1	
Staining tray	1	
Immersion oil	as needed	
Lens paper	as needed	
Bibulous paper	as needed	
Microscope	1	
Glassware marking pencil	1	

Procedural Points to Emphasize

1. For the performance of this independent identification procedure, the laboratory, as well as the necessary media, staining and test reagents, and Bergey's Manual should be made available to the students for approximately 1 week.
2. The following reminders may serve to make this identification procedure an enjoyable experience rather than a frustrating one for the students:

   a. The initial step in this procedure should be the preparation of a subculture from the number-coded unknown. This will serve as a backup culture if contamination of the original unknown is suspected or for the repetition of biochemical tests if necessary.

   b. Adherence to the prescribed incubation periods, which vary for different procedures, is important in order to obtain the proper reaction.

   c. Strain variations may be responsible for unexpected test results. Therefore, students may have to make judgment calls based on the importance of the test procedure for the identification of the organism.
3. Contamination of the numbered unknown culture is a common problem facing students. Be sure to have them inoculate a subculture from the original unknown culture to be used in the event that contamination occurs.
4. Absolute aseptic technique is imperative in order to avoid any possible source of contamination.
5. Students should record their observations as soon as they observe them.
6. There is a possibility that variants are organisms that resemble the other members of their species and may not produce all of the expected test results exactly.

Tip

- If performing the Gram stain, a young culture should be used to ensure the best morphologic characteristics of the organism and the correct Gram reaction.

Additional Reading

- Sharma, K. K., & Kalawat, U. (2010). Emerging infections: shewanella—a series of five cases. *Journal of Laboratory Physicians*, 2(2):61–5.