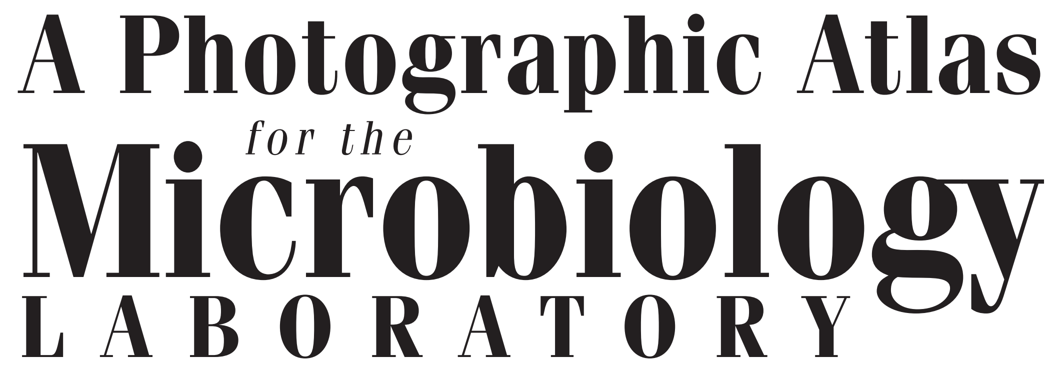
A Photographic Atlas for the Microbiology Laboratory

4TH EDITION



A Photographic Atlas for the Microbiology Laboratory

4th EDITION

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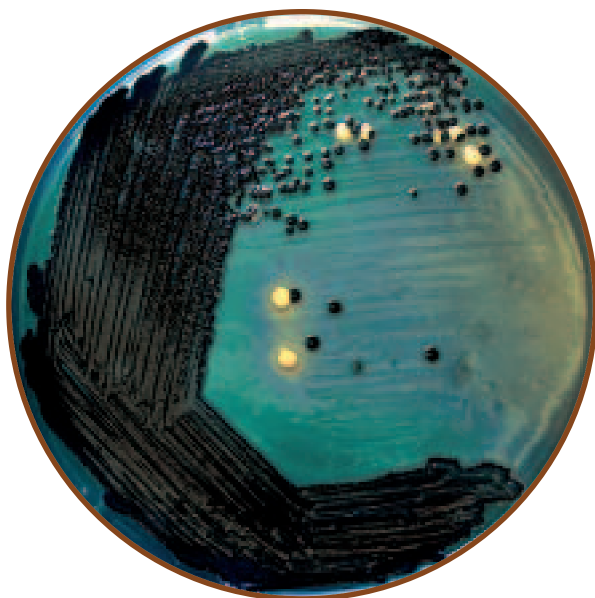


2-18 Hektoen Enteric Agar Streaked for Isolation HE agar streaked with *Salmonella typhimurium* and *Escherichia coli*. Note the black *Salmonella* colonies due to sulfur reduction and the yellow *E. coli* colonies due to lactose fermentation with acid end-products.

MacConkey Agar

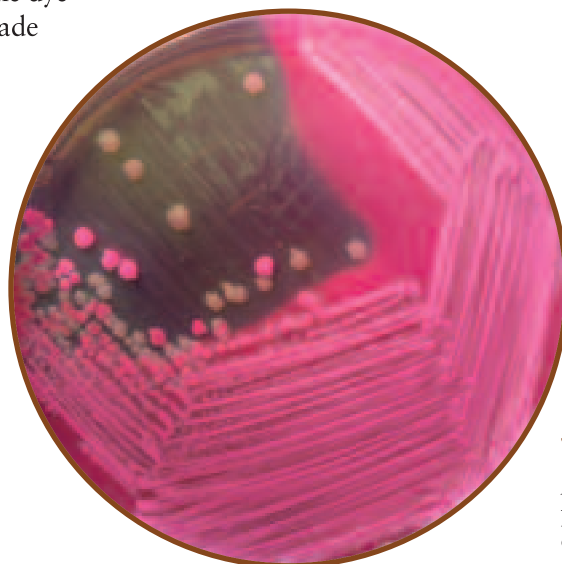
Purpose

MacConkey Agar is used to isolate and differentiate members of the *Enterobacteriaceae* based on the ability to ferment lactose. Variations on the standard medium include MacConkey Agar w/o CV (without crystal violet) to allow detection of Gram-positive cocci or MacConkey Agar CS to control swarming bacteria (such as *Proteus*) that interfere with other results.

2-19 MacConkey Agar MacConkey Agar inoculated with (clockwise from top) *Escherichia coli, Enterobacter aerogenes, Shigella sonnei,* and *Proteus mirabilis. E. coli* and *E. aerogenes* produce pink color from acid-producing lactose fermentation. *S. sonnei* and *P. mirabilis*, both lactose nonfermenters, remain their normal color. Note the precipitated bile salts around the *E. coli*, also shown in Figure 2-20.

Principle

MacConkey Agar is a selective and differential medium containing lactose, bile salts, neutral red, and crystal violet. Bile salts and crystal violet inhibit growth of Gram-positive bacteria. Neutral red dye is a pH indicator that is colorless above a pH of 6.8 and red at a pH below 6.8. Acid accumulating from lactose fermentation turns the dye red. Lactose fermenters turn a shade of red on MacConkey agar whereas lactose nonfermenters remain their normal color or the color of the medium (Figures 2-19 and 2-20). Formulations without crystal violet allow growth of *Enterococcus* and some species of *Staphylococcus*, which ferment the lactose and appear pink on the medium.



2-20 MacConkey Agar Streaked for Isolation MacConkey Agar inoculated with *Escherichia coli* and *Shigella flexneri*. Note the precipitated bile salts around the *E. coli* caused by acid from lactose fermentation.

Mannitol Salt Agar

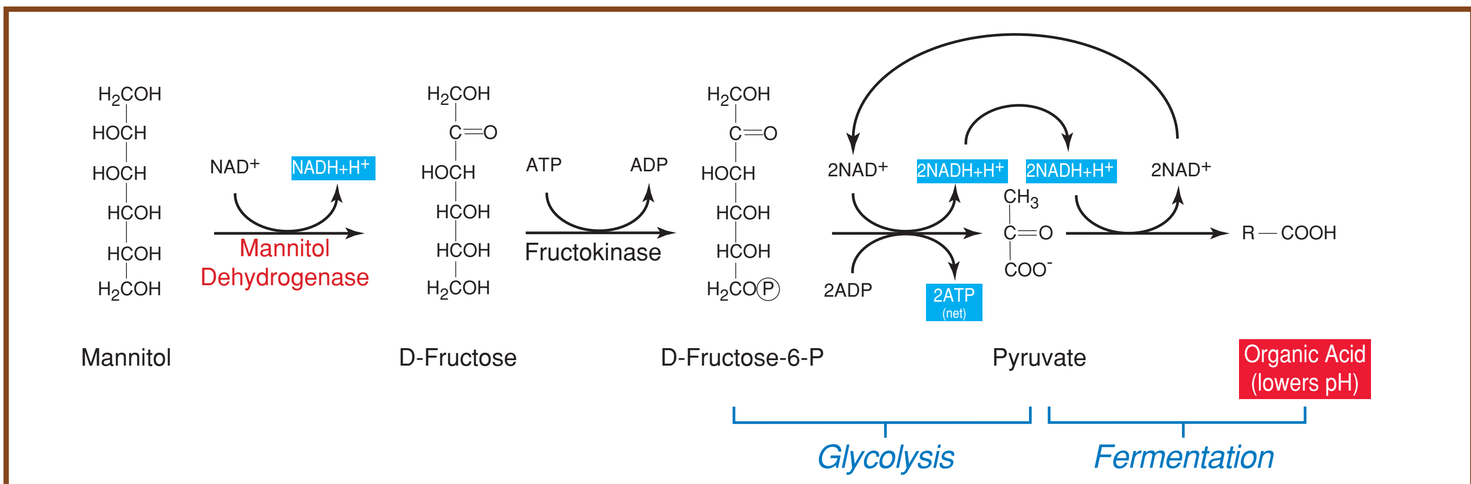
Purpose

Mannitol Salt Agar (MSA) is used for isolation and differentiation of pathogenic staphylococci, principally *Staphylococcus aureus*.

Principle

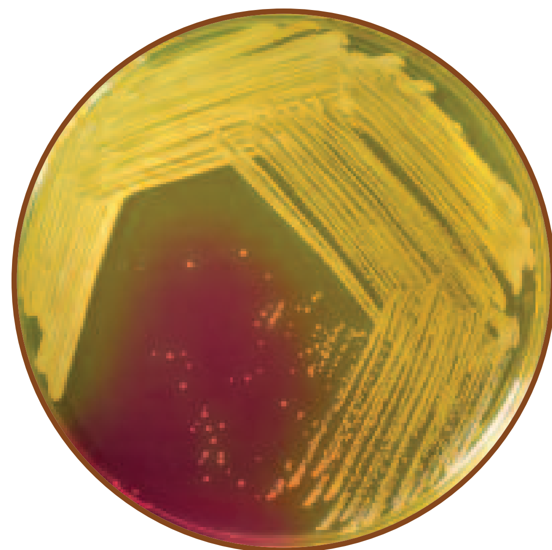
Mannitol Salt Agar contains the carbohydrate mannitol, 7.5% sodium chloride (NaCl), and the pH indicator phenol red. Phenol red is yellow below pH 6.8, red at pH 7.4 to 8.4, and pink above 8.4. The sodium chloride makes this medium selective for staphylococci since most other bacteria cannot survive in this level of salinity. The pathogenic species of *Staphylococcus* ferment mannitol (Figure 2-21) and produce acid, which turns the pH indicator yellow. Nonpathogenic staphylococcal species grow, but produce no color change. Refer to pages 71–73 and Figure A-5 in the Appendix for more information on fermentation.

The development of yellow halos around the bacterial growth is presumptive evidence that the organism is a pathogenic *Staphylococcus* (usually *S. aureus*). Good growth that produces no color change is presumptive evidence for nonpathogenic *Staphylococcus* (Figures 2-22 and 2-23). With few exceptions, organisms that grow poorly on the medium are not staphylococci.

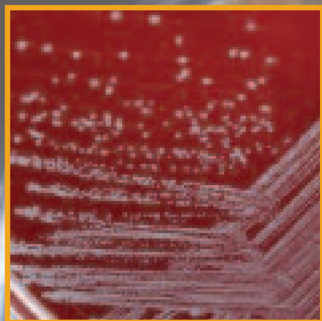


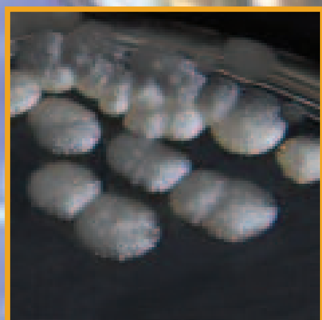
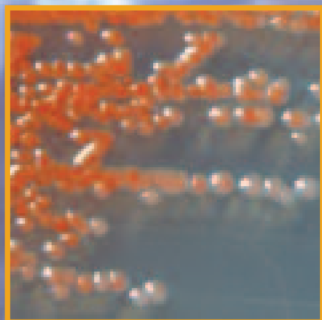
2-21 Mannitol Fermentation with Acid End-Products The organic acids produced lower the pH and turn the medium yellow.

2-22 Mannitol Salt Agar MSA inoculated with *Staphylococcus aureus* (top) and *S. epidermidis* (bottom). (*Note:* Some strains of *S. epidermidis* are inhibited by this medium). The yellow halo around *S. aureus* is due to mannitol fermentation with acid end products.



2-23 Mannitol Salt Agar Streaked for Isolation MSA inoculated with *Staphylococcus aureus* and *Staphylococcus epidermidis*. The growth shown in this photo is typical of the two species on this medium; the colonies of *S. epidermidis* are small and red whereas those of *S. aureus* are slightly larger and yellow.



SECTION 3

Bacterial Growth

Growth Patterns on Agar

Purpose

Recognizing different bacterial growth morphologies on agar plates is a useful and often crucial step in the identification process. Agar slants are typically used for cultivation of pure cultures. Bacteria also frequently display distinct morphological color and texture on agar slants.

Principle

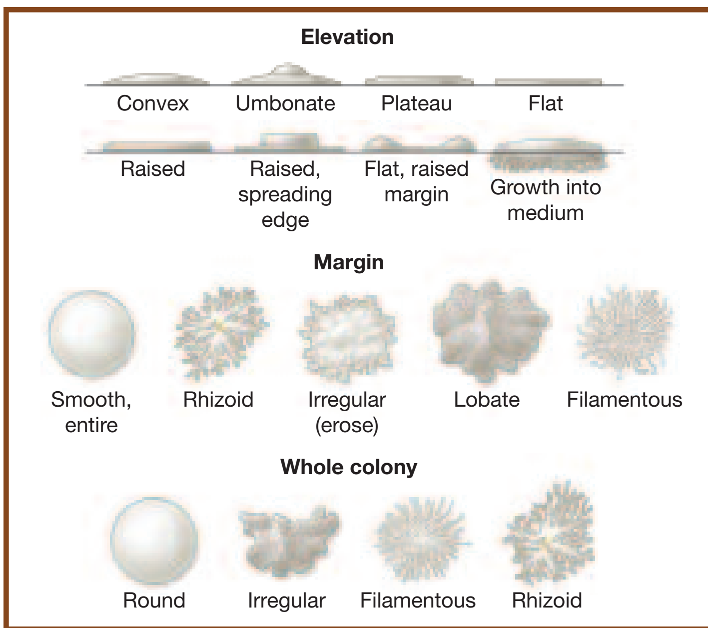
When a single bacterial cell is deposited on a solid nutrient medium, it begins to divide. One cell makes two, two make four, four make eight . . . one million make two million, and so on. Eventually a **colony** appears where the original cell was deposited. Once the purity of a colony has been confirmed by an appropriate staining procedure (Sections 5 and 6), cells can then be transferred to a sterile medium to begin a **pure culture**.

Color, size, shape, and texture of microbial growth are determined by the genetic makeup of the organism. However, organismal genetic expression is also greatly influenced by environmental factors including nutrient availability, temperature, and incubation time. Colony characteristics may be viewed with the naked eye or with the assistance of a colony counter (Figure 3-1).

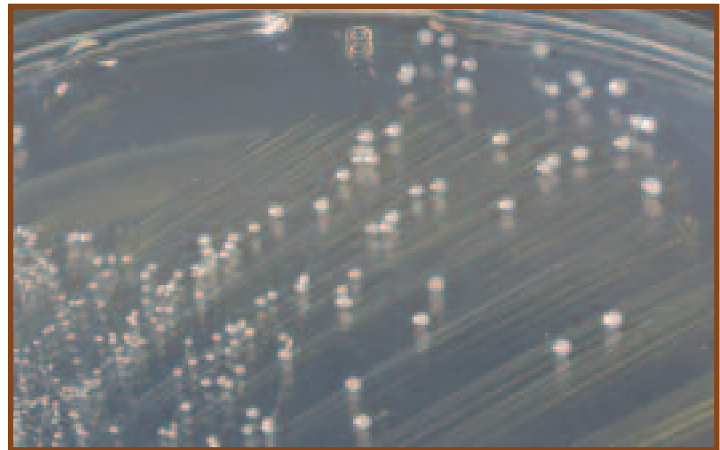
The basic categories of growth include colony shape, margin (edge), elevation, color, and texture (Figure 3-2). Colony shape may be described as **circular**, **irregular**, or **punctiform** (tiny). The margin may be **entire** (smooth, with no irregularities), **undulate** (wavy), **lobate** (lobed), **filamentous**, or **rhizoid** (branched like roots). Colony elevations include **flat**, **raised**, **convex**, **pulvinate** (very convex), and **umbonate** (raised in the center). Colony texture may be **moist**, **mucoid**, or **dry**. Pigment production is another useful characteristic and may be combined with optical properties such as **opaque**, **translucent**, **shiny**, or **dull**.

3-1 The Colony Counter Subtle differences in colony shape and size can best be viewed on the colony counter. The **transmitted light** and magnifying glass allow observation of greater detail, however, colony color is best determined with **reflected** light. The grid in the background is used as a counting aid. Each big square is a square centimeter.

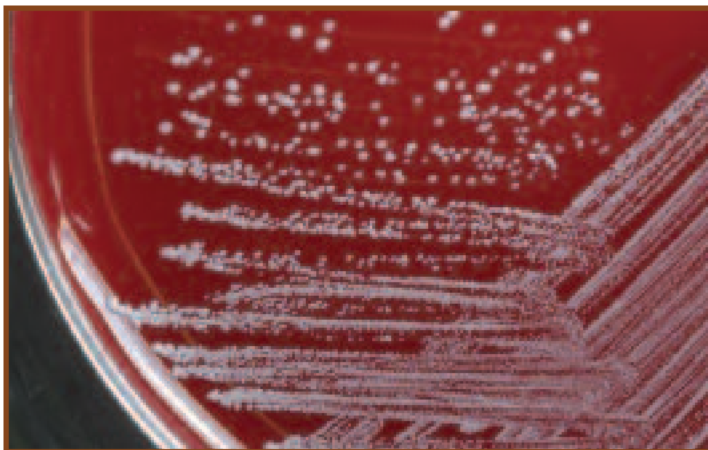
Figures 3-3 through 3-31 show a variety of bacterial colony forms and characteristics. Where applicable, contrasting environmental factors are indicated. Figures 3-32 and 3-34 show growth characteristics on agar slants.



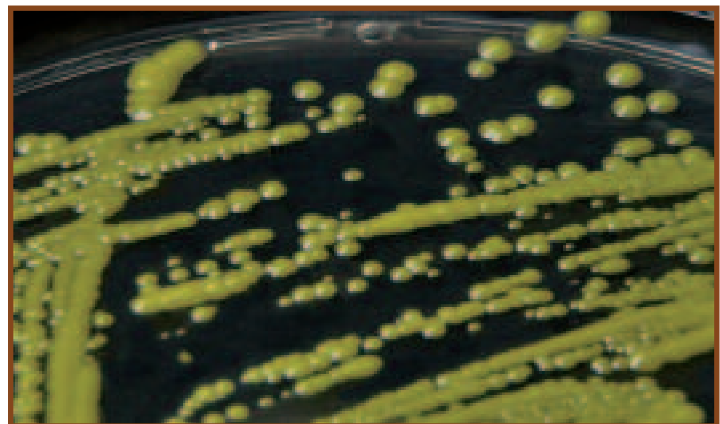
3-2 A SAMPLING OF BACTERIAL COLONY FEATURES These terms are used to describe colonial morphology. Descriptions also should include color, surface characteristics (dull or shiny), consistency (dry, butyrous-buttery, or moist) and optical properties (opaque or translucent).



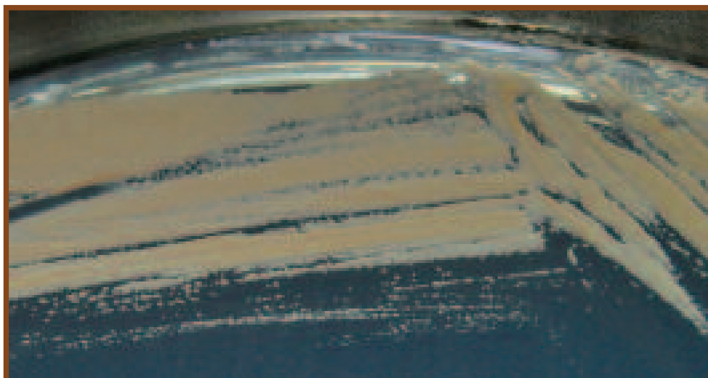
3-3 *ENTEROCOCCUS FAECIUM* GROWN ON NUTRIENT AGAR The colonies are white, circular, convex, smooth, and have an entire margin. *E. faecium* (formerly known as *Streptococcus faecium*) is found in human and animal feces.



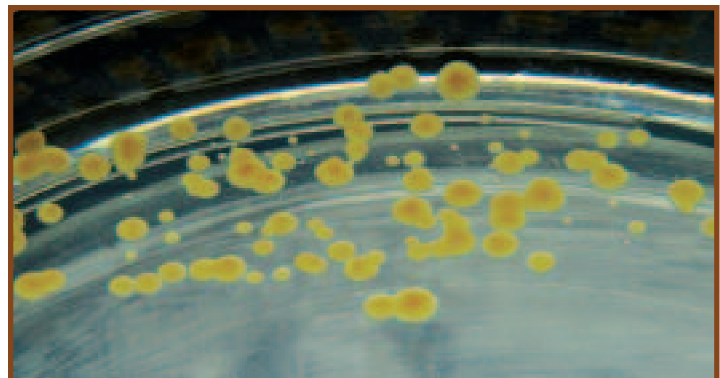
3-4 *STAPHYLOCOCCUS EPIDERMIDIS* GROWN ON SHEEP BLOOD AGAR The colonies are white, raised, circular, and entire. *S. epidermidis* is an opportunistic pathogen.



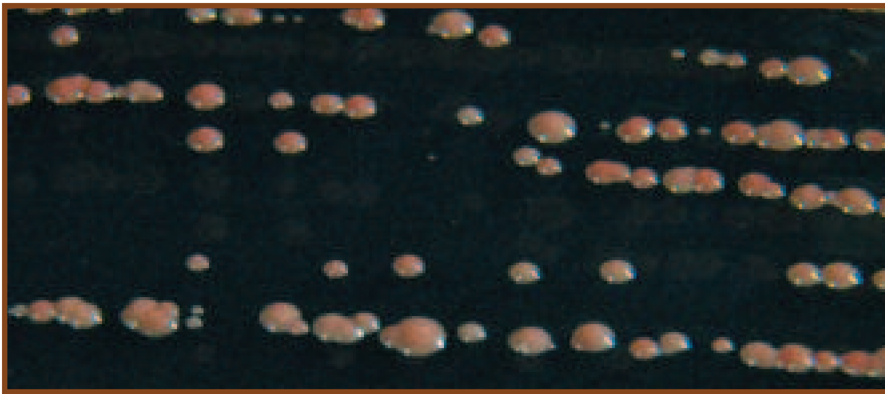
3-5 *MICROCOCCUS LUTEUS* GROWN ON BRAIN HEART INFUSION AGAR These colonies are yellow, smooth, and convex with a regular margin. They range in size from 1 to 3 mm. *M. luteus* is common in soil, dust, and on human skin.



3-6 *KOCURIA ROSEA* GROWN ON BRAIN HEART INFUSION AGAR Pink, punctiform (these are less than 1 mm in diameter), smooth, regular colonies typify *K. rosea*, an inhabitant of water, dust, and salty foods.

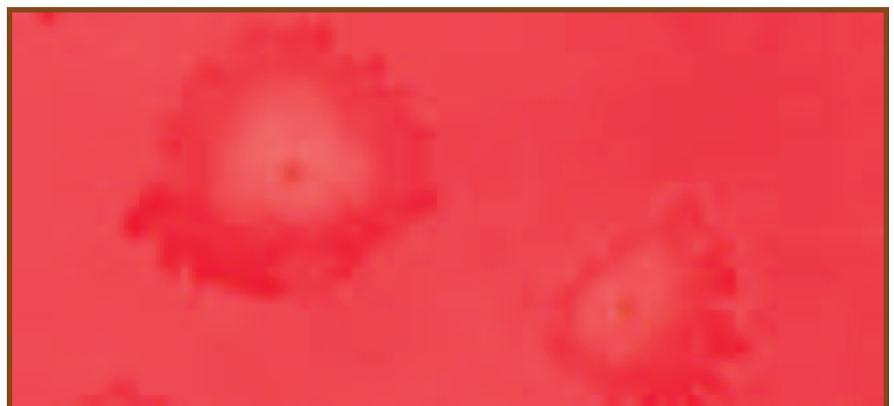


3-7 *SARCINA AURANTIACA* GROWN ON BRAIN HEART INFUSION AGAR *S. aurantiaca* produced yellow-orange, smooth, convex, regular colonies on BHIA. These are between 1 to 3 mm in diameter.



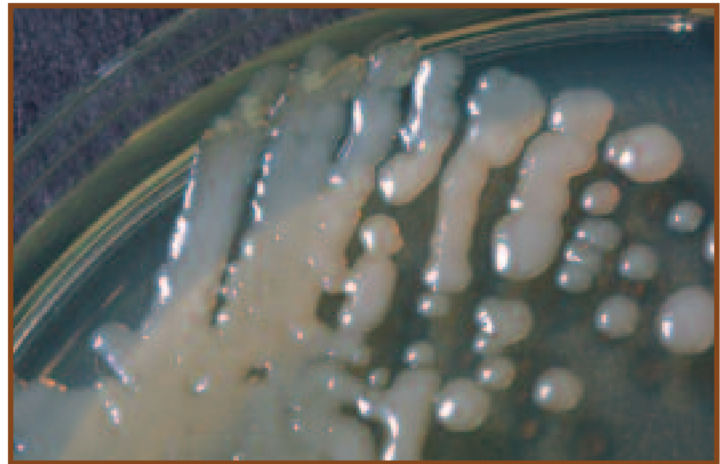
3-8 *Rhodococcus rhodochrous* Grown on Brain Heart Infusion Agar These colonies are reddish-pink, smooth, and convex with a regular margin. They are about 1 mm in diameter. *Rhodococcus* species are soil organisms.

3-9 Comparison of Four *Bacillus* Species **A** *B. cereus* grown on Blood Agar produces distinctively large (up to 7 mm), gray, granular, irregular colonies. They often produce a "mousy" smell. Also note the extensions of growth along the streak line. **B** *B. anthracis* colonies resemble *B. cereus*, but are usually smaller and adhere to the medium more tenaciously. **C** *B. mycoides* produces rapidly spreading, rhizoid colonies. **D** This unknown *Bacillus* isolated as a laboratory contaminant produced wrinkled, irregular colonies with an undulate (wavy) margin.

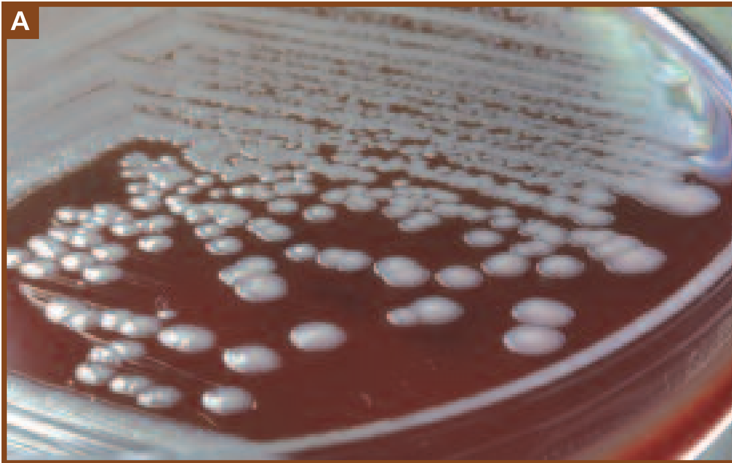


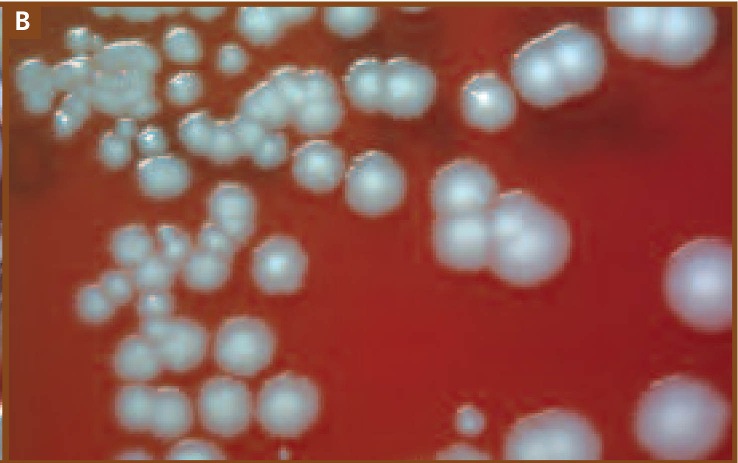
3-10 "Medusa Head" Colonies of *Clostridium sporogenes* on Blood Agar These irregularly circular colonies have a raised, yellow center and a flat, spreading edge of tangled filaments (reminiscent of the mythological creature Medusa, who had snakes for hair!). They vary in size from 2 to 6 mm.

3-11 *Providencia stuartii* Grown on Nutrient Agar The colonies are shiny, buff, and convex. *P. stuartii* is a frequent isolate in urine samples obtained from hospitalized and catheterized patients. *P. stuartii* is highly resistant to antibiotics.



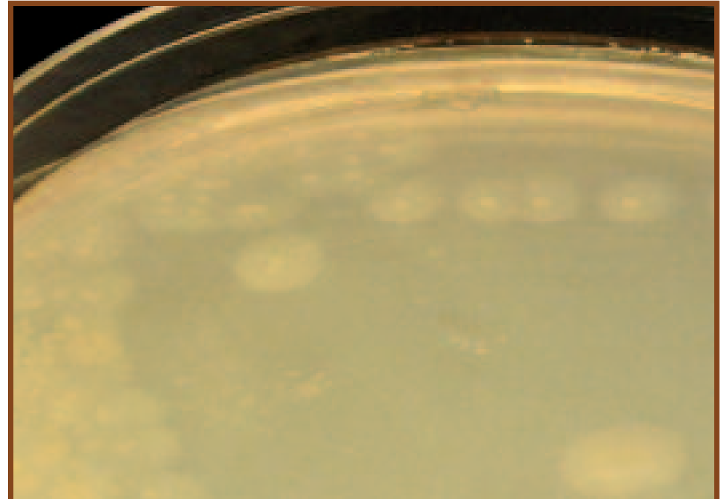
3-12 *Klebsiella pneumoniae* Grown on Nutrient Agar The colonies are mucoid, raised, and shiny. While it is a normal inhabitant of the human intestinal tract, it is associated with community-acquired pneumonia and nososomial urinary tract infections.



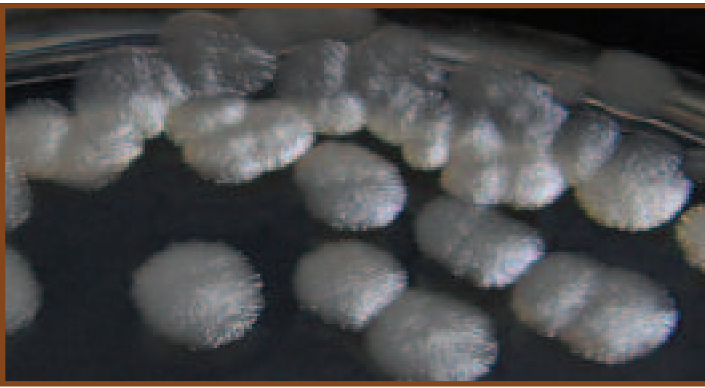


3-13 *Alcaligenes faecalis* Colonies on Sheep Blood Agar The colonies of this opportunistic pathogen are umbonate with an opaque center and a spreading edge. **A** Side view. Note the raised center. **B** Close-up of the *A. faecalis* colonies showing spreading edge.

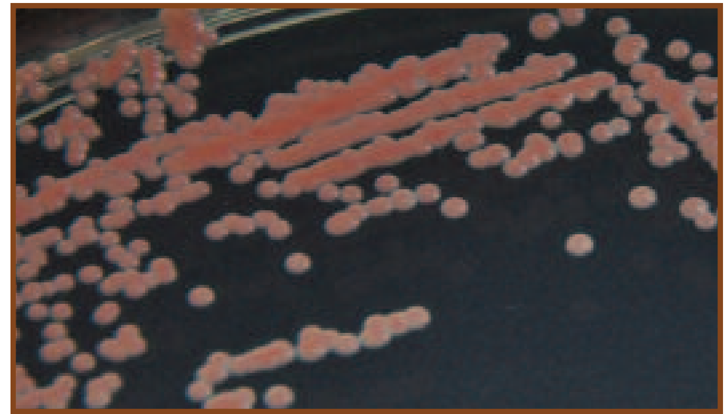
3-14 *Citrobacter koseri* Grown on Sheep Blood Agar The colonies are round, smooth, and opaque with a regular margin. This species is also able to partially hemolyze red blood cells (α-hemolytic), as evidenced by the greening around each colony. They range in size from 1 to 2 mm.



3-15 *Erwinia amylovora* Grown on Brain Heart Infusion Agar These colonies are whitish, transluscent, spreading, and umbonate. *Erwinia* species are plant pathogens.

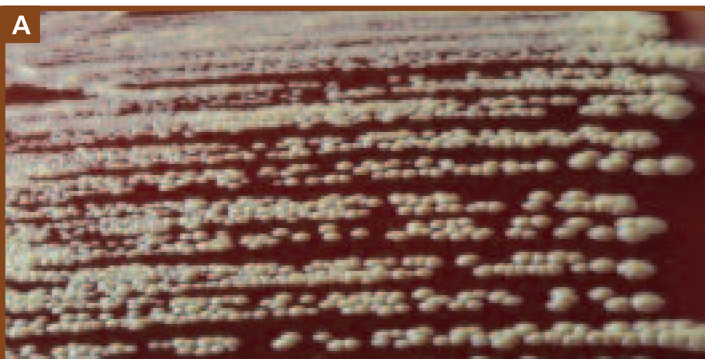


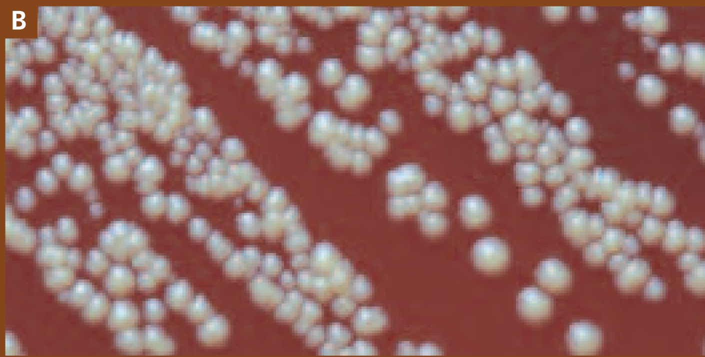
3-16 ***Rhizobium leguminosarum*** **Grown on Brain Heart Infusion Agar** The colonies are convex, circular, and filamentous. They are translucent at the edges and about 5 mm in diameter. *R. leguminosarum* is capable of producing root nodules (tumors) in many legumes and subsequently fixing atmospheric nitrogen.



3-17 ***Deinococcus radiodurans*** **Grown on Trytpicase Soy Agar** These small (between 1 and 2 mm in diameter), round, convex, and regular colonies took 36 hours to develop the orange color. This species is highly resistant to ionizing radiation.

3-18 ***Mycobacterium smegmatis*** **Grown on Sheep Blood Agar** The colonies of this slow growing relative of *M. tuberculosis* are punctiform.

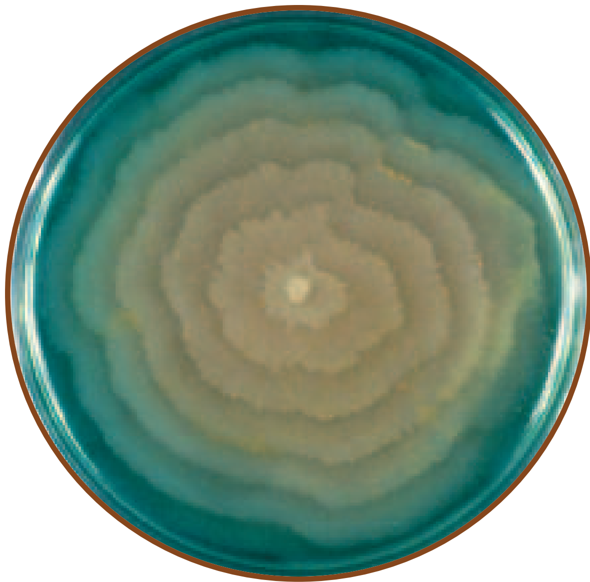




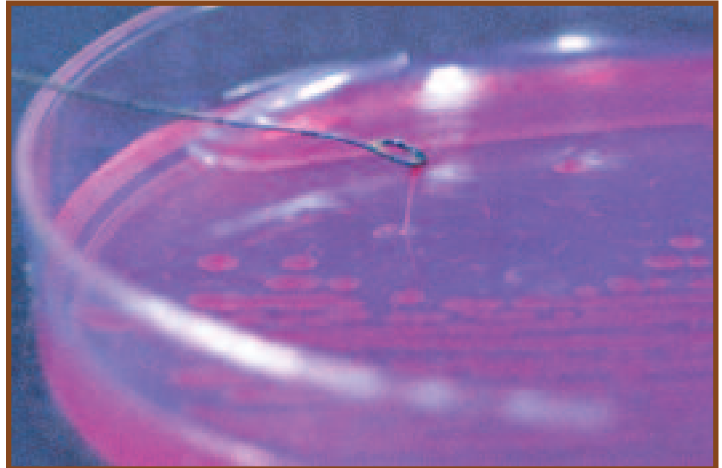
3-19 ***Corynebacterium xerosis*** **Grown on Sheep Blood Agar** **A** As seen in this view from the side, the colonies are dull, buff, and convex. **B** Close-up of circular *C. xerosis* colonies. *C. xerosis* is rarely an opportunistic pathogen.

3-20 **Umbonate Colony of an Anaerobic Lab Contaminant** The colony on the left is truly umbonate. The one on the right is getting there. Their diameters are about 3 mm.

3-21 ***Streptomyces griseus*** **Grown on Brain Heart Infusion Agar** These colonies are circular and ridged with a granular appearance. At a later stage of development, they produce yellow reproductive spores. Growth of streptomycetes is associated with an "earthy" smell. This one plate fragranced the entire incubator!



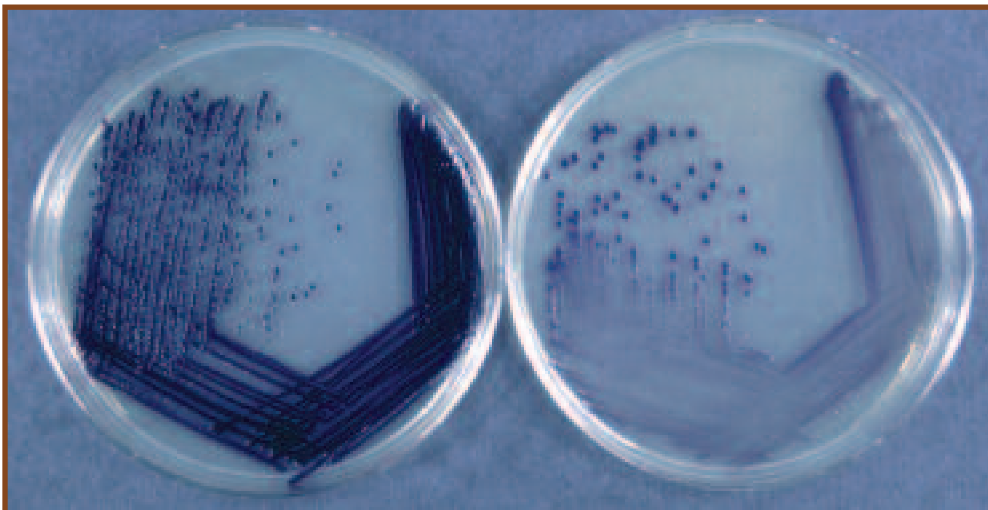
3-22 Swarming Growth Pattern Members of the genus *Proteus* will swarm at certain intervals and produce a pattern of concentric rings due to their motility. This is a photograph of *P. vulgaris* demonstrating swarming behavior on DNase agar.



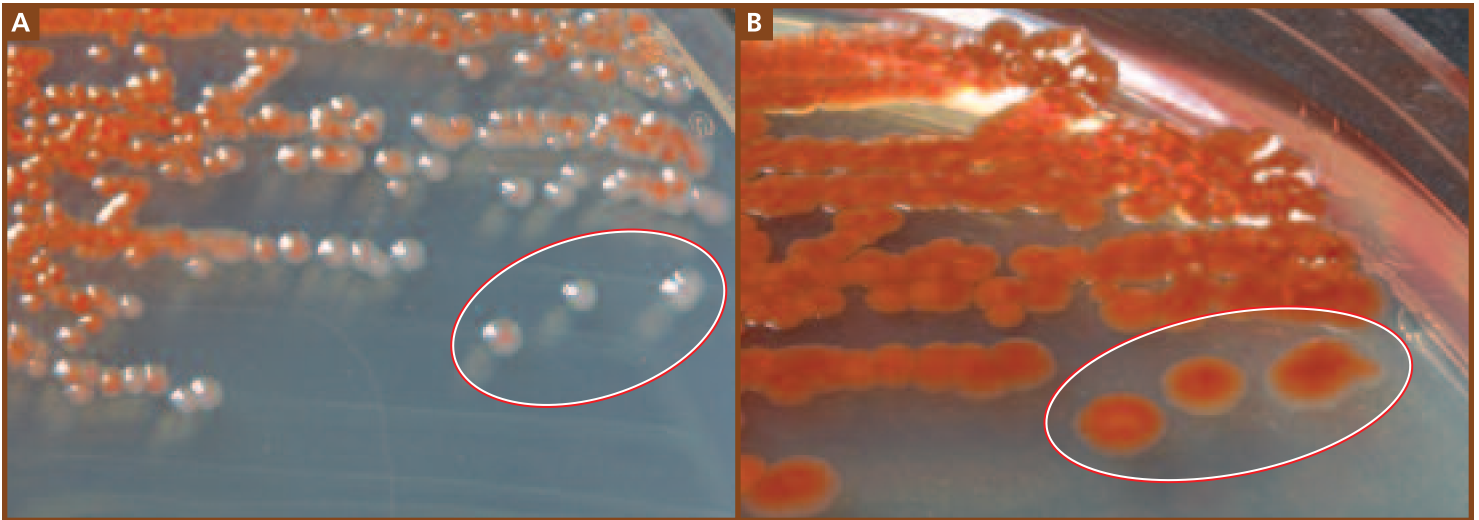
3-23 Mucoid Colonies *Pseudomonas aeruginosa* grown on Endo agar demonstrates a mucoid texture. *P. aeruginosa* is found in soil and water, and can cause infections in burn patients.

3-24 Butyrous Colony of an Unknown Soil Isolate This 12 mm colony was found on a Glycerol Yeast Extract plate inoculated with a diluted soil sample. It was almost liquid in composition, something that is indicated by its contact with the yellow colony to its right.

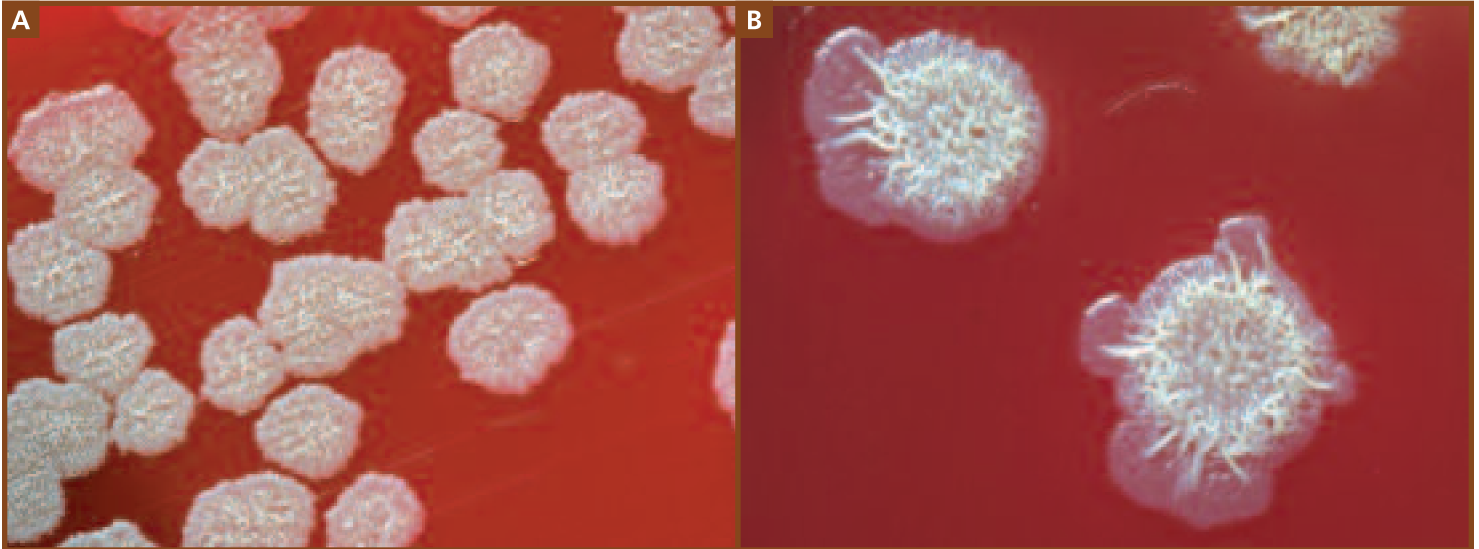
3-25 *Chromobacterium violaceum* Grown on Sheep Blood Agar *C. violaceum* produces shiny, purple, convex colonies. It is found in soil and water, and rarely produces infections in humans.



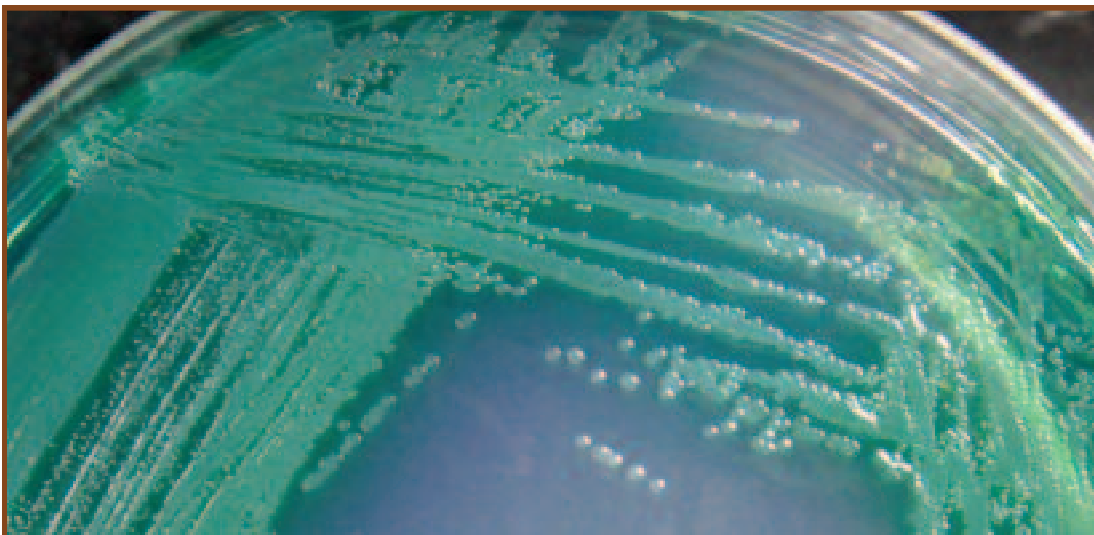
3-26 Influence of Nutrient Availability on Pigment Production Pigment production may be influenced by environmental factors such as nutrient availability. *Chromobacterium violaceum* produces a much more intense purple pigment when grown on Trypticase Soy Agar (left) than when grown on Nutrient Agar, a less nutritious medium (right).



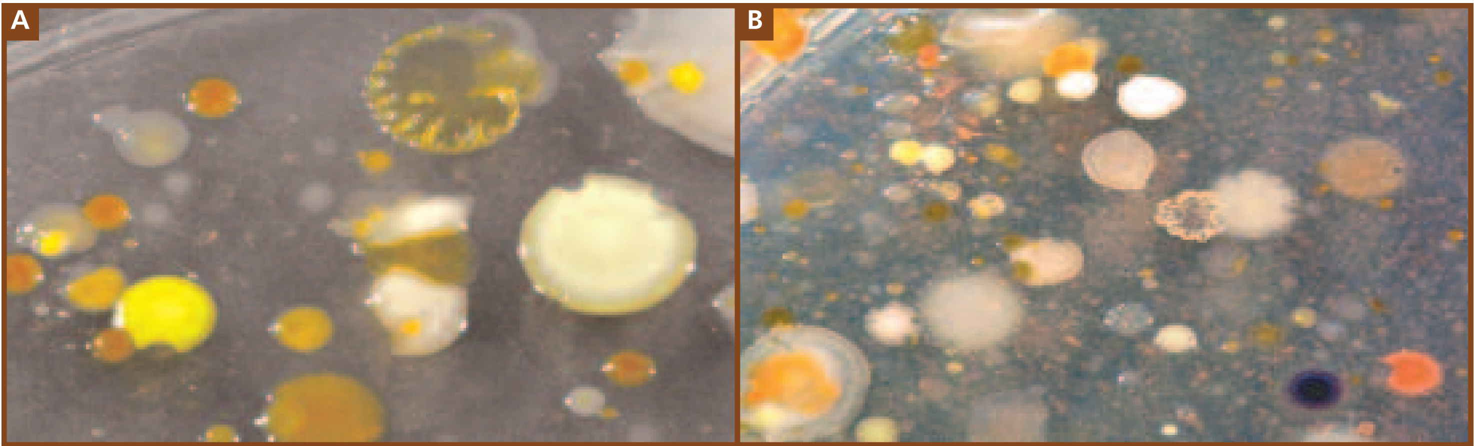
3-27 INFLUENCE OF AGE ON PIGMENT PRODUCTION **A** *Serratia marcescens* grown on Sheep Blood Agar after 24 hours. **B** The same plate of *S. marcescens* after 48 hours. Note in particular the change in the 3 colonies in the lower right (encircled).



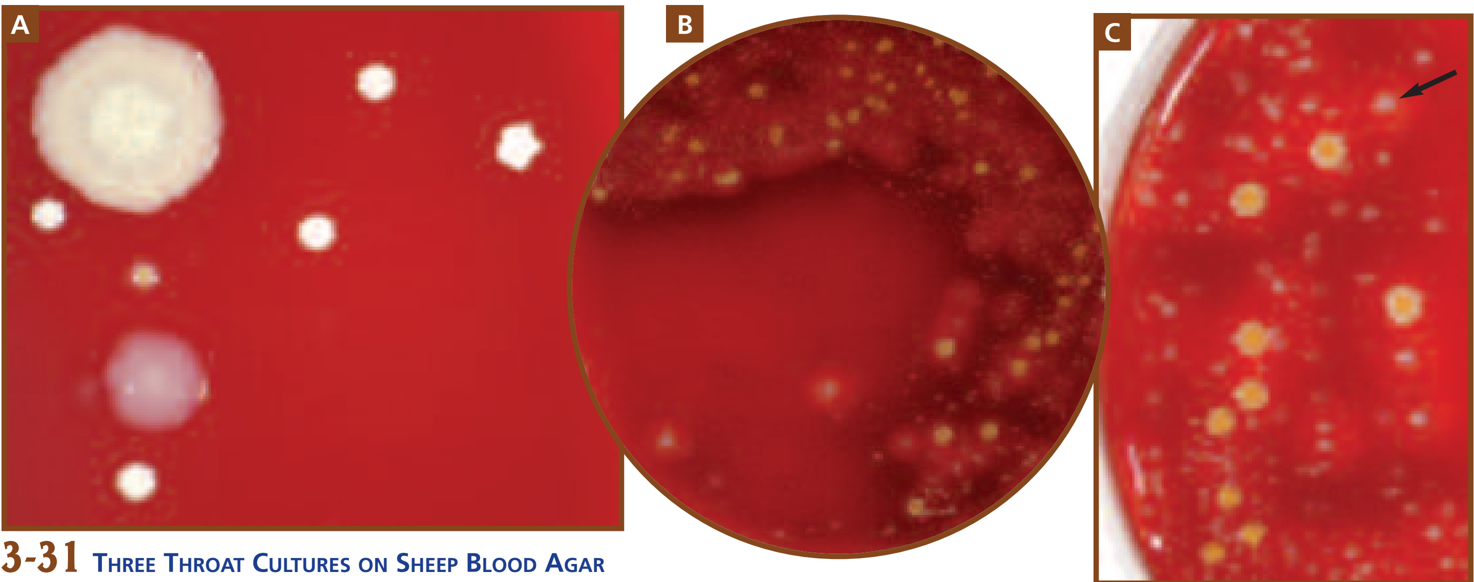
3-28 EFFECT OF AGE ON COLONY MORPHOLOGY **A** Close-up of *Bacillus subtilis* on Sheep Blood Agar after 24 hours of incubation. **B** Close-up of *B. subtilis* on Sheep Blood Agar after 48 hours. Note the wormlike appearance.



3-29 DIFFUSIBLE PIGMENT OF *PSEUDOMONAS AERUGINOSA* The blue-green pigment pyocyanin often makes *P. aeruginosa* easy to identify.

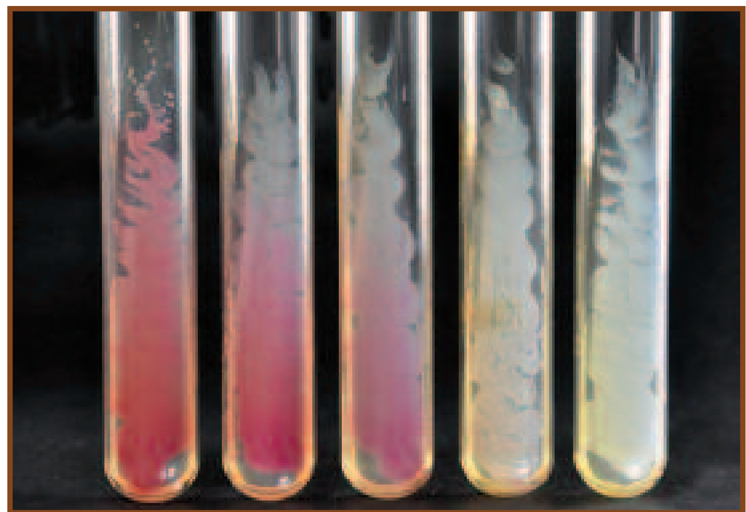


3-30 Two Mixed Soil Cultures on Nutrient Agar These plates show the morphological diversity present in two soil samples.



3-31 Three Throat Cultures on Sheep Blood Agar
A There are probably five different species on this plate. **B** Note the α-hemolysis (darkening of the agar; see page 61 for more information) shown by much of the growth. **C** This is a close-up of the same plate as in **B**. Note the weak β-hemolysis of the white colony in the upper right (**arrow**). White growth with β-hemolysis is characteristic of *Staphylococcus aureus*.

3-32 Pigment Production on Slants From left to right, *Staphylococcus epidermidis* (white), *Pseudomonas aeruginosa* (green), *Chromobacterium violaceum* (violet), *Serratia marcescens* (red/orange), *Kocuria rosea* (rose), and *Micrococcus luteus* (yellow).



3-33 Influence of Temperature on Pigment Production *Serratia marcescens* was grown for 48 hours on Trypticase Soy Agar slants at five different temperatures. From left to right: 25°C, 30°C, 33°C, 35°C, and 37°C. A difference of 2°C makes the difference between being pigmented or not!

3-34 Growth Texture on Slants From left to right, *Bacillus* spp. (flat, dry), *Alcaligenes faecalis* (spreading edge), *Mycobacterium phlei* (crusty/friable), *Lactobacillus plantarum* (transparent, barely visible).

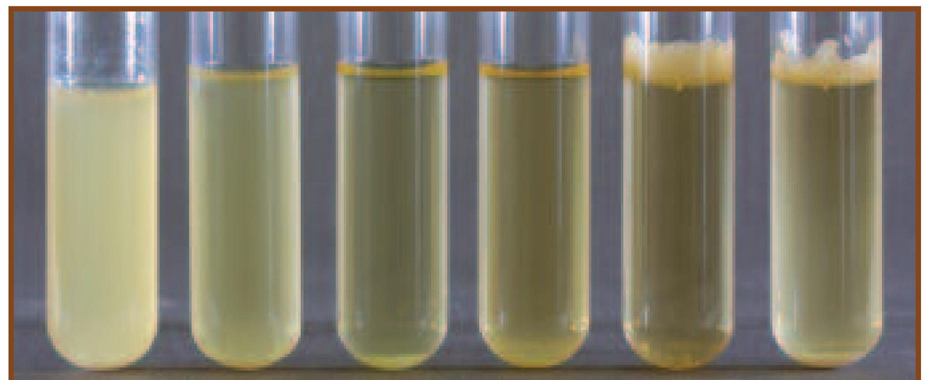
Growth Patterns in Broth

Purpose

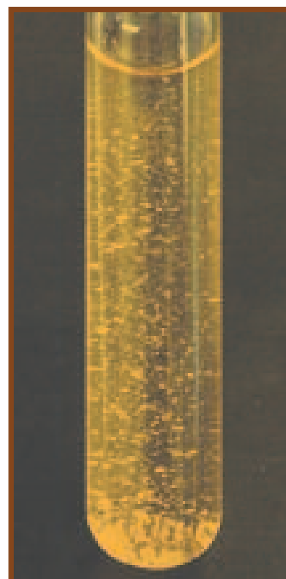
Bacterial genera—and frequently different species within a genus—demonstrate characteristic growth patterns in broth that provide useful information when attempting to identify an organism.

Principle

Microorganisms cultivated in broth display a variety of growth characteristics. Some organisms float on top of the medium and produce a type of surface membrane called a **pellicle;** others sink to the bottom as **sediment.** Some bacteria produce **uniform fine turbidity** while others appear to clump in what is called **flocculent** growth. Refer to Figures 3-35 and 3-36. Figure 3-37 shows an example of a pigmented species (*Rhodospirillum rubrum*) in broth.

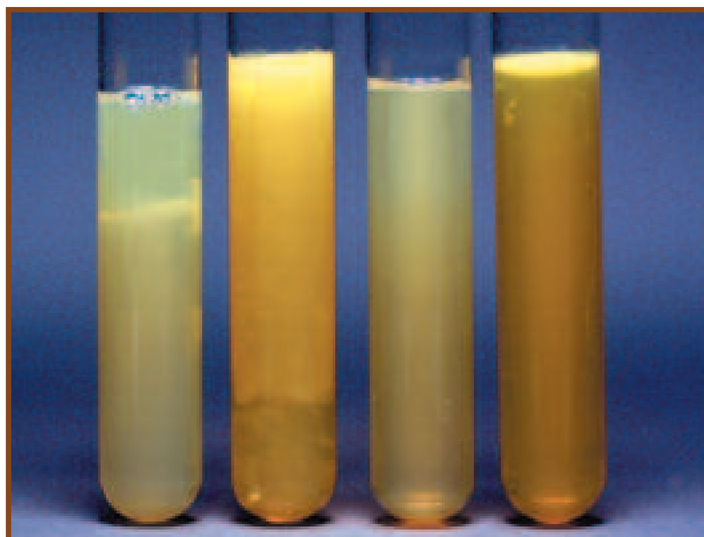


3-35 Growth Patterns in Broth From left to right in pairs (by type of organism): *Enterobacter aerogenes* and *Citrobacter diversus*—motile members of *Enterobacteriaceae* (uniform fine turbidity), *Enterococcus faecalis* and *Staphylococcus aureus*—nonmotile Gram-positive cocci (sediment), *Mycobacterium phlei* and *Mycobacterium smegmatis* (relatives of *Mycobacterium tuberculosis*)—nonmotile with a waxy cell wall (pellicle).



3-36 Flocculence in Broth This is a *Streptococcus* species from a throat culture demonstrating flocculence in Todd-Hewitt Broth.

3-37 Pigment in Broth *Rhodospirillum rubrum* has a red color due to carotenoid pigments. It grows as a photoheterotroph in the presence of light and the absence of oxygen.



3-39 Agar Shake Tubes From left to right, *Clostridium butyricum* (strict anaerobe), *Escherichia coli* (facultative anaerobe), uninoculated control, and *Pseudomonas aeruginosa* (strict aerobe).

Cultivation of Anaerobes—Anaerobic Jar

Purpose

Cultivation of obligate anaerobes and microaerophiles requires providing an environment in which oxygen is either absent or considerably reduced. Various methods have been devised to provide these environments, three of which are covered in the remainder of Section 3.

The anaerobic jar (Figure 3-40) is used to grow obligate anaerobes and microaerophiles. Because it is the atmosphere within the jar that is anaerobic, the jar can be incubated in a normal incubator alongside aerobically grown cultures.

3-40 The Anaerobic Jar Note the sachet and the white indicator strip inside the jar. The sachet has performed properly, reducing the oxygen level within the jar to less than 1%, as evidenced by the indicator strip. If the indicator were blue, it would mean free oxygen remained in the jar and the resulting growth would be in question relative to its ability to survive in anaerobic conditions.

Principle

Inoculated plates or tubes are placed in the jar and the appropriate gas-generating sachet is activated. In the case of the Anaerogen™ Gas Generating System by Oxoid, simply opening the packet inside the jar and immediately clamping the lid on the jar is all that is necessary. Ascorbic acid in the packet reacts with free oxygen and in turn releases CO_2. Within 30 minutes, the atmosphere inside the jar is less than 1% O_2 and between 9 and 13% CO_2. A methylene blue (or some other) indicator strip is also placed inside the jar. It will turn blue if exposed to air, thus acting as a control to ensure anaerobic conditions have been produced. Figure 3-41 shows two plates inoculated with the same organisms, but one was incubated anaerobically while the other was incubated aerobically.

The Oxoid Campygen™ sachet works in a similar way, but produces 5% O_2, 10% CO_2, and 85% N_2. It is designed for growing microaerophiles, such as *Campylobacter jejuni*.

3-41 Plates Incubated Inside and Outside the Anaerobic Jar Both Nutrient Agar plates were inoculated with *Staphylococcus aureus* (top), *Pseudomonas aeruginosa* (right), and *Clostridium sporogenes* (left). The plate on the left was incubated aerobically outside the jar; the plate on the right was incubated inside the anaerobic jar. Note the relative amounts of growth of the three organisms.

Cultivation of Anaerobes—Thioglycollate Broth

Purpose

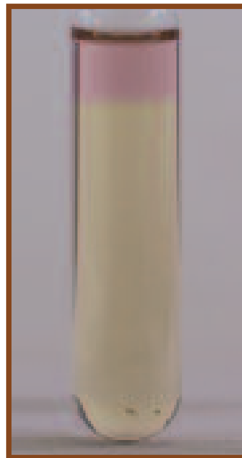
Fluid Thioglycollate Medium is a simple, inexpensive system for cultivating small numbers of anaerobic or microaerophilic bacteria. It is a liquid medium formulated to promote growth of a wide variety of fastidious anaerobic and microaerophilic microorganisms.

Principle

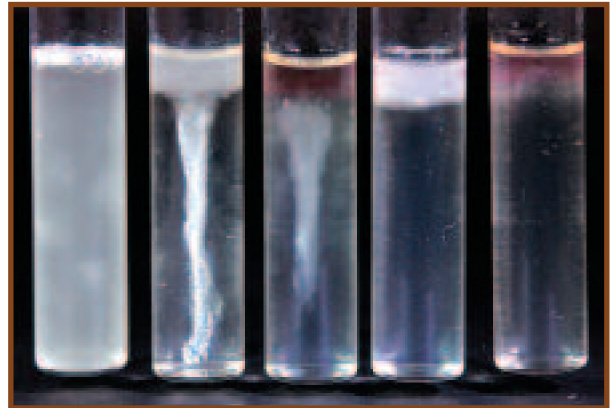
Fluid Thioglycollate Medium is prepared as a basic medium or with a variety of supplements, depending on the specific needs of organisms being cultivated. As such, it is appropriate for a broad variety of aerobic and anaerobic, fastidious and nonfastidious organisms. It is particularly well adapted for cultivation of strict anaerobes and microaerophiles.

Key components of the medium are yeast extract, pancreatic digest of casein, dextrose, sodium thioglycollate, L-cystine, and resazurin. Yeast extract and pancreatic digest of casein provide nutrients; sodium thioglycollate and L-cystine reduce oxygen to water; and resazurin (pink when oxidized, colorless when reduced) acts as an indicator. A small amount of agar is included to slow oxygen diffusion.

Oxygen is removed from the medium during autoclaving but begins to diffuse back in as the tubes cool to room temperature. This produces a gradient of concentrations from fully aerobic at the top to anaerobic at the bottom. Thus, fresh media will appear clear to straw colored with a pink region at the top where the dye has become oxidized (Figure 3-42). Figure 3-43 demonstrates some basic bacterial growth patterns in the medium as influenced by the oxygen gradient.



3-42 Aerobic zone in Thioglycollate Medium Note the pink region in the top (oxidized) portion of the broth. The bottom (reduced) portion of the medium remains colorless.



3-43 Growth Patterns in Thioglycollate Medium Growth patterns of a variety of organisms are shown in these Fluid Thioglycollate Broths. Pictured from left to right are: aerotolerant anaerobe, facultative anaerobe, strict anaerobe, strict aerobe, and microaerophile. Compare these tubes with the uninoculated broth in Figure 3-42.

Cultivation of Anaerobes—Cooked Meat Broth

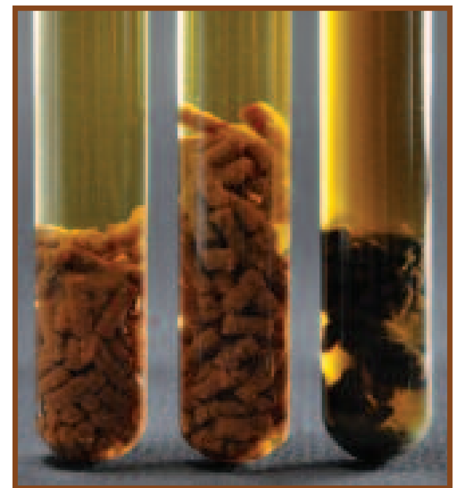
Purpose

The purpose of Cooked Meat Broth is to grow anaerobes, especially pathogenic clostridia such as *Clostridium perfringens*, *C. tetani*, *C. botulinum*, and *C. difficile*. Certain clostridia are proteolytic, whereas others are saccharolytic.

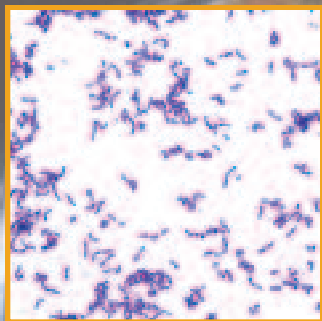
Because it is the medium that becomes anaerobic, these tubes can be incubated in an aerobic incubator, thus eliminating the need for expensive equipment.

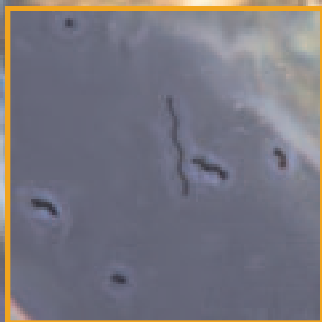
Principle

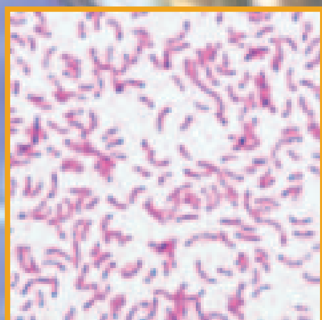
Cooked Meat Broth (Figure 3-44) is a nutrient-rich medium, with beef heart, peptone, and dextrose acting as carbon and nitrogen sources. The beef heart is in the form of meat particles, whereas the other ingredients are dissolved in the broth. Anaerobic conditions occur as a result of several factors. One, cardiac muscle contains glutathione, a tripeptide that can reduce free molecular oxygen in the medium. Two, the meat is cooked prior to use. This denatures proteins and exposes their sulfhydryl groups, which perform the same function—oxygen reduction. Lastly, the medium with caps loosened is either incubated in an anaerobic jar for 24 hours to remove O_2 or boiled to drive off the O_2. Caps are immediately tightened to prevent the reentry of O_2. Blackening and disintegration of the meat particles indicate proteolytic growth. Acid (not indicated directly) and gas production indicate saccharolytic growth.

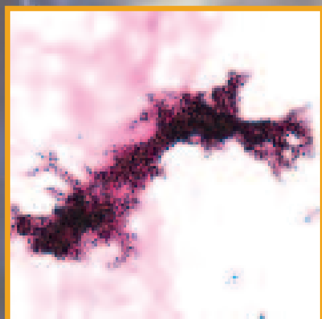


3-44 Cooked Meat Broth The meat particles are visible in each broth. From left to right: *Clostridium butyricum*, uninoculated, *C. sporogenes*. Blackening of the meat particles by *C. sporogenes* is indicative of proteolytic activity. *C. butyricum* grew, but is not proteolytic.









Bacterial Cellular Morphology and Simple Stains

SECTION **5**

Simple Stains

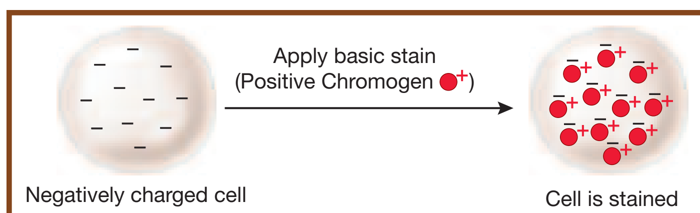
Purpose

In Section 4 you were introduced to two of the three important features of a microscope and microscopy: magnification and resolution. A third feature is **contrast**. To be visible, the specimen must contrast with the background of the microscope field. Because cytoplasm is essentially transparent, viewing cells with the standard light microscope is difficult without stains to provide that contrast. Once stained, cell morphology, size, and arrangement then may be determined.

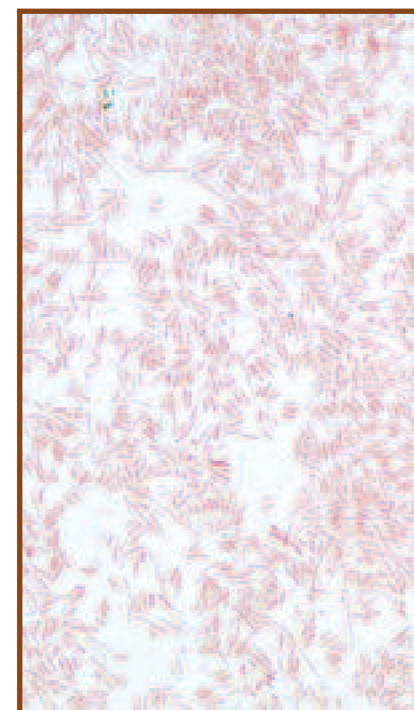
Principle

Stains are solutions consisting of a solvent (usually water or ethanol) and a colored molecule (often a benzene derivative), the **chromogen**. The portion of the chromogen that gives it its color is the **chromophore**. A chromogen may have multiple chromophores, with each intensifying the color. The **auxochrome** is the charged portion of a chromogen and allows it to stain the cell through ionic or covalent bonds. **Basic stains**[1] (where the auxochrome becomes positively charged as a result of picking up a hydrogen ion or losing a hydroxide ion) are attracted to the negative charges on the surface of most bacterial cells. Thus, the cell becomes colored (Figure 5-1). Common basic stains include methylene blue, crystal violet, and safranin. Examples of basic stains may be seen in Figures 5-2 and 5-3, and in A Gallery of Bacterial Cell Diversity (pages 39–44).

[1] Notice that the term "basic" means "alkaline," not "elementary," although coincidentally basic stains can be used for simple staining procedures.

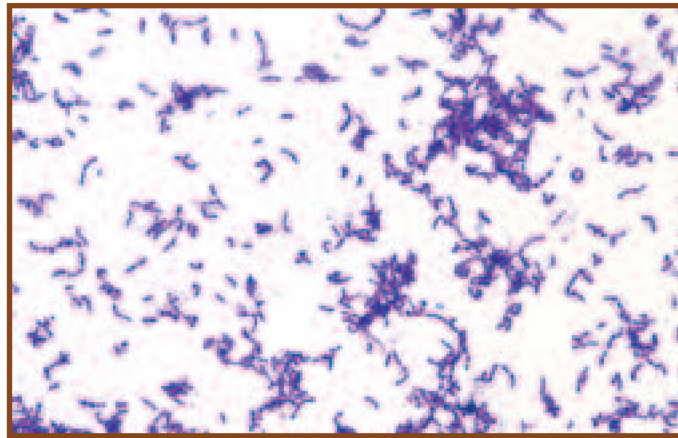


5-1 Chemistry of Basic Stains Basic stains have a positively charged chromogen (●+), which forms an ionic bond with the negatively charged bacterial cell, thus colorizing the cell.



5-2 A Simple Stain Using Safranin Safranin is a basic stain. Notice that the stain is associated with the cells and not the background. The organism is *Klebsiella mobilis* (formerly *Enterobacter aerogenes*), grown in culture. Cell dimensions are 0.3–1.0 µm wide by 0.6–6.0 µm long.

Basic stains are applied to bacterial smears that have been **heat-fixed**. Heat-fixing kills most of the bacteria, makes them adhere to the slide, and coagulates cytoplasmic proteins to make them more visible. It also distorts the cells to some extent.



5-3 A Simple Stain Using Crystal Violet This micrograph shows *Propionibacterium acnes* stained with the basic stain crystal violet. *P. acnes* is a commensal living on the skin of most humans. It has been associated with the skin condition acne. Cell dimensions are 0.5–0.8 µm wide by 1–5 µm long.

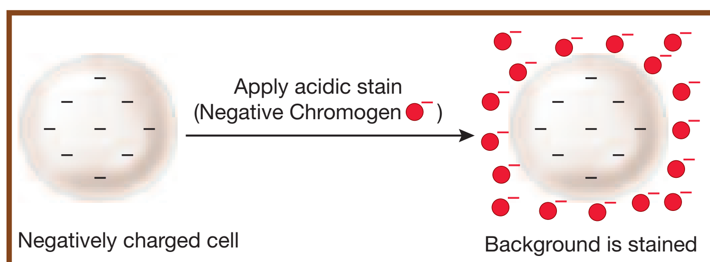
Negative Stain

Purpose

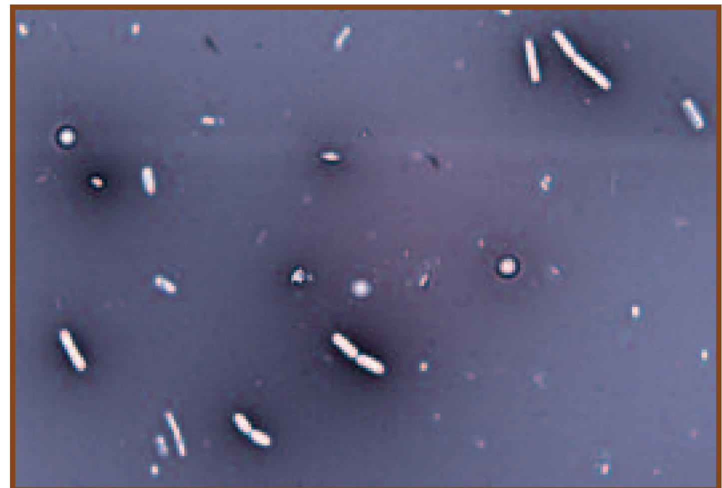
The negative staining technique is used to determine morphology and cellular arrangement in bacteria that are too delicate to withstand heat-fixing. A primary example is the spirochete *Treponema*, which is distorted by the heat-fixing of other staining techniques. Also, where determining the accurate size is crucial, a negative stain can be used because it produces minimal cell shrinkage.

Principle

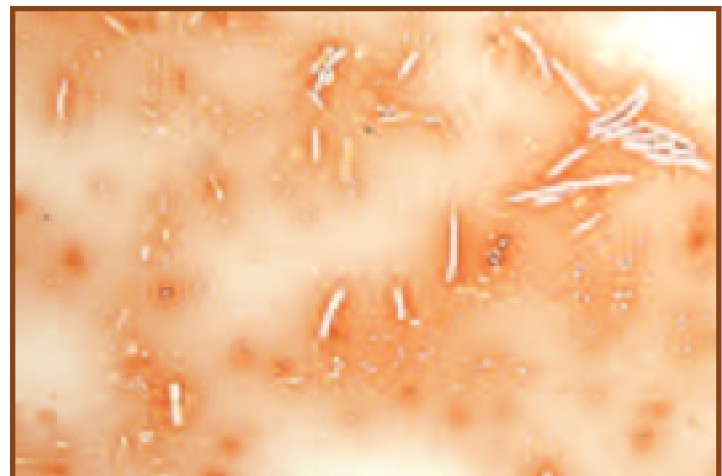
The negative staining technique uses a dye solution in which the chromogen is acidic and carries a negative charge. (An acidic chromogen gives up a hydrogen ion, which leaves it with a negative charge.) The negative charge on the bacterial surface repels the negatively charged chromogen, so the cell remains unstained against a colored background (Figure 5-4). Examples of acidic staining solutions used in negative stains are Nigrosin and Congo red (Figures 5-5 and 5-6).



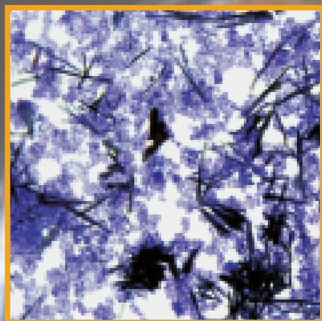
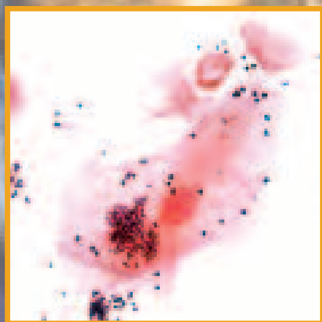
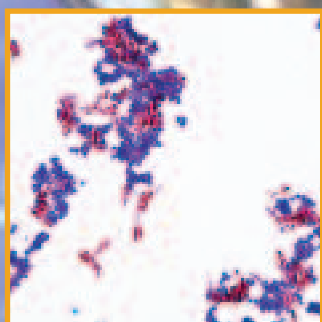
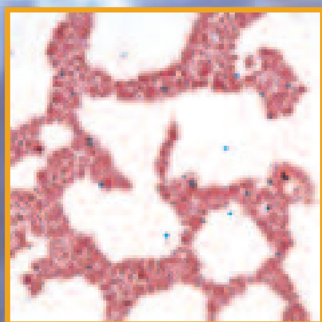
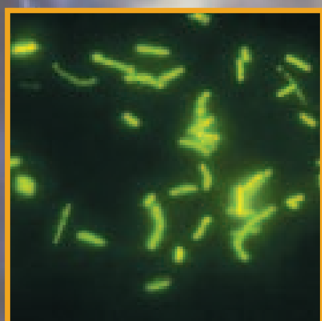
5-4 Chemistry of Acidic Stains Acidic stains have a negatively charged chromogen (●⁻) that is repelled by negatively charged cells. Thus, the background is colored and the cell remains transparent.



5-5 A Nigrosin Negative Stain Notice that the *Bacillus megaterium* cells are unstained against a dark background. Cell dimensions are 1.2–1.5 µm wide by 2.0–5.0 µm long. The small, irregularly-shaped white spots are bubbles or other artifacts.



5-6 A Negative Stain with Congo Red Compare this negative stain of *Bacillus megaterium* to Figure 5-5. *B. megaterium* is a soil organism. Cell dimensions are 1.2–1.5 µm wide by 2.0–5.0 µm long.

Bacterial Cell Structures and Differential Stains

SECTION 6

Gram Stain

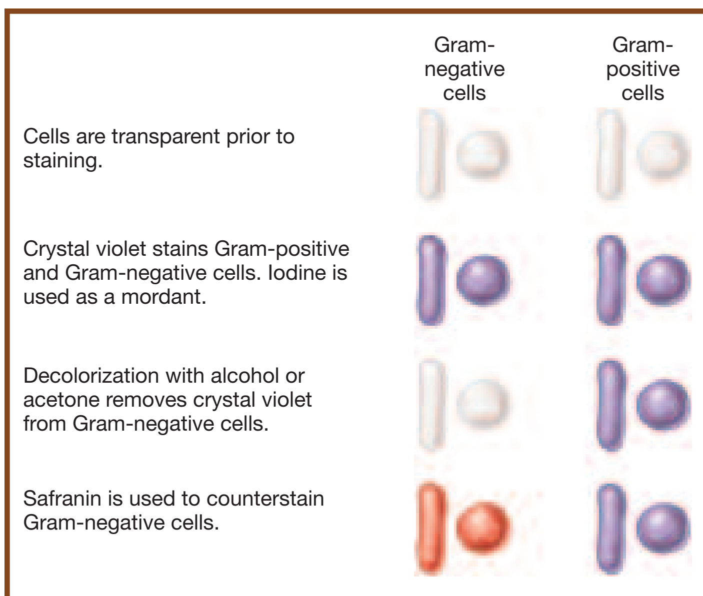
Purpose

The Gram stain, used to distinguish between Gram-positive and Gram-negative cells, is the most important and widely used microbiological differential stain. In addition to Gram reaction, this stain also allows determination of cell morphology, size, and arrangement. It is typically the first differential test run on a specimen brought into the laboratory for identification. In some cases, a rapid, presumptive identification of the organism or elimination of a particular organism is possible.

Principle

The Gram stain is a differential stain in which a **decolorization** step occurs between the application of two basic stains. The Gram stain has many variations, but they all work in basically the same way (Figure 6-1). The **primary stain** is crystal violet. Iodine is added as a **mordant** to enhance crystal violet staining by forming a **crystal violet–iodine complex**. Decolorization follows and is the most critical step in the procedure. Gram-negative cells are decolorized by the solution (of variable composition—generally alcohol or acetone) whereas Gram-positive cells are not. Gram-negative cells can thus be colorized by the **counterstain** safranin. Upon successful completion of a Gram stain, Gram-positive cells appear purple and Gram-negative cells appear reddish-pink (Figure 6-2).

Electron microscopy and other evidence indicate that the ability to resist decolorization or not is based on the different wall constructions of Gram-positive and Gram-negative cells. Gram-negative cell walls have a higher lipid content (because of the outer membrane) and a thinner peptidoglycan layer than Gram-positive cell walls (Figure 6-3). The alcohol/acetone in the decolorizer



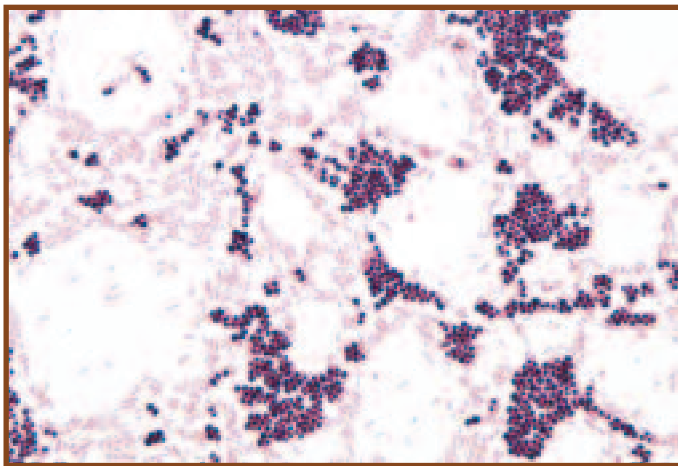
6-1 GRAM STAIN After application of the primary stain (crystal violet), decolorization, and counterstaining with safranin, Gram-positive cells stain violet and Gram-negative cells stain pink/red. Notice that crystal violet and safranin are both basic stains, and that it is the decolorization step that makes the Gram stain differential.

extracts the lipid, making the Gram-negative wall more porous and incapable of retaining the crystal violet–iodine complex, thereby decolorizing it. The thicker peptidoglycan and greater degree of cross-linking (because of teichoic acids) trap the crystal violet–iodine complex more effectively, making the Gram-positive wall less susceptible to decolorization.

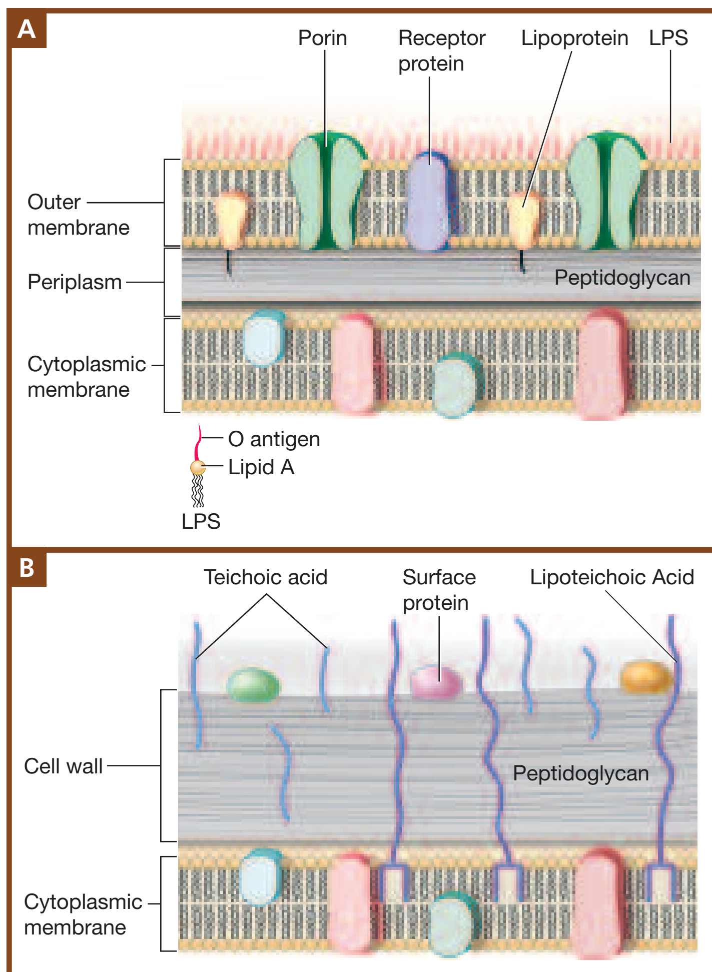
Although some organisms give Gram-variable results, most variable results are a consequence of poor technique. The decolorization step is the most crucial and most likely source of Gram stain inconsistency. It is possible to **over-decolorize** by leaving the alcohol on too long and get reddish Gram-*positive* cells. It also is possible to **under-decolorize** and produce purple Gram-*negative* cells. Neither of these situations changes the actual Gram reaction for the organism being stained. Rather, these are false results because of poor technique.

A second source of poor Gram stains is inconsistency in preparation of the emulsion. Remember, a good emulsion dries to a faint haze on the slide.

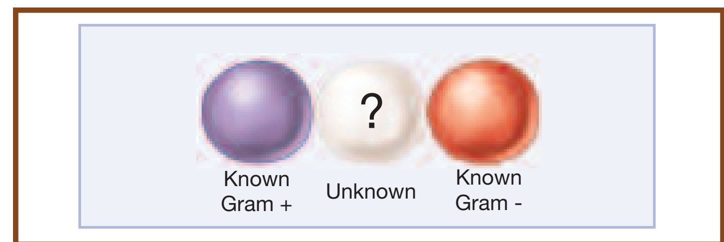
Until correct results are obtained consistently, it is recommended that control smears of Gram-positive and Gram-negative organisms be stained along with the organism in question (Figure 6-4). As an alternative control, a direct smear made from the gumline may be Gram-stained (Figure 6-5) with the expectation that both Gram-positive and



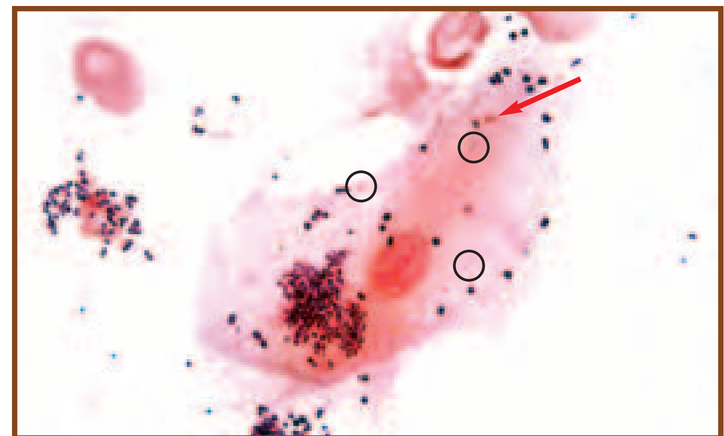
6-2 Gram Stain of *Staphylococcus epidermidis* (+) and *Citrobacter diversus* (−) *S. epidermidis* has a staphylococcal arrangement, whereas *C. diversus* is a bacillus of varying lengths.



6-3 Bacterial Cell Walls **A** The Gram-negative wall is composed of less peptidoglycan (as little as a single layer) and more lipid (due to the outer membrane) than the Gram-positive wall **B**.



6-4 Positive Controls to Check Your Technique Staining known Gram-positive and Gram-negative organisms on either side of your unknown organism act as positive controls for your technique. Try to make the emulsions as close to one another as possible. Spreading them out across the slide makes it difficult to stain and decolorize them equally.

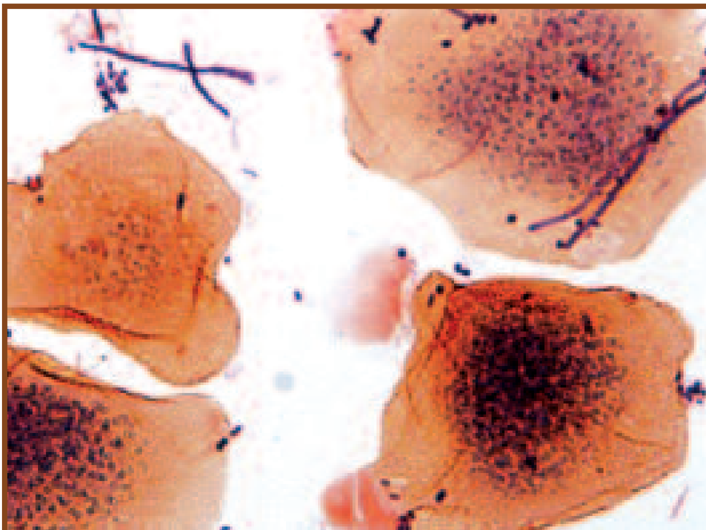


6-5 Direct Smear Positive Control (Gram Stain) A direct smear made from the gumline may also be used as a Gram stain control. Expect numerous Gram-positive bacteria (especially cocci) and some Gram-negative cells, including your own epithelial cells. In this slide, Gram-positive cocci predominate, but a few Gram-negative cells are visible, including Gram-negative rods (circled) and a Gram-negative diplococcus (**arrow**) on the surface of the epithelial cell.

Gram-negative organisms will be seen. Over-decolorized and under-decolorized gumline direct smears are shown for comparison (Figures 6-6 and 6-7). Positive controls also should be run when using new reagent batches.

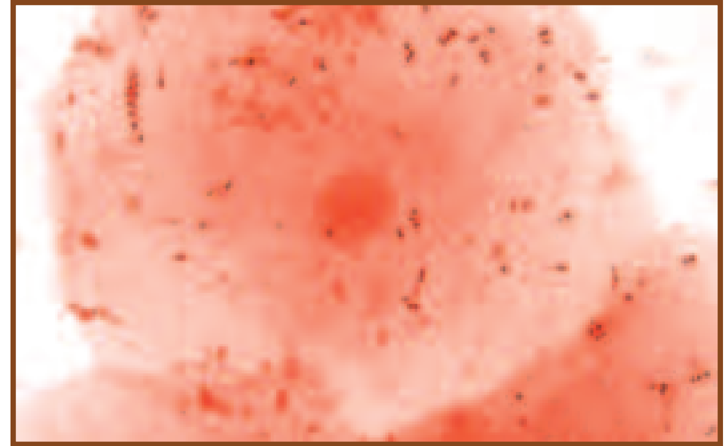
Age of the culture also affects Gram stain consistency. Older Gram-positive cultures may lose their ability to resist decolorization and give an artifactual Gram-negative result. The genus *Bacillus* is notorious for this. *Staphylococcus* can also be a culprit. Cultures 24 hours old or less are best for this procedure.

Potassium hydroxide provides a nonstain test to confirm Gram reaction for particularly difficult species. Part of a colony is emulsified in a drop of KOH for one minute, then the loop is slowly withdrawn. Release of chromosomal material by Gram-negative cells makes the suspension viscous, stringy, and adhesive (Figure 6-8). Gram-positives are unchanged and the emulsion remains watery.

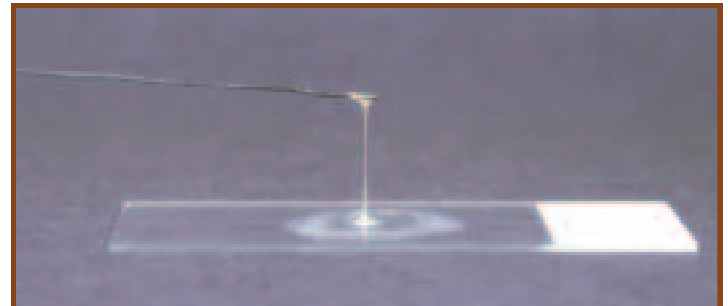


6-6 AN UNDER-DECOLORIZED GRAM STAIN This is a direct smear from the gum line. Notice the purple patches of stain on the epithelial cells. Also notice the variable quality of this stain—the epithelial cell to the left of center is stained better than the others.

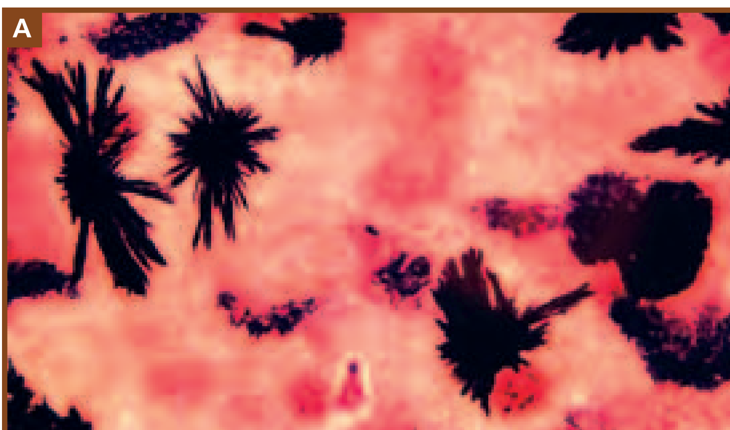
Interpretation of Gram stains can be complicated by nonbacterial elements. For instance, stain crystals from an old or improperly made stain solution can disrupt the field (Figure 6-9) or stain precipitate may be mistakenly identified as bacteria (Figure 6-10). In direct smears host cells or noncellular material may be seen (Figures 6-11 to 6-14).

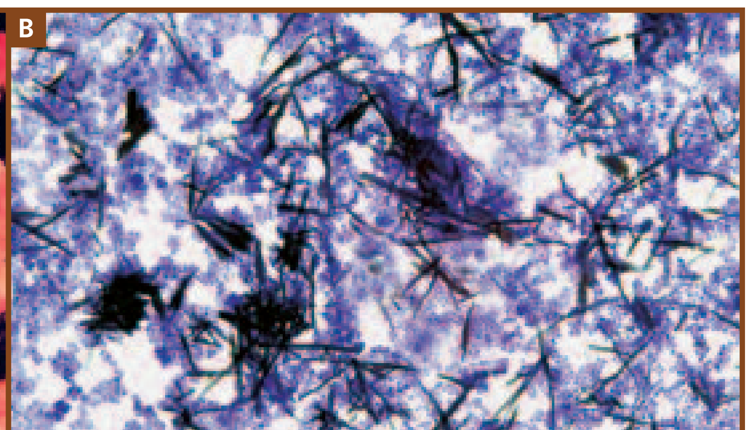


6-7 AN OVER-DECOLORIZED GRAM STAIN This also is a direct smear from the gumline. Notice the virtual absence of any purple cells, a certain indication of over-decoloration.

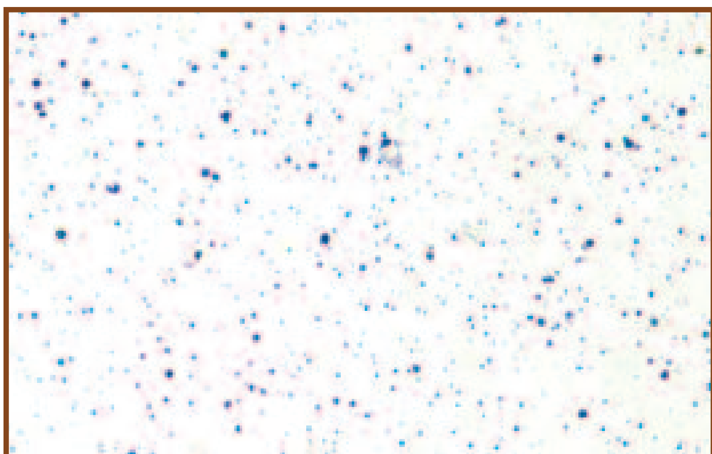


6-8 THE KOH TEST FOR GRAM REACTION This preparation of *Escherichia coli*, a Gram-negative organism, has been emulsified in KOH for one minute. The solution has become viscous and stringy due to the release of chromosomal material from the cells.

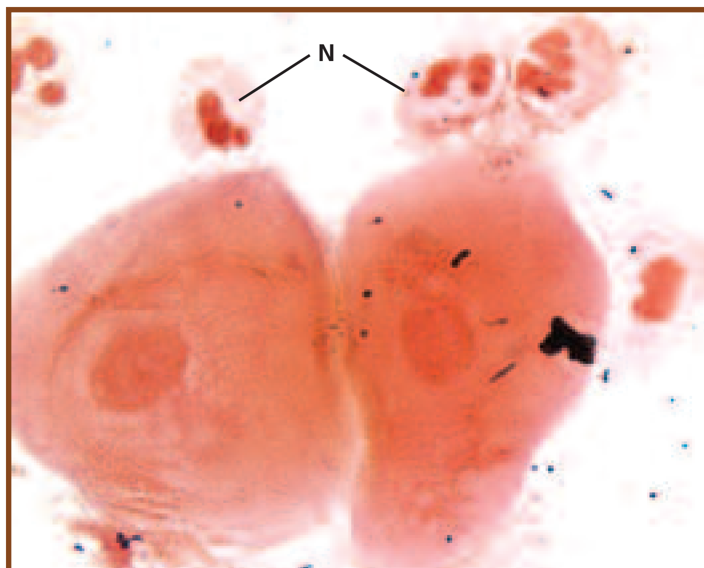




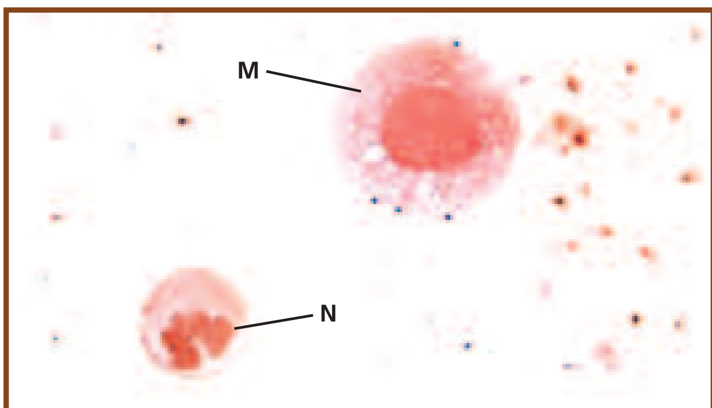
6-9 CRYSTAL VIOLET CRYSTALS (GRAM STAIN) If the staining solution is old or inadequately filtered, crystal violet crystals may appear. Although they are pleasing to the eye, they obstruct your view of the specimen. Crystals from two different Gram stains are shown here. **A** This specimen is of a gum line direct smear; **B** *Micrococcus roseus* grown in culture.



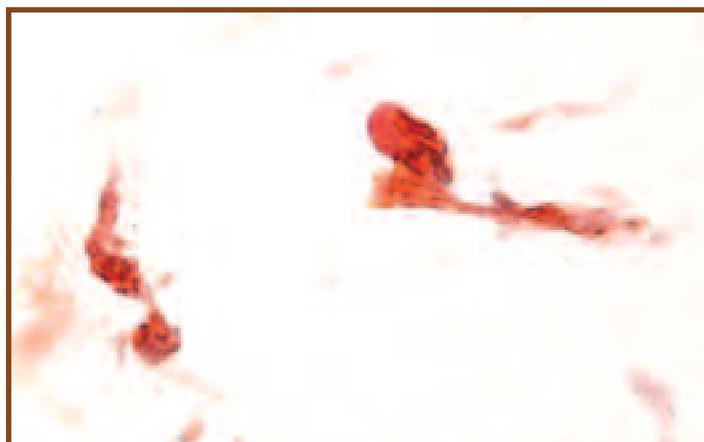
6-10 Stain Precipitate (Gram Stain) If the slide is not rinsed thoroughly or the stain is allowed to dry on the slide, spots of stain precipitate may form and may be confused with bacterial cells. Their variability in size is a clue that they are not bacteria.



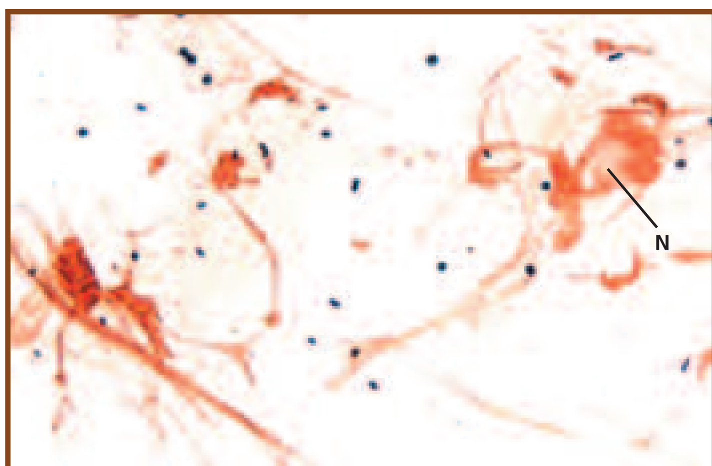
6-11 Neutrophils in Direct Smears (Gram Stain) This gum line smear illustrates neutrophils (**N**), cells typically found in inflamed tissue. Notice their size relative to the epithelial cells, their lobed nucleus, and Gram-negative staining reaction. In some preparations, they are very distorted and only the nuclei make them identifiable (Figure 6-14).



6-12 Macrophage (Gram Stain) In some direct smears, macrophages (**M**) are visible. Compare the size of this macrophage to the single neutrophil (**N**) below it. Notice its spherical nucleus and vacuolated cytoplasm (containing bacteria in the process of digestion). Also notice the Gram-positive cocci on its surface, probably caught in the act of being engulfed.



6-13 Respiratory Epithelial Cells (Gram Stain) Two distorted respiratory epithelial cells are seen in this direct smear of a nasal swab. Their columnar and irregular shape, nucleus, and cilia (what are left) provide clues to their identity.



6-14 Mucus (Gram Stain) Mucus strands dominate the field in this Gram-stained nasal swab. Gram-positive cocci and a deteriorating neutrophil (**N**) are also visible.

Capsule Stain

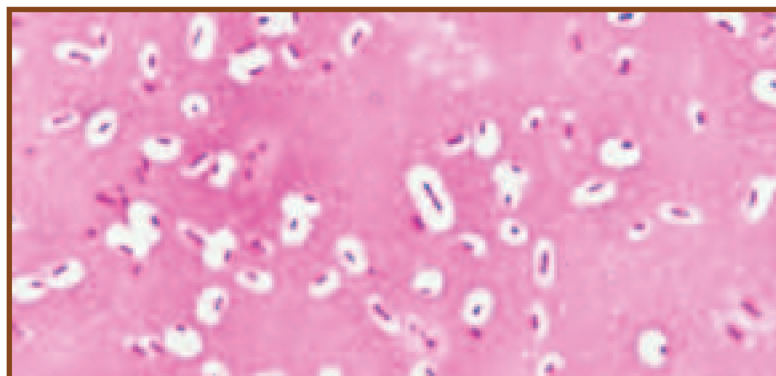
Purpose

The capsule stain is a differential stain used to detect cells capable of producing an extracellular capsule. Capsule production increases virulence in some microbes (such as the anthrax bacillus *Bacillus anthracis* and the pneumococcus *Streptococcus pneumoniae*) by making them less vulnerable to phagocytosis.

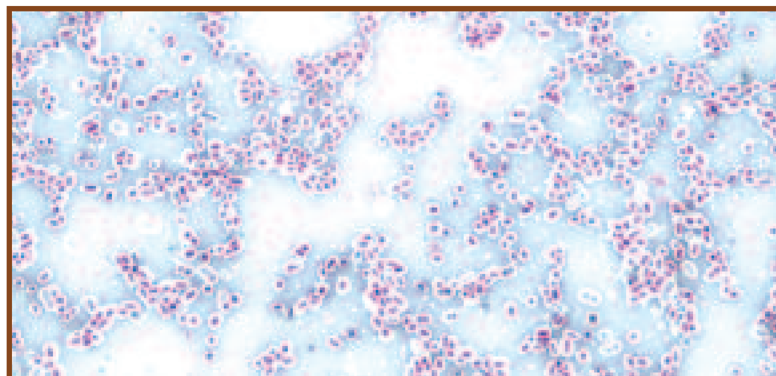
Principle

Capsules are composed of mucoid polysaccharides or polypeptides that repel most stains. The capsule stain technique takes advantage of this phenomenon by staining *around* the cells. Typically, an acidic stain, such as Congo red or nigrosin that stains the background, and a basic stain that colorizes the cell proper, are used. The capsule remains unstained and appears as a white halo between the cells and the colored background (Figures 6-20 and 6-21).

This technique begins by spreading the cells in a film of an acidic stain and serum. The smear is allowed to air dry and is not heat-fixed. Heat-fixing causes shrinkage of the cells, leaving an artifactual white halo around them that might be interpreted as a capsule when counterstained. In place of heat-fixing, cells may be emulsified in a drop of serum to promote adherence to the glass slide.



6-20 Capsule Stain of *Klebsiella pneumoniae* The acidic stain colorizes the background while the basic stain colorizes the cell, leaving the capsules as unstained, and white clearings around the cells. Notice the lack of uniform capsule size, and even the absence of a capsule in some cells. Compare this micrograph to Figure 6-21. The difference in cell size between the two photos is due to enlargement of the micrograph, not to the staining.



6-21 An Alternative Capsule Stain of *Klebsiella pneumoniae* In this capsule stain, Congo red is the acidic stain and Maneval's is the basic stain. After staining, the Congo red often looks bluish or gray. *K. pneumoniae* is an inhabitant of the intestinal tract of humans and is associated with urinary and respiratory tract infections, but this specimen was grown on an agar slant. The cells are approximately 1 µm wide by 2–4 µm long. Compare this micrograph to Figure 6-20.

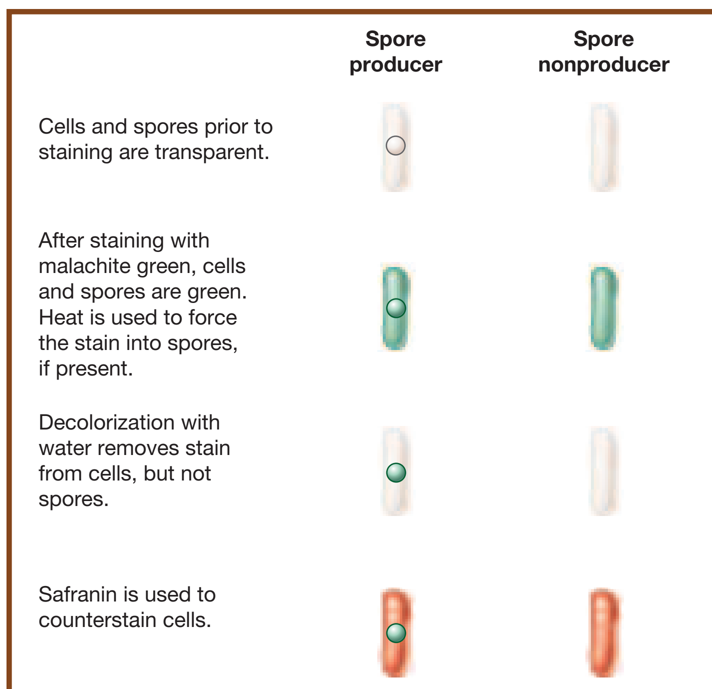
Endospore Stain

Purpose

The spore stain is a differential stain used to detect the presence and location of spores in bacterial cells. Only a few genera produce spores. Among them are the genera *Bacillus* and *Clostridium*. Most members of *Bacillus* are soil, freshwater, or marine **saprophytes**, but a few are pathogens, such as *B. anthracis*, the causative agent of anthrax. Most members of *Clostridium* are soil or aquatic saprophytes or inhabitants of human intestines, but four pathogens are fairly well known: *C. tetani*, *C. botulinum*, *C. perfringens*, and *C. difficile,* which produce tetanus, botulism, gas gangrene, and pseudomembranous colitis, respectively.

Principle

An **endospore** is a dormant form of the bacterium that allows it to survive poor environmental conditions. Spores are resistant to heat and chemicals because of a tough outer covering made of the protein **keratin**. The keratin also resists staining, so extreme measures must be taken to stain the spore. In the Schaeffer-Fulton method (Figure 6-22), a primary stain of malachite green is forced into the spore by steaming the bacterial emulsion. Alternatively, malachite green can be left on the slide for 15 minutes or more to stain the spores. Malachite green is water-soluble and has a low affinity for cellular material, so **vegetative cells** and **spore**

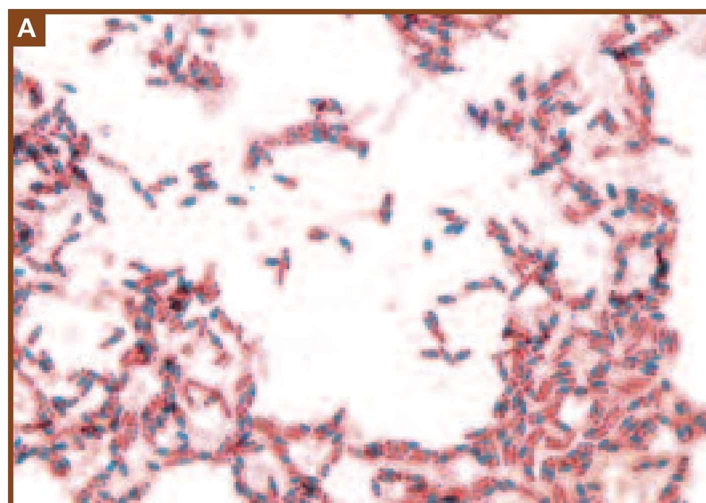


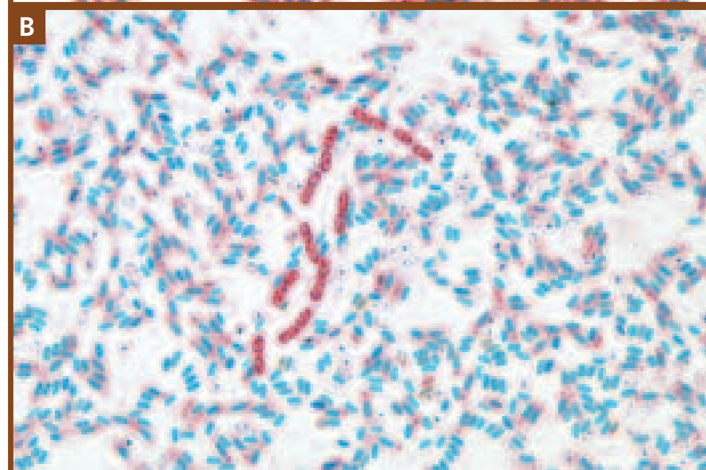
6-22 The Schaeffer-Fulton Spore Stain Upon completion, spores are green, and vegetative and spore mother cells are red.

mother cells can be decolorized with water and counterstained with safranin (Figures 6-23 and 6-24).

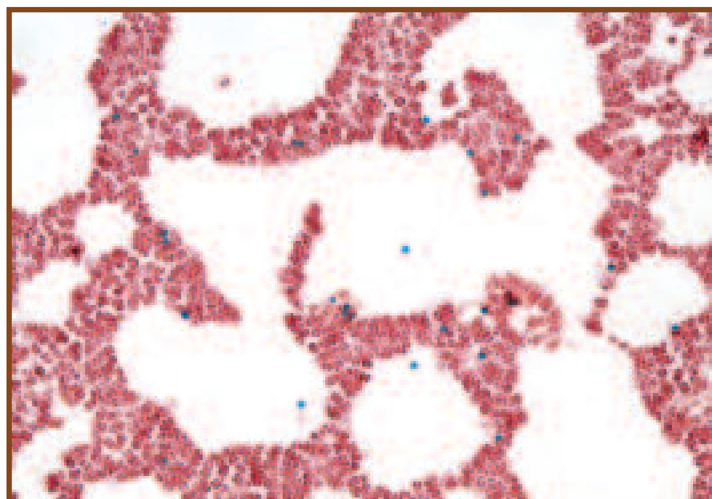
Spores may be located in the middle of the cell (**central**), at the end of the cell (**terminal**), or between the end and middle of the cell (**subterminal**). Spores also may be differentiated based on shape—either **spherical** or **elliptical** (**oval**)—and size relative to the cell (*i.e.*, whether they cause the cell to look swollen or not). These structural features are shown in Figures 6-25 through 6-27.

A special stain is not required to visualize endospores. Figure 6-28 is a phase contrast micrograph of estuarine mud

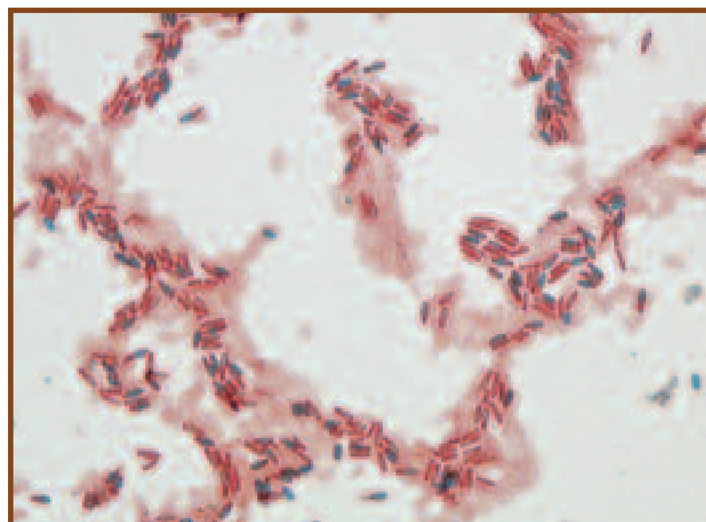




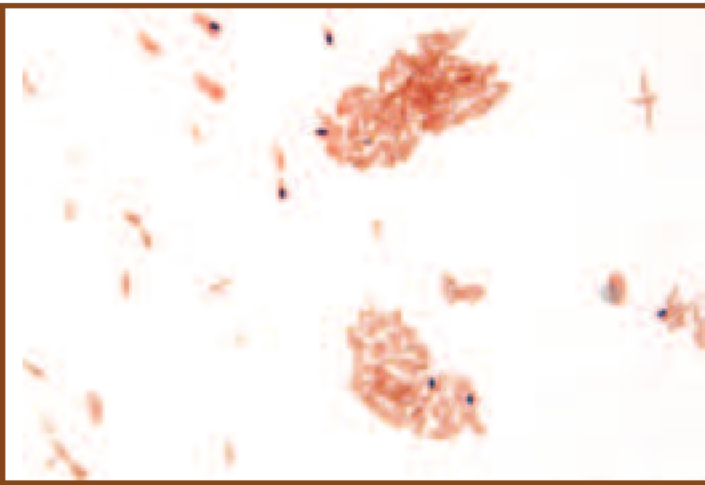
6-23 Culture Age Can Affect Sporulation Bacteria capable of producing spores don't do so uniformly during their culture's growth. Sporulation is done in response to nutrient depletion, and so is characteristic of older cultures. These two *Bacillus* cultures illustrate different degrees of sporulation. **A** Most cells in this specimen contain spores; very few have been released. **B** This specimen consists mostly of released spores.



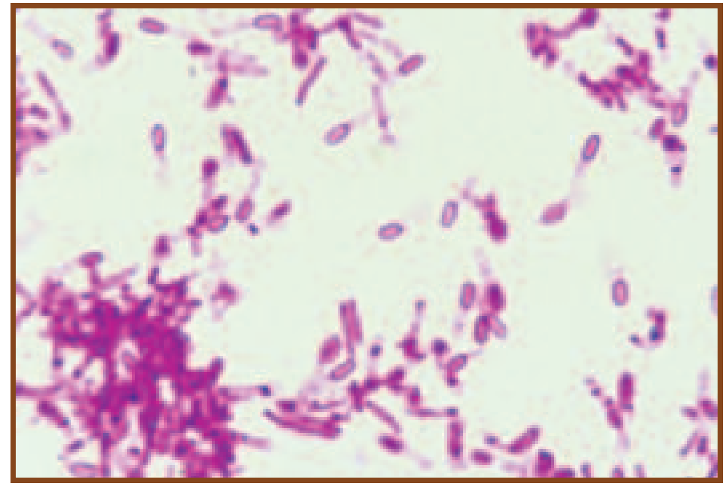
6-24 *Sporosarcina ureae* Spores *Clostridium* and *Bacillus* are the largest and most commonly encountered endospore forming genera, but there are at least a half-dozen others. *Sporosarcina* cells are spherical or slightly elongated and are approximately 2 µm in diameter.



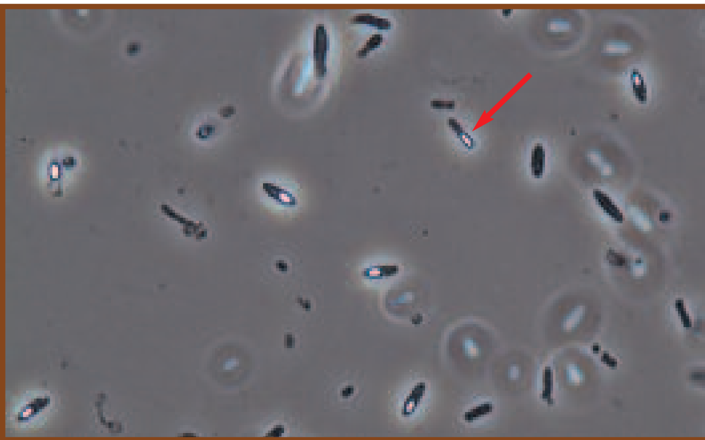
6-25 Central Elliptical Endospores Most of these *Bacillus megaterium* spores are centrally positioned, but all are elliptical. The spore does not distend the mother cell.



6-26 Subterminal Spores This is a stained preparation of *Clostridium botulinum* using an alternative procedure. The black spores slightly distend the cell.

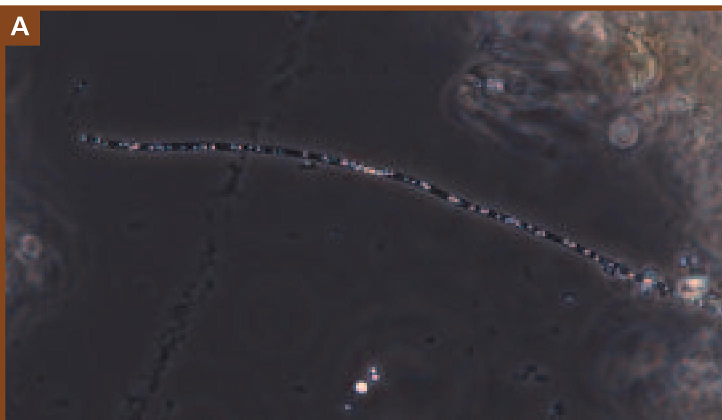


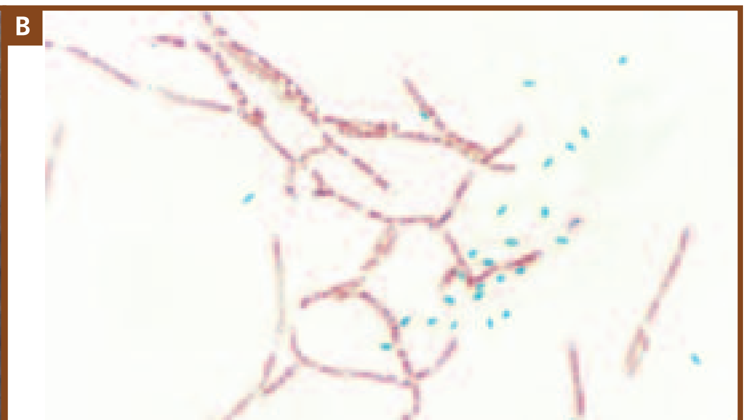
6-27 Elliptical Terminal Spores *Clostridium tetani* stained by a different spore stain protocol using carbolfuchsin. Notice how the spores have caused the ends of the cells to swell.



6-28 Spores as Seen with Phase Contrast Microscopy The white, elliptical spores are easily seen in these unidentified, anaerobic rods found in an estuarine mud sample. Because they are anaerobic, it is likely they are one or more species of *Clostridium*. Note the difference in morphology between the cell marked with the **arrow** and the others.

in which several spore-forming bacteria are visible. Without staining, however, one must be careful not to confuse inclusions, such as sulfur (Figure 6-29A) or lipid (Figure 6-29B) granules, with true endospores. The spore stain will definitively identify true endospores. *Corynebacterium* species may also be a source of confusion, because they often have club-shaped swellings (Figure 6-30).





6-29 The Endospore Stain Allows Differentiation Between True Endospores and Cellular Inclusions **A** The bright spots in this filamentous bacterium (possibly *Thiothrix*) are sulfur granules, but they might be confused with endospores in this phase contrast micrograph. Their irregular size and the presence of more than one per cell are clues that they are not endospores, but an endospore stain would remove any doubt. **B** This micrograph is an endospore stain of *Bacillus cereus* grown in culture. The spores are green, but notice all the unstained, white spots inside the cells! They are lipid granules. Now, imagine looking at this specimen using a simple stain or a Gram stain. Would you be able to identify the spores or would the lipid granules mislead you?

Blood Agar

Purpose

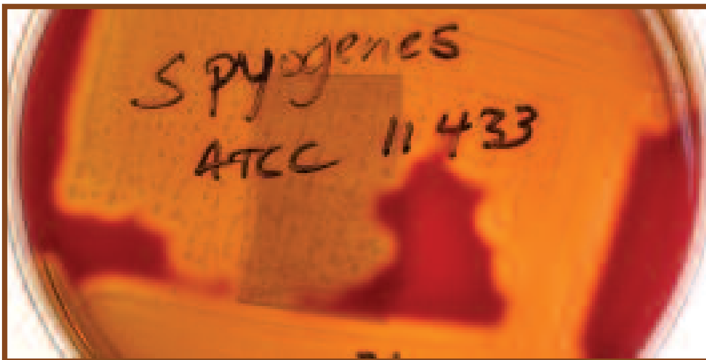
Blood agar is used for isolation and cultivation of many types of fastidious bacteria. It is also used to differentiate bacteria based on their hemolytic characteristics, especially within the genera *Streptococcus*, *Enterococcus*, and *Aerococcus*.

Principle

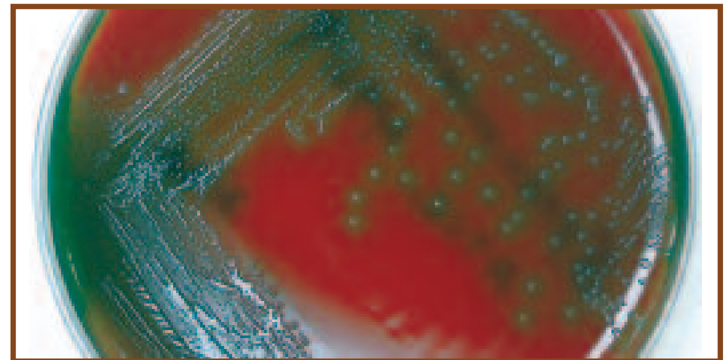
Several species of Gram-positive cocci produce exotoxins called **hemolysins**, which are able to destroy red blood cells (RBCs) and hemoglobin. Blood Agar, sometimes called Sheep Blood Agar because it includes 5% sheep blood in a Tryptic Soy Agar base, allows differentiation of bacteria based on their ability to hemolyze RBCs.

The three major types of hemolysis are β-hemolysis, α-hemolysis, and γ-hemolysis. β-hemolysis, the complete destruction of RBCs and hemoglobin, results in a clearing of the medium around the colonies (Figure 7-10). α-hemolysis is the partial destruction of RBCs and produces a greenish discoloration of the agar around the colonies (Figure 7-11). γ-hemolysis is actually nonhemolysis and appears as simple growth with no change to the medium (Figure 7-12).

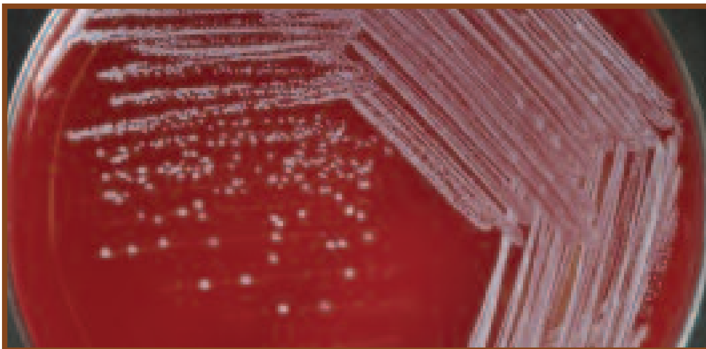
Hemolysins produced by streptococci are called **streptolysins**. They come in two forms—type O and type S. **Streptolysin O** is oxygen-labile and expresses maximal activity under anaerobic conditions. **Streptolysin S** is oxygen-stable but expresses itself optimally under anaerobic conditions as well. The easiest method of providing an environment favorable for streptolysins on Blood Agar is what is called the **streak–stab technique.** In this procedure, the Blood Agar plate is streaked for isolation and then stabbed with a loop. The stabs encourage streptolysin activity because of the reduced oxygen concentration of the subsurface environment (Figure 7-13).



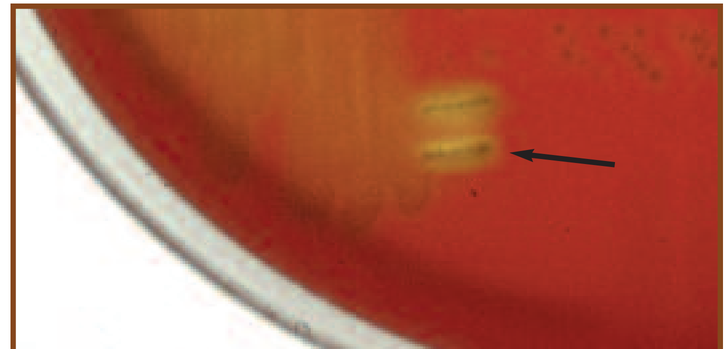
7-10 β-Hemolysis *Streptococcus pyogenes* demonstrates β-hemolysis. The clearing around the growth is a result of complete lysis of red blood cells. This photograph was taken with transmitted light.



7-11 α-Hemolysis This is a streak plate of *Streptococcus pneumoniae* demonstrating α-hemolysis. The greenish zone around the colonies results from incomplete lysis of red blood cells.



7-12 γ-Hemolysis This streak plate of *Staphylococcus epidermidis* on a Sheep Blood Agar illustrates no hemolysis.



7-13 Aerobic *vs.* Anaerobic Hemolysis An unidentified throat culture isolate demonstrates α-hemolysis when growing on the surface, but β-hemolysis beneath the surface surrounding the stabs (**arrow**). This results from production of an oxygen-labile hemolysin.

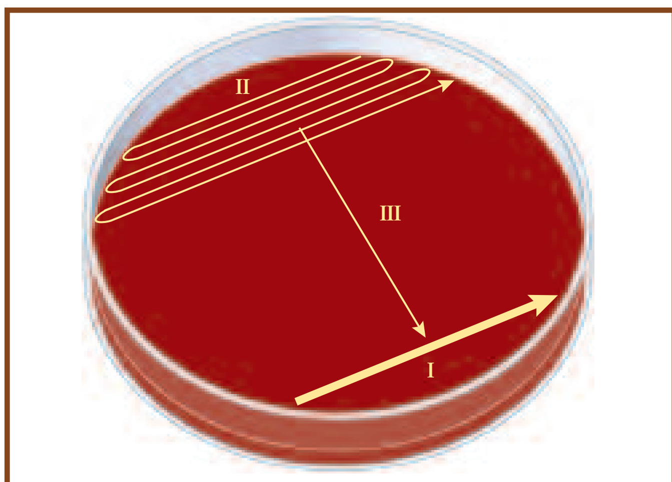
CAMP Test

Purpose

The CAMP test (an acronym of the developers of the test—Christie, Atkins, and Munch-Peterson) is used to differentiate Group B *Streptococcus agalactiae* (+) from other *Streptococcus* species (−).

Principle

Group B *Streptococcus agalactiae* produces the CAMP factor—a hemolytic protein that acts synergistically with the β-hemolysin of *Staphylococcus aureus* subsp. *aureus*. When streaked perpendicularly to an *S. aureus* subsp. *aureus* streak on blood agar (Figure 7-14), an arrowhead-shaped zone of hemolysis forms and is a positive result (Figure 7-15).



7-14 CAMP Test Inoculation Two inoculations are made. First *Staphylococcus aureus* subsp. *aureus* is streaked along one edge of a fresh Blood Agar plate (I). Then the isolate (when testing an unknown organism) is inoculated densely in the other half of the plate opposite *S. aureus* (II). Finally, a single streak is made from inside Streak II toward, but not touching, *S. aureus* (III).

7-15 Positive CAMP Test Results Note the arrowhead zone of clearing in the region where the CAMP factor of *Streptococcus agalactiae* acts synergistically with the β-hemolysin of *Staphylococcus aureus* subsp. *aureus*.

Casein Hydrolysis Test

Purpose

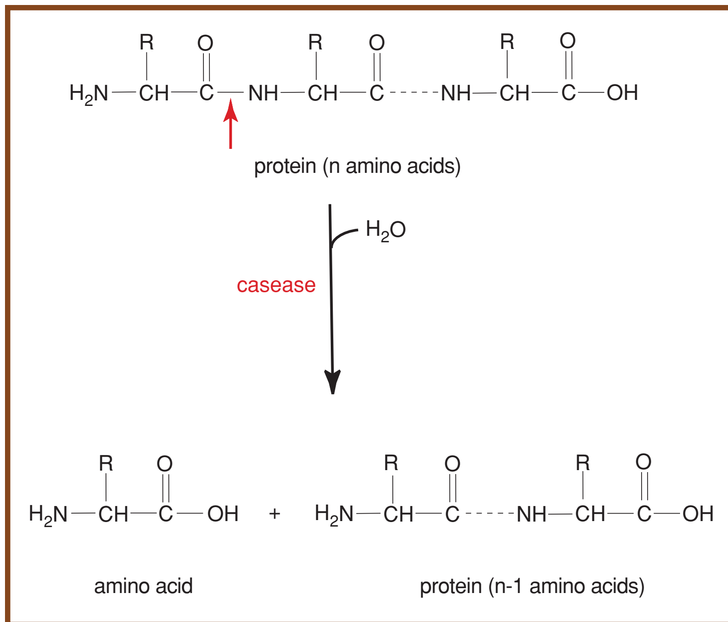
The Casein Hydrolysis Test is used to identify bacteria capable of hydrolyzing casein with the enzyme **casease**.

Principle

Many bacteria require proteins as a source of amino acids and other components for synthetic processes. Some bacteria have the ability to produce and secrete enzymes (exoenzymes) into the environment that catalyze the hydrolysis (break-down) of large proteins to smaller peptides or individual amino acids, thus enabling their uptake across the membrane.

Casease is an enzyme some bacteria produce to hydrolyze the milk protein **casein** (Figure 7-16), the molecule that gives milk its white color. When broken down into smaller fragments, the ordinarily white casein loses its opacity and becomes clear.

The presence of casease can be detected easily with the test medium Milk Agar (Figure 7-17). Milk Agar is an undefined medium containing pancreatic digest of casein, yeast extract, dextrose, and powdered milk. When plated Milk Agar is inoculated with a casease-positive organism, secreted casease will diffuse into the medium around the colonies and create a zone of clearing where the casein has been hydrolyzed. Casease-negative organisms do not secrete casease and, thus, do not produce clear zones around the growth.



7-16 Casein Hydrolysis Hydrolysis of any protein occurs by breaking peptide bonds (**red arrow**) between adjacent amino acids to produce short peptides or individual amino acids.

7-17 Casein Hydrolysis Test Results This Milk Agar plate was inoculated with *Bacillus megaterium* (casease-positive) above and *Micrococcus roseus* (casease-negative) below.

Catalase Test

Purpose

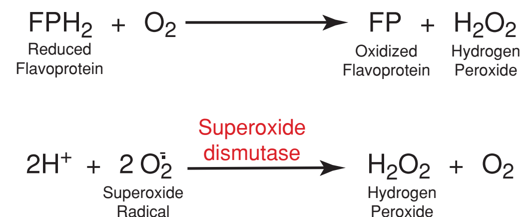
The Catalase Test is used to identify organisms that produce the enzyme **catalase**. It is most commonly used to differentiate members of the catalase-positive *Micrococcaceae* from the catalase-negative *Streptococcaceae*. Variations on this test may also be used in identification of *Mycobacterium* species.

Principle

The electron transport chains of aerobic and facultatively anaerobic bacteria are composed of molecules capable of accepting and donating electrons as conditions dictate. As such, these molecules alternate between the oxidized and reduced form, passing electrons down the chain to the final electron acceptor (FEA). Energy lost by electrons in this sequential transfer is used to perform oxidative phosphorylation (*i.e.*, produce ATP from ADP).

One carrier molecule in the ETC called **flavoprotein** can bypass the next carrier in the chain and transfer electrons directly to oxygen (Figure 7-18). This alternate pathway produces two very potent cellular toxins—hydrogen peroxide (H_2O_2) and superoxide radical (O_2^-).

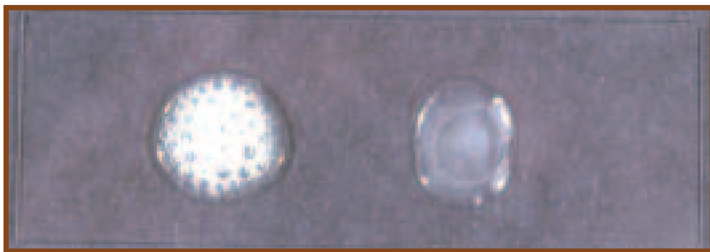
Aerobic and facultatively anaerobic bacteria produce enzymes capable of detoxifying these compounds. **Superoxide dismutase** catalyzes conversion of superoxide radicals (the more lethal of the two compounds) to hydrogen peroxide (Figure 7-18). Catalase converts hydrogen peroxide into water and gaseous oxygen (Figure 7-19).



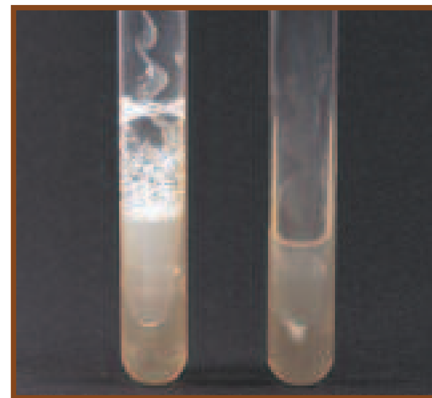
7-18 Microbial Production of H_2O_2 Hydrogen peroxide may be formed through the transfer of electrons from reduced flavoprotein to oxygen or from the action of superoxide dismutase.

7-19 Catalase Mediated Conversion of H_2O_2 Catalase is an enzyme of aerobes, microaerophiles, and facultative anaerobes that converts hydrogen peroxide to water and oxygen gas.

Bacteria that produce catalase can be detected easily using typical store-grade hydrogen peroxide. When hydrogen peroxide is added to a catalase-positive culture, oxygen gas bubbles form immediately (Figures 7-20 and 7-21). If no bubbles appear, the organism is catalase-negative. This test can be performed on a microscope slide or by adding hydrogen peroxide directly to the bacterial growth.



7-20 Catalase Slide Test Shown is the catalase slide test in which visible bubble production indicates a positive result. *Staphylococcus aureus* (+) is on the left, *Enterococcus faecium* (−) is on the right. It is a good idea to cover the slide with a Petri dish lid immediately after addition of peroxide to contain aerosols produced in positive reactions.



7-21 Catalase Tube Test The catalase test may also be performed on an agar slant. *Staphylococcus aureus* (+) is on the left, *Enterococcus faecium* (−) is on the right.

Citrate Utilization Test

Purpose

The Citrate Utilization Test is used to determine the ability of an organism to use citrate as its sole source of carbon. Citrate utilization is one part of a test series referred to as the IMViC (*I*ndole, *M*ethyl Red, *V*oges-Proskauer and *C*itrate tests) that distinguishes between members of the family *Enterobacteriaceae* and differentiates them from other Gram-negative rods.

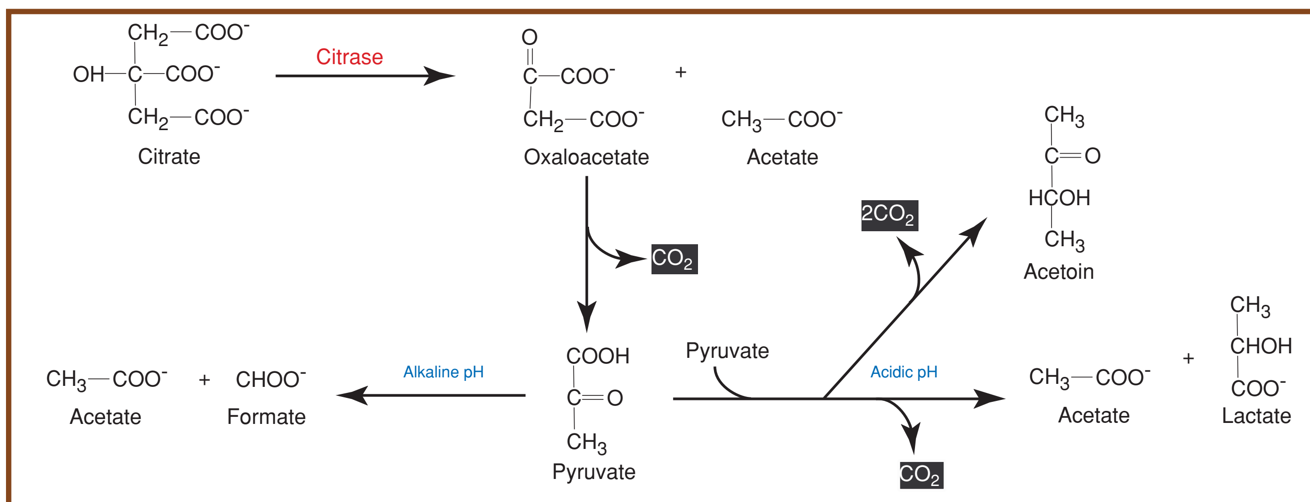
Principle

In many bacteria, citrate (citric acid) is produced as acetyl coenzyme A (from the oxidation of pyruvate or the β-oxidation of fatty acids) reacts with oxaloacetate at the entry to the Krebs cycle. Citrate is then converted through a complex series of reactions back to oxaloacetate, which begins the cycle anew. Refer to the Appendix (Figures A-1 and A-4) and Figure 7-51 for more information on the Krebs cycle and fatty acid metabolism.

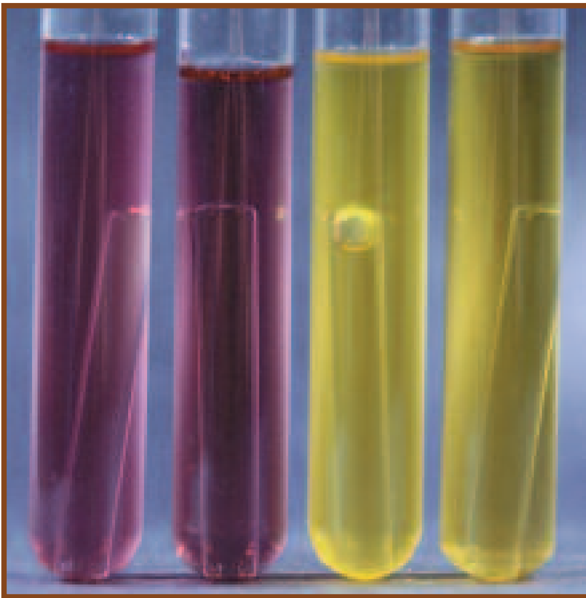
In a medium containing citrate as the only available carbon source, bacteria that possess **citrate-permease** can transport the molecules into the cell and enzymatically convert it to pyruvate. Pyruvate can then be converted to a variety of products, depending on the pH of the environment (Figure 7-22).

Simmons Citrate Agar is a defined medium that contains sodium citrate as the sole carbon source and ammonium phosphate as the sole nitrogen source. Bromthymol blue dye, which is green at pH 6.9 and blue at pH 7.6, is added as an indicator. Bacteria that survive in the medium and utilize the citrate also convert the ammonium phosphate to ammonia (NH_3) and ammonium hydroxide (NH_4OH), both of which tend to alkalinize the agar. As the pH goes up, the medium changes from green to blue (Figure 7-23). Thus, conversion of the medium to blue is a positive citrate test result.

Occasionally a citrate-positive organism will grow on a Simmons Citrate slant without producing a change in color.



7-22 Citrate Chemistry In the presence of citrate-permease enzyme, citrate enters the cell and is converted to pyruvate. The pyruvate is then converted to a variety of products depending on the pH of the environment.



7-41 Purple Lactose Broth Results From left to right are *Proteus vulgaris* (–/–), an uninoculated control, *Escherichia coli* (A/G), and *Staphylococcus aureus* (A/–).

7-42 PR Glucose Broth Results From left to right are *Escherichia coli* (A/G), *Staphylococcus aureus* (A/–), uninoculated control, *Micrococcus luteus* (–/–) and *Alcaligenes faecalis* (K).

Gelatin Hydrolysis Test

Purpose

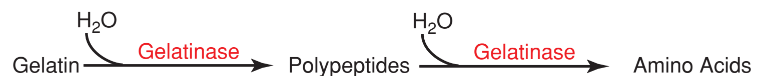
The Gelatin Hydrolysis Test is used to determine the ability of a microbe to produce **gelatinases**. *Staphylococcus aureus*, which is gelatinase-positive can be differentiated from *S. epidermidis* (gelatinase-negative). *Serratia* and *Proteus* species are positive members of *Enterobacteriaceae* while most others in the family are negative. *Bacillus anthracis*, *B. cereus*, and several other members of the genus are gelatinase-positive, as are *Clostridium tetani* and *C. perfringens*.

Principle

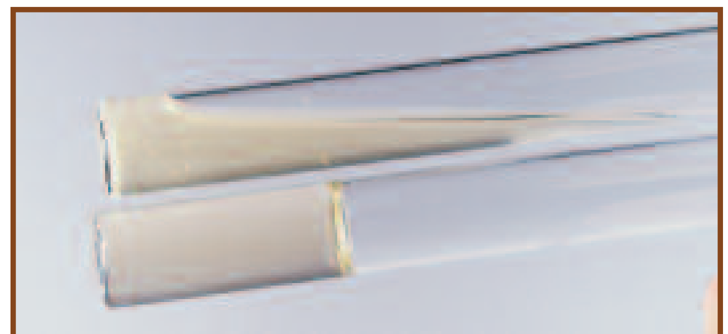
Gelatin is a protein derived from collagen—a component of vertebrate connective tissue. Gelatinases comprise a family of extracellular enzymes produced and secreted by some microorganisms to hydrolyze gelatin. Subsequently, the cell can take up individual amino acids and use them for metabolic purposes. Bacterial hydrolysis of gelatin occurs in two sequential reactions, as shown in Figure 7-43.

The presence of gelatinases can be detected using Nutrient Gelatin, a simple test medium composed of gelatin, peptone, and beef extract. Nutrient Gelatin differs from most other solid media in that the solidifying agent (gelatin) is also the substrate for enzymatic activity. Consequently, when a tube of Nutrient Gelatin is stab-inoculated with a gelatinase-positive organism, secreted gelatinase (or gelatinases) will liquefy the medium. Gelatinase-negative organisms do not secrete the enzyme and do not liquefy the medium (Figure 7-44 and Figure 7-45). A 7-day incubation period is usually sufficient to see liquefaction of the medium. However, gelatinase activity is very slow in some organisms. All tubes still negative after 7 days should be incubated an additional 7 days.

A slight disadvantage of Nutrient Gelatin is that it melts at 28°C (82°F). Therefore, inoculated stabs are typically incubated at 25°C along with an uninoculated control to verify that any liquefaction is not temperature-related.



7-43 Gelatin Hydrolysis Gelatin is hydrolyzed by the gelatinase family of enzymes.



7-44 Nutrient Gelatin Stabs *Aeromonas hydrophila* (+) above and *Micrococcus roseus* (–) below.

7-45 Crateriform Liquefaction This form of liquefaction may also be of diagnostic use because not all gelatinase-positive microbes liquefy the gelatin completely. Shown here is *Micrococcus luteus* liquefying the gelatin in the shape of a crater.

Indole Test (SIM Medium)

Purpose

The Indole Test identifies bacteria capable of producing indole using the enzyme **tryptophanase.** The Indole Test is one component of the IMViC battery of tests (*I*ndole, *M*ethyl Red, *V*oges-Proskauer, and *C*itrate) used to differentiate the *Enterobacteriaceae*.

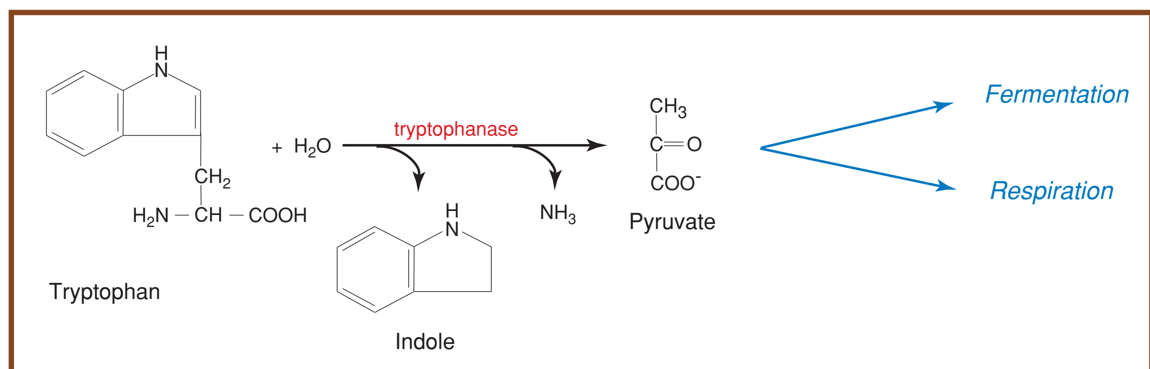
Principle

The Indole Test, as it appears in this manual, is performed using SIM medium. SIM medium also tests for motility and sulfur reduction (SIM is an acronym for Sugar-Indole-Motility). It is a semi-solid medium that is formulated with casein and animal tissue as sources of amino acids, an iron-containing compound, and sulfur in the form of sodium thiosulfate.

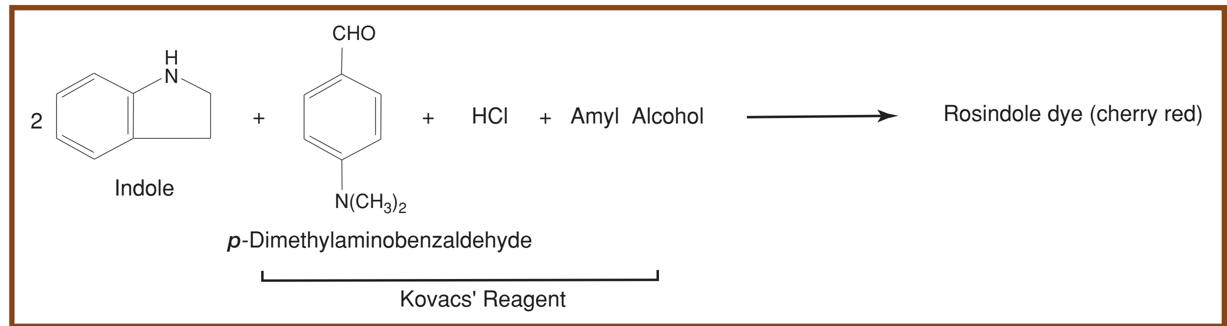
Indole production in the medium is made possible by the presence of tryptophan (contained in casein and animal protein). Bacteria possessing the enzyme tryptophanase can hydrolyze tryptophan to pyruvate, ammonia (by deamination), and indole (Figure 7-46).

The hydrolysis of tryptophan in SIM medium can be detected by the addition of Kovacs' reagent after a period of incubation. Kovacs' reagent contains dimethylaminobenzaldehyde (DMABA) and HCl dissolved in amyl alcohol. When a few drops of Kovacs' reagent are added to the tube, it forms a liquid layer over the solid medium. DMABA then reacts with any indole present and produces a quinoidal compound that turns the reagent layer red (Figures 7-47 and 7-48). The formation of red color in the reagent layer indicates a positive reaction and the presence of tryptophanase. No red color is indole-negative.

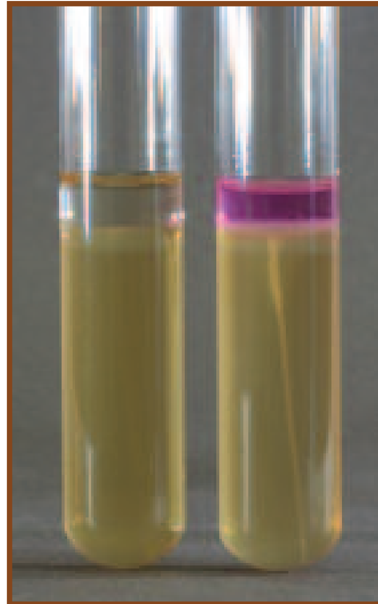
An instantaneous indole test is available and done by placing bacterial growth on a paper slide impregnated with 5% DMABA (Figure 7-49). A positive result is formation of pink on the paper slide.



7-46 Tryptophan Catabolism in Indole-Positive Organisms Tryptophanase hydrolyzes the amino acid tryptophan to indole, ammonia, and pyruvate. Subsequently, pyruvate can be used in the Krebs Cycle.



7-47 Indole reaction with Kovacs' Reagent Kovacs' Reagent is added to the medium following incubation. If the organism is indole-positive, a red color is produced by this reaction.



7-48 Indole Test Results This is SIM medium inoculated with *Morganella morganii* (+) on the right and *Enterobacter aerogenes* (−) on the left.

7-49 Rapid Indole Test BBL™ *Dry*Slide™ (Available from Becton-Dickinson, Sparks, MD.) This slide was inoculated with *Escherichia coli* (+) on the left and *Enterobacter aerogenes* (−) on the right.

Kligler's Iron Agar

Purpose

Kligler's Iron Agar (KIA) is primarily used to differentiate members of *Enterobacteriaceae* and to distinguish them from other Gram-negative bacilli such as *Pseudomonas* or *Alcaligenes*.

Principle

KIA is a rich medium designed to differentiate bacteria on the basis of glucose fermentation, lactose fermentation, and sulfur reduction. In addition to the two carbohydrates, it includes beef extract, yeast extract, and peptone as carbon and nitrogen sources, and sodium thiosulfate as an electron acceptor. Phenol red is the pH indicator and ferrous sulfate is the hydrogen sulfide indicator.

The medium is prepared as a shallow agar slant with a deep butt, thereby providing both aerobic and anaerobic growth environments. It is inoculated by a stab in the agar butt followed by a fishtail streak of the slant. The incubation period is 18 to 24 hours for carbohydrate fermentation and up to 48 hours for hydrogen sulfide reactions. Many reactions in various combinations are possible (Figure 7-50 and Table 7-1).

When KIA is inoculated with a glucose-only fermenter, acid products lower the pH and turn the entire medium yellow within a few hours. Because glucose is in short supply (0.1%), it will be exhausted within about 12 hours. As the glucose is used up, the organisms located in the aerobic region (slant) will begin to break down available amino acids, producing NH_3 and raising the pH. This process, which takes 18 to 24 hours to complete, is called a **reversion** and only occurs in the slant because of the anaerobic conditions in the butt. Thus, a KIA with a red slant and yellow butt after a 24-hour incubation period indicates that the organism ferments glucose but not lactose.

Organisms that are able to ferment glucose *and* lactose also turn the medium yellow throughout. However, because the lactose concentration is ten times higher than that of

Lipase Test

Purpose

The Lipase Test is used to detect and enumerate lipolytic bacteria, especially in high-fat dairy products. A variety of other lipid substrates, including corn oil, olive oil, and soybean oil, are used to detect differential characteristics among members of *Enterobacteriaceae*, *Clostridium*, *Staphylococcus*, and *Neisseria*. Several fungal species also demonstrate lipolytic ability.

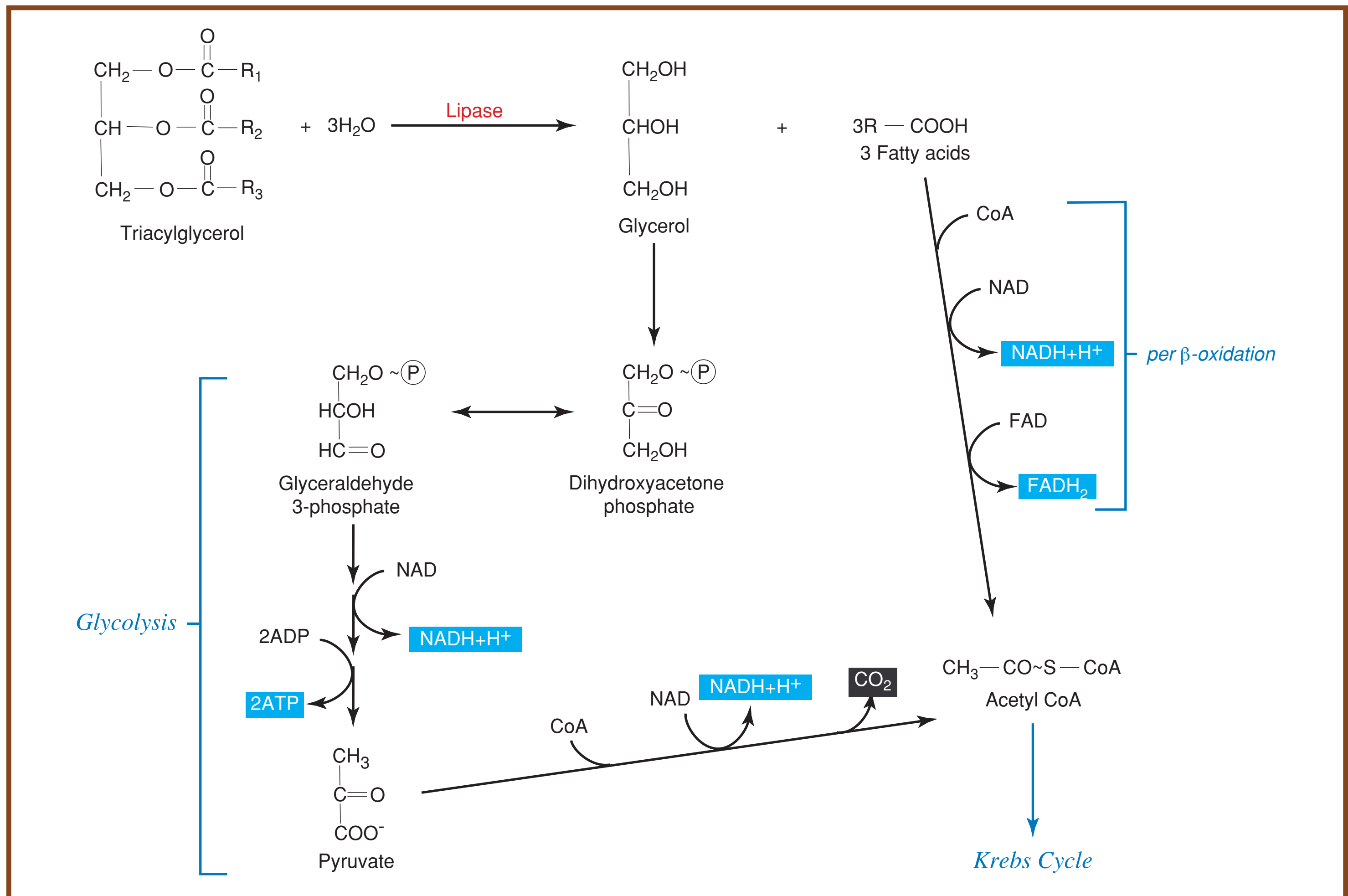
Principle

Lipid is the word generally used to describe all types of fats. The enzymes that hydrolyze fats are called **lipases**. Bacteria can be differentiated based on their ability to produce and secrete lipases. Although a variety of simple fats can be used for this determination, tributyrin oil is the most common constituent of lipase-testing media because it is the simplest triglyceride found in natural fats and oils.

Simple fats are known as **triglycerides,** or **triacylglycerols** (Figure 7-51). Triglycerides are composed of glycerol and three long-chain fatty acids. As is true of many biochemicals, tributyrin is too large to enter the cell, so some cells have the ability to secrete a lipase to break it down prior to cellular uptake. After lipolysis (hydrolysis), the glycerol can be converted to dihydroxyacetone phosphate, an intermediate of glycolysis (see Appendix Figure A-1). The fatty acids are catabolized by a process called **β-oxidation**. Two carbon fragments from the fatty acid are combined with Coenzyme A to produce Acetyl-CoA, which then may be used in the Krebs cycle to produce energy. Each Acetyl-CoA produced by this process also yields one NADH and one $FADH_2$ (important coenzymes in the electron transport chain). Glycerol and fatty acids may be used alternatively in anabolic pathways.

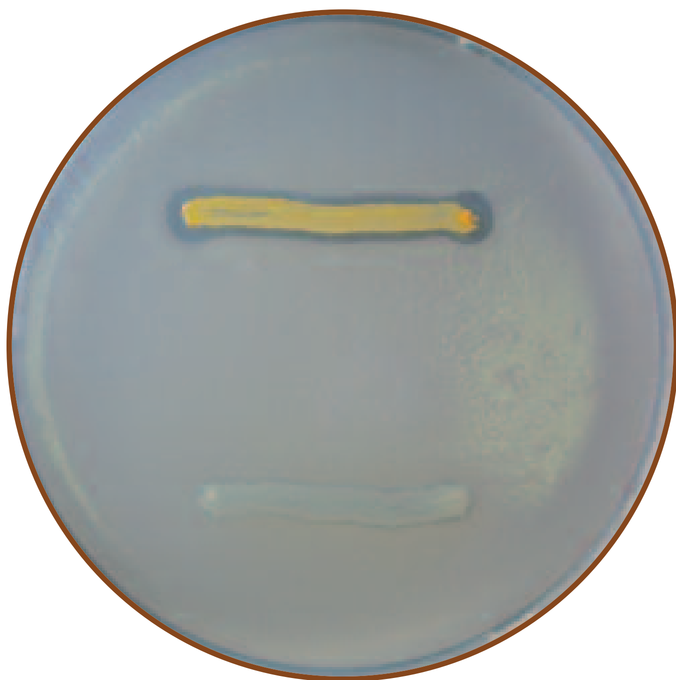
Tributyrin Agar is prepared as an emulsion that makes the agar appear opaque. When the plate is inoculated with a lipase-positive organism, clear zones will appear around the growth as evidence of lipolytic activity. If no clear zones appear, the organism is lipase-negative (Figure 7-52).

Spirit Blue Agar is prepared as an emulsion with tributyrin oil, but also contains spirit blue dye as a color

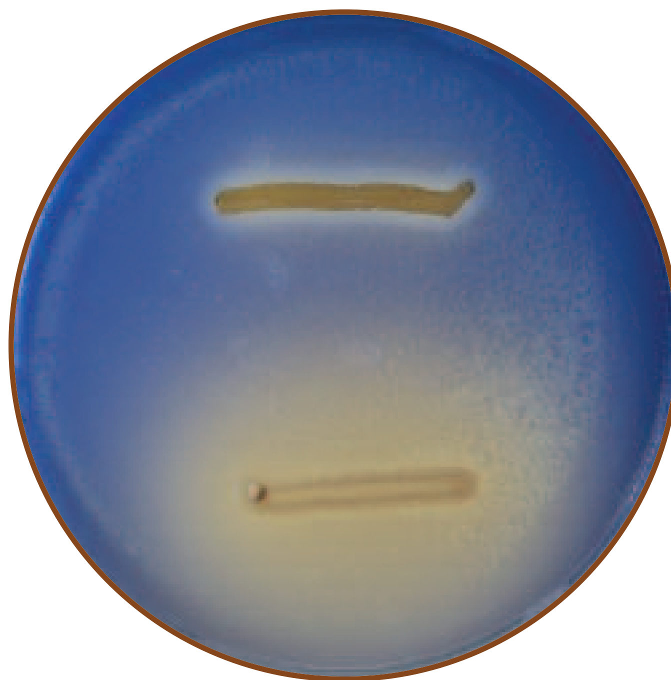


7-51 Lipid Metabolism Triacylglycerols can be hydrolyzed by lipase into glycerol and three fatty acids. Glycerol can then be converted into dihydroxyacetone phosphate, an intermediate of glycolysis. The fatty acids can be broken down, two carbons at a time (by β-oxidation), to form Acetyl-CoA, a reactant of the Krebs Cycle.

indicator. The oil and dye form a complex that gives the medium an opaque light blue appearance. Lipase-positive bacteria growing on the medium hydrolyze the oil and produce clear halos surrounding the growth. Lightening of the medium such as that produced around *P. mirabilis* is not a positive result (Figure 7-53).



7-52 TRIBUTYRIN AGAR *Staphylococcus aureus* (lipase-positive) is above and *Proteus mirabilis* (lipase-negative) is below.



7-53 SPIRIT BLUE AGAR *Staphylococcus aureus* (lipase-positive) is above and *Proteus mirabilis* (lipase-negative) is below. Clearing, not lightening of the medium (as with *P. mirabilis*) is considered a positive result.

Litmus Milk Medium

Purpose

Litmus Milk is used primarily to differentiate members within the genus *Clostridium*. It differentiates *Enterobacteriaceae* from other Gram-negative bacilli based on the ability of enterics to reduce litmus. Litmus Milk also is used to cultivate and maintain cultures of lactic acid bacteria.

Principle

Litmus Milk is an undefined medium consisting of skim milk and the pH indicator azolitmin. Skim milk provides nutrients for growth, lactose for fermentation, and protein in the form of casein. Azolitmin (litmus) is pink at pH 4.5 and blue at pH 8.3. Between these extremes it is purple.

Four basic reactions occur in Litmus Milk: lactose fermentation, reduction of litmus, casein coagulation, and casein hydrolysis. In combination these reactions yield a variety of results, each of which can be used to differentiate bacteria. Several possible combinations are described in Table 7-2.

Lactose fermentation acidifies the medium and turns the litmus pink (Figure 7-54, second tube from the right). This **acid reaction** typically begins with the splitting of the disaccharide into the monosaccharides glucose and galactose by the enzyme β-galactosidase (Figure 7-40). Accumulating acid may cause the casein to precipitate and form an **acid clot** (Figures 7-56 and 7-57). Acid clots solidify the medium and can appear pink or white with a pink band at the top (Figure 7-54, tube on far right) depending on the oxidation-reduction status of litmus. Reduced litmus is white; oxidized litmus is purple. Acid clots can be dissolved in alkaline conditions. Fissures or cracks in the clot are evidence of **gas** production (Figure 7-54, third tube from right). Heavy gas production that breaks up the clot is called **stormy fermentation.**

In addition to being a pH indicator, litmus is an E_h (oxidation-reduction) indicator. As mentioned above, reduced litmus is white. If litmus becomes reduced during lactose fermentation it will turn the medium white in the lower portion of the tube where the reduction rate is greatest.

Some bacteria produce proteolytic enzymes (caseases) such as rennin, pepsin, or chymotrypsin that coagulate casein and produce a **curd** (Figure 7-58). A curd differs from an acid clot in that it will not dissolve in alkaline conditions

Methyl Red Test

Purpose

The Methyl Red Test is a component of the IMViC battery of tests (*I*ndole, *M*ethyl Red, *V*oges-Proskauer, and *C*itrate) used to differentiate the *Enterobacteriaceae*. It identifies bacterial ability to produce stable acid end products by means of a **mixed-acid fermentation** of glucose.

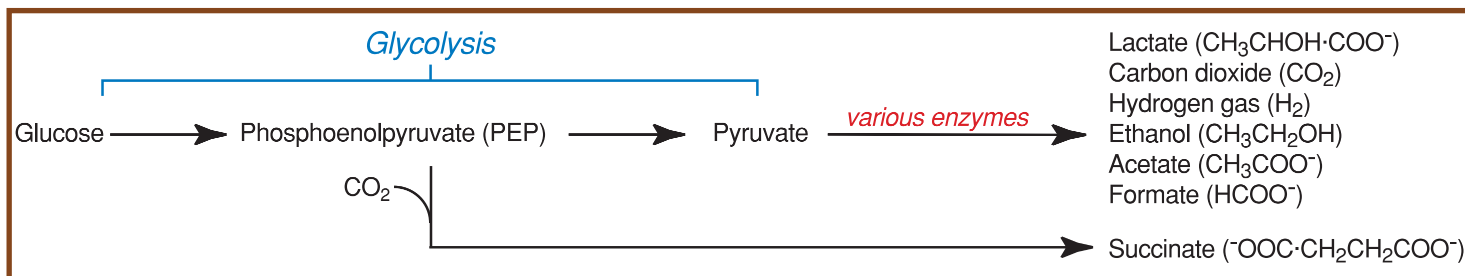
Principle

MR-VP Broth is a combination medium used for both Methyl Red (MR) and Voges-Proskauer (VP) tests. (Refer to page 98 for the VP test.) It is a simple solution containing only peptone, glucose, and a phosphate buffer. The peptone and glucose provide protein and a fermentable carbohydrate, respectively, and the potassium phosphate resists pH changes in the medium.

The MR test is designed to detect organisms capable of performing a **mixed acid fermentation**, which overcomes the phosphate buffer in the medium and lowers the pH (Figure 7-62). The acids produced by these organisms tend to be stable, whereas acids produced by other organisms tend to be unstable and subsequently are converted to more neutral products.

Mixed acid fermentation is verified by the addition of methyl red indicator dye following incubation. Methyl red is red at pH 4.4 and yellow at pH 6.2. Between these two pH values, it is various shades of orange. Red color is the only true indication of a positive result. Orange is negative or inconclusive. Yellow is negative (Figure 7-63).

7-63 The Methyl Red Test *Escherichia coli* (MR-positive) on the left and *Enterobacter aerogenes* (MR-negative) on the right.



7-62 Mixed Acid Fermentation of *E. coli* *E. coli* is a representative Methyl Red-positive organism and is recommended as a positive control for the test. Its mixed acid fermentation produces the end products listed in order of abundance. Most of the formate is converted to H_2 and CO_2 gases. Note: The amount of succinate falls between acetate and formate, but is derived from PEP, not pyruvate. *Salmonella* and *Shigella* are also Methyl Red positive.

Motility Test

Purpose

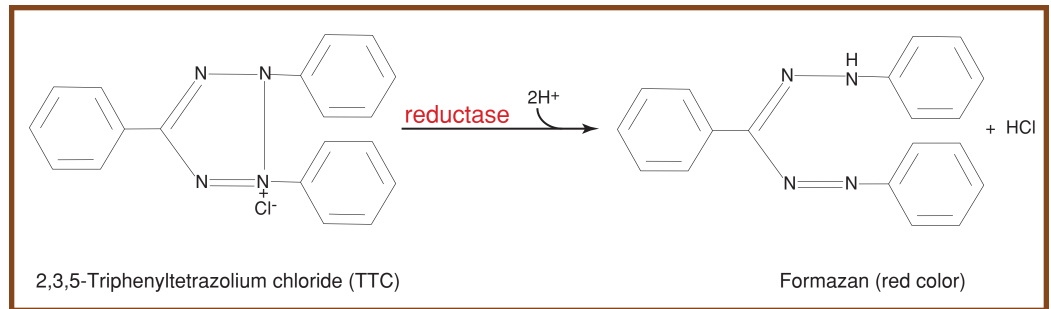
This test is used to detect bacterial motility. Motility is an important differential characteristic of *Enterobacteriaceae* and many other groups.

Principle

Motility Test Agar is a semisolid medium designed to detect bacterial motility. Its agar concentration is reduced from the typical 1.5% to 0.4%—just enough to maintain its form while allowing movement of motile bacteria. It is inoculated by stabbing with a straight transfer needle. Motility is detectable as diffuse growth radiating from the central stab line (Figure 7-64).

A tetrazolium salt (TTC) is sometimes added to the medium to make interpretation easier. TTC is used by the bacteria as an electron acceptor. In its oxidized form, TTC is colorless and soluble; when reduced it is red and insoluble (Figure 7-65). A positive result for motility is indicated when the red (reduced) TTC is seen radiating outward from the central stab. A negative result shows red only along the stab line (Figure 7-66).

7-64 Motility Test in SIM Medium Without TTC On the left is *Proteus vulgaris* (motile); *Shigella sonnei* (nonmotile) is on the right. Notice that motility of *P. vulgaris* is seen only as haziness in the medium. Tubes must be compared to uninoculated controls to discriminate between faint haziness and motility. Compare with Figure 7-66.



7-65 Reduction of TTC Reduction of 2,3,5-Triphenyltetrazolium chloride by metabolizing bacteria results in its conversion from colorless and soluble to the red and insoluble compound formazan. The location of the growing bacteria can be easily determined by the location of the formazan in the medium.

7-66 Motility Test Results Motility Test Medium tubes containing TTC inoculated with *Enterobacter aerogenes* (+) on the left and *Micrococcus luteus* (−) on the right. Compare with Figure 7-64.

Nitrate Reduction Test

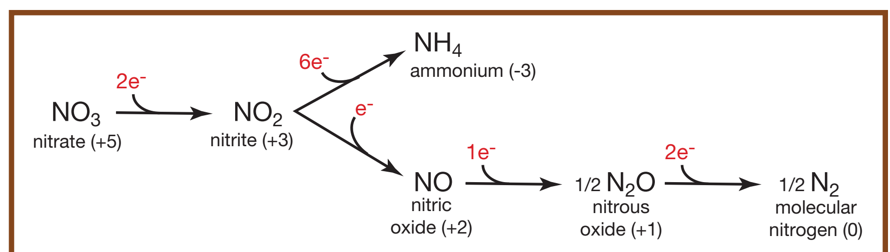
Purpose

Virtually all members of *Enterobacteriaceae* perform a one-step reduction of nitrate to nitrite. The Nitrate Test differentiates them from Gram-negative rods that either do not reduce nitrate or reduce it beyond nitrite to N_2 or other compounds.

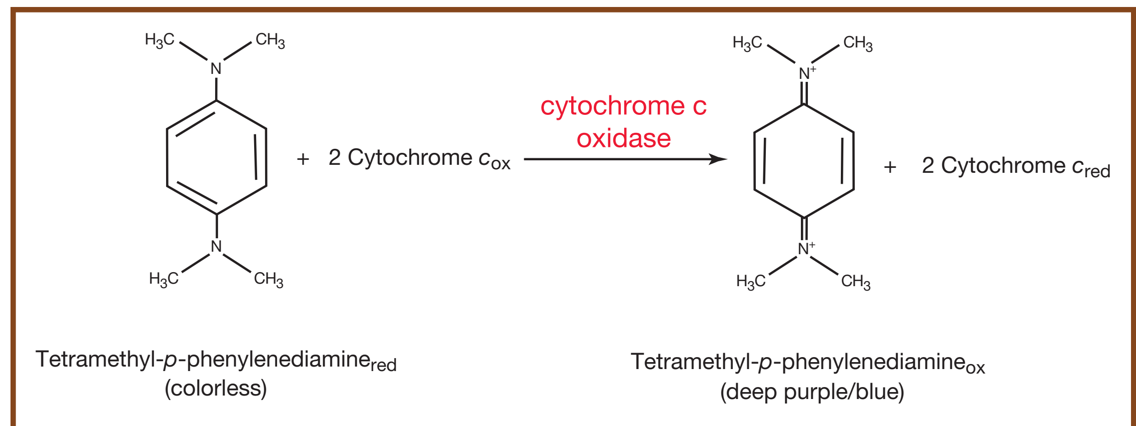
Principle

Anaerobic respiration involves the reduction of (*i.e.*, transfer of electrons to) an inorganic molecule other than oxygen. Nitrate reduction is one such example. Many Gram-negative bacteria (including most *Enterobacteriaceae*) contain the enzyme **nitrate reductase** and perform a single-step reduction of nitrate to nitrite ($NO_3 \rightarrow NO_2$). Other bacteria, in a multi-step process known as **denitrification**, are capable of enzymatically converting nitrate to molecular nitrogen (N_2). Some products of nitrate reduction are shown in Figure 7-67.

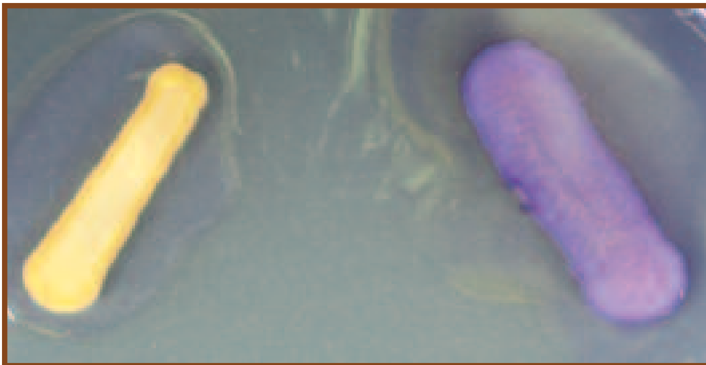
Nitrate broth is an undefined medium of beef extract, peptone, and potassium nitrate (KNO_3). An inverted Durham tube is placed in each broth to trap a portion of any gas produced. In contrast to many differential media, no color indicators are included. The color reactions obtained in Nitrate Broth take place as a result of reactions between



7-67 Possible End Products of Nitrate Reduction Nitrate reduction is complex. Many different organisms under many different circumstances perform nitrate reduction with many different outcomes. Members of the *Enterobacteriaceae* simply reduce NO_3 to NO_2. Other bacteria, functionally known as "denitrifiers," reduce NO_3 all the way to N_2 via the intermediates shown, and are important ecologically in the nitrogen cycle. Both of these are anaerobic respiration pathways (also known as "nitrate respiration"). Other organisms are capable of assimilatory nitrate reduction, in which NO_3 is reduced to NH_4, which can be used in amino acid synthesis. The oxidation state of nitrogen in each compound is shown in parentheses.



7-77 Chemistry of the Oxidase Reaction The oxidase enzyme shown is not involved directly in the indicator reaction as shown. Rather, it removes electrons from cytochrome c, making it available to react with the phenylenediamine reagent.



7-78 Oxidase Test on Bacterial Growth A few drops of reagent on oxidase-positive bacteria will produce a purple-blue color immediately. Oxidase-negative organisms will not turn purple. The bacterium on the left is its natural color, not the color of an oxidase-negative organism.

7-79 Oxidase Slide Test Positive results with this test should appear within 20 seconds. The dark blue is a positive result (left upper square). No color change is a negative result (right upper square).
(BBL™ DrySlide™ systems available from Becton Dickinson, Sparks, MD.)

saturated with the reagent (Figure 7-79). A dramatic color change occurs within seconds if the reducing agent becomes oxidized, thus indicating that cytochrome c oxidase is present. Lack of color change within the allotted time means that cytochrome c oxidase is not present and signifies a negative result.

Oxidation–Fermentation Test

Purpose

The Oxidation-Fermentation (O–F) Test is used to differentiate bacteria based on their ability to oxidize or ferment specific sugars. It allows presumptive separation of the fermentative *Enterobacteriaceae* from the oxidative *Pseudomonas* and *Bordetella*, and the nonreactive *Alcaligenes* and *Moraxella*.

Principle

The O–F Test is designed to differentiate bacteria on the basis of fermentative or oxidative metabolism of carbohydrates. In oxidation pathways a carbohydrate is directly oxidized to pyruvate and further converted to CO_2 and energy by way of the Krebs cycle and the electron transport chain, where an inorganic molecule such as oxygen is required to act as the final electron acceptor. Fermentation also converts carbohydrates to pyruvate, but uses it to produce one or more acids (as well as other compounds). Consequently, fermenters identified by this test acidify O-F medium to a greater extent than do oxidizers.

Hugh and Leifson's O–F medium includes a high sugar-to-peptone ratio to reduce the possibility that alkaline products from peptone utilization will neutralize weak acids produced by oxidation of the carbohydrate. Bromthymol blue dye, which is yellow at pH 6.0 and green at pH 7.1, is

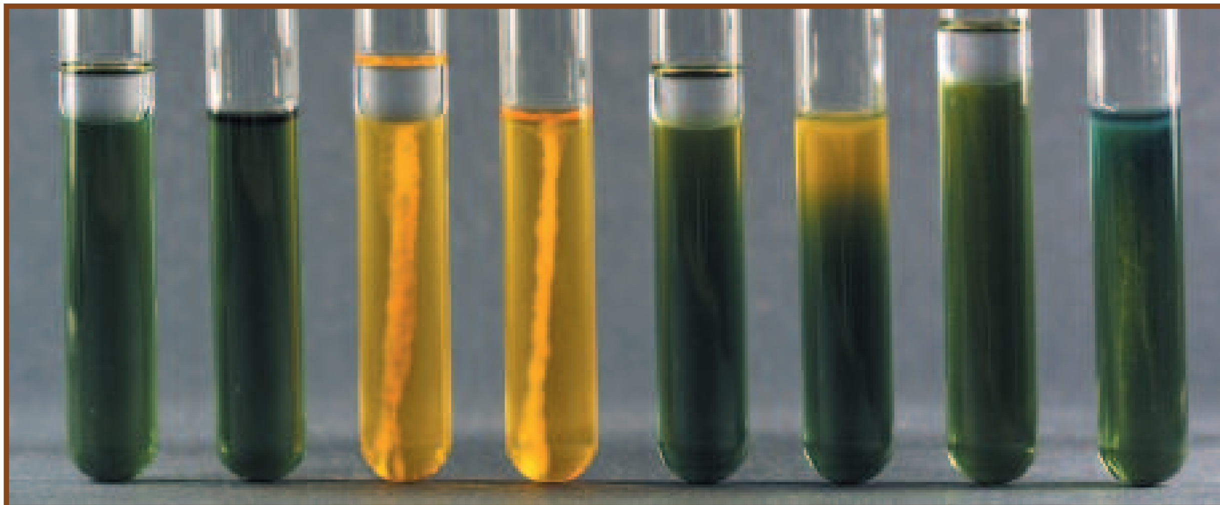
added as a pH indicator. A low agar concentration makes it a semi-solid medium that allows determination of motility.

The medium is prepared with glucose, lactose, sucrose, maltose, mannitol, or xylose and is not slanted. Two tubes of the specific sugar medium are stab-inoculated several times with the test organism. After inoculation, one tube is sealed with a layer of sterile mineral oil to promote anaerobic growth and fermentation. The other tube is left unsealed to allow aerobic growth and oxidation. (***Note:*** Tubes of O–F medium are heated in boiling water and then cooled prior to inoculation. This removes free oxygen from the medium and ensures an anaerobic environment in all tubes. The tubes covered with oil will remain anaerobic, whereas the uncovered medium quickly will become aerobic as oxygen diffuses back in.)

Organisms able to ferment the carbohydrate or ferment *and* oxidize the carbohydrate will turn the sealed and unsealed media yellow throughout. Organisms that are able to oxidize only will turn the unsealed medium yellow (or partially yellow) and leave the sealed medium green or blue. Slow or weak fermenters will turn both tubes slightly yellow at the top. Organisms that are not able to metabolize the sugar will either produce no color change or turn the medium blue because of alkaline products from amino acid degradation. The results are summarized in Table 7-4 and shown in Figure 7-80.

TABLE 7-4 O–F Medium Results and Interpretations

TABLE OF RESULTS			
Sealed	**Unsealed**	**Interpretation**	**Symbol**
Green or blue	Any amount of yellow	Oxidation	O
Yellow throughout	Yellow throughout	Oxidation and fermentation or fermentation only	O–F or F
Slightly yellow at the top	Slightly yellow at the top	Oxidation and slow fermentation or slow fermentation only	O–F or F
Green or blue	Green or blue	No sugar metabolism; organism is nonsaccharolytic	N



7-80 Oxidation–Fermentation Test These pairs of tubes represent three possible results in the Oxidation–Fermentation (O–F) Test. Each pair contains one tube sealed with an overlay of mineral oil and one unsealed tube. The mineral oil creates an environment unsuitable for oxidation because it prevents diffusion of oxygen from the air into the medium. The result is that an organism capable of fermentation will turn both tubes yellow, whereas an organism capable only of oxidizing glucose will turn only the oxygen-containing portion of the unsealed medium yellow. An organism incapable of utilizing glucose by any means either will not change the color of the medium or will turn it blue-green as a result of alkaline products from protein degradation. Reading from left to right, the first pair of tubes on the left are uninoculated controls for color comparison. The second pair of tubes was inoculated with *Shigella flexneri*, an organism capable of both oxidative and fermentative utilization of glucose (O–F). Unfortunately, this determination cannot be made simply by visual examination, as the results of a fermentative organism (F) look exactly the same as an organism capable of both oxidation and fermentation (O–F). Therefore, when both tubes are yellow, the organism is assumed to be either (F) *or* (O–F). The third pair of tubes was inoculated with *Pseudomonas aeruginosa*, a glucose nonfermenter. This organism is capable only of oxidation. Note the yellowing only of the oxygenated portion of the unsealed tube. The fourth pair of tubes (right) was inoculated with *Alcaligenes faecalis*, an organism incapable of utilizing glucose. The blue color in the oxygenated portion of the unsealed tube suggests that the organism is both nonsaccharolytic (N) and a strict aerobe.

Phenylalanine Deaminase Test

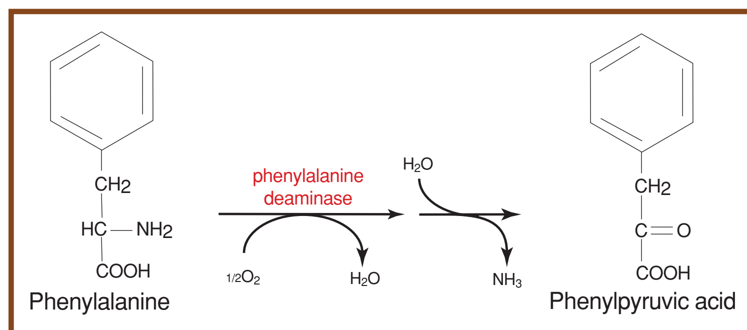
Purpose

The Phenylalanine Deaminase Test is used to differentiate the genera *Morganella*, *Proteus* and *Providencia* (phenylalanine deaminase-positive) from other members of the *Enterobacteriaceae* (phenylalanine deaminase-negative).

Principle

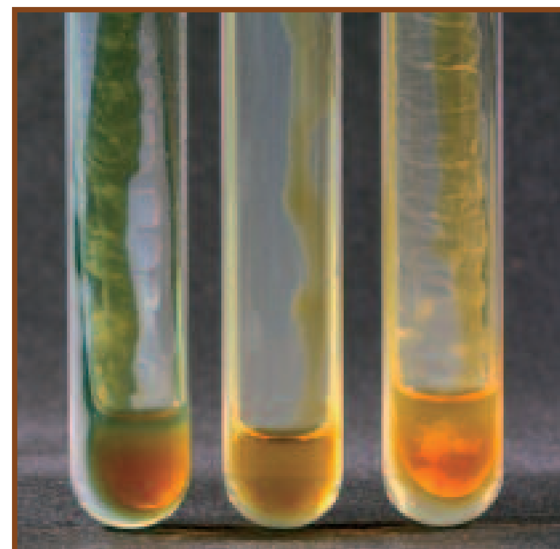
Organisms that produce phenylalanine deaminase can be identified by their ability to remove the amine group (NH_2) from the amino acid phenylalanine. The reaction, as shown in Figure 7-81, requires oxygen and produces ammonia (NH_3) and phenylpyruvic acid. Deaminase activity is evidenced by the presence of phenylpyruvic acid.

Phenylalanine Agar provides a rich source of phenylalanine. A reagent containing ferric chloride ($FeCl_3$) is added to the medium after incubation. The normally colorless phenylpyruvic acid reacts with the ferric chloride and turns a dark green color almost immediately (Figure 7-82). Formation of green color indicates the presence of phenylpyruvic acid and, hence, the presence of phenylalanine deaminase. Yellow is negative (Figure 7-83).



7-81 DEAMINATION OF PHENYLALANINE

7-82 INDICATOR REACTION Phenylpyruvic acid produced by positive organisms reacts with $FeCl_3$ to produce a green color. The test must be read immediately because the color may fade.



7-83 PHENYLALANINE DEAMINASE TEST Note the color produced by the stream of ferric chloride in each tube. *Proteus mirabilis* (+) is on the left, an uninoculated control is in the middle, and *Escherichia coli* (−) is on the right.

PYR Test

Purpose

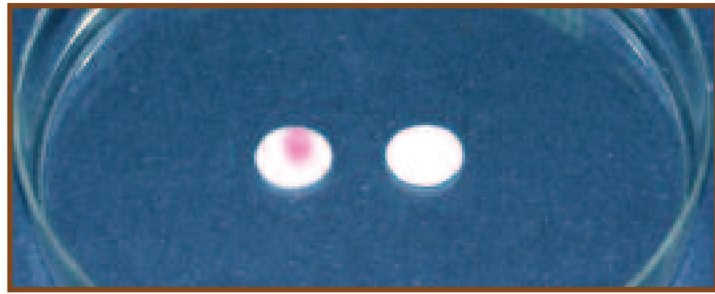
The PYR Test is designed for presumptive identification of group A streptococci (*Streptococcus pyogenes*) and enterococci by determining the presence of the enzyme *L*-pyrrolidonyl arylamidase (PYR).

Principle

Group A streptococci and enterococci produce the enzyme *L*-pyrrolidonyl arylamidase. This enzyme hydrolyzes the amide pyroglutamyl-β-naphthylamide to produce *L*-pyrrolidone and β-naphthylamine, both of which are colorless. β-naphthylamine will react with *p*-dimethylaminocinnamaldehyde and form a red precipitate.

PYR may be performed as an 18-hour agar test, a four hour broth test or, as used in this example, a rapid disk test. In each case the medium (or disk) contains pyroglutamyl-β-naphthylamide (the PYR substrate) to which is added a heavy inoculum of the test organism. After the appropriate incubation or waiting period, a 0.01% *p*-dimethylaminocinnamaldehyde solution is added. Formation of a deep red color within a few minutes is interpreted as PYR-positive. Yellow or orange is PYR-negative (Figure 7-84).

7-84 PYR Disk Test The disk on the left was inoculated with *Streptococcus pyogenes* (PYR-positive); the disk on the right contains *Streptococcus agalactiae* (PYR–negative).



Starch Hydrolysis

Purpose

Starch Agar originally was designed for cultivating *Neisseria*. It no longer is used for this, but with pH indicators, it is used to isolate and presumptively identify *Gardnerella vaginalis*. It aids in differentiating species of the genera *Corynebacterium, Clostridium, Bacillus, Bacteroides, Fusobacterium*, and *Enterococcus*, most of which have positive and negative species.

Principle

Starch is a polysaccharide made up of α-D-glucose subunits. It exists in two forms—linear (amylose) and branched (amylopectin)—usually as a mixture with the branched configuration being predominant. The α-D-glucose molecules in both amylose and amylopectin are bonded by 1,4-α-glycosidic (acetal) linkages (Figure 7-85). The two forms differ in that amylopectin contains polysaccharide side chains connected

H_2O α-Amylase

α-Amylase
[1,4-α-glucosidic (acetal) linkages]

α-D-Glucose
(many)

H_2O α-Amylase
Oligo-1,6-glucosidase

Amylopectin
[1,4-α-glucosidic (acetal) linkages and 1,6-α-glucosidic (acetal) branch linkages]

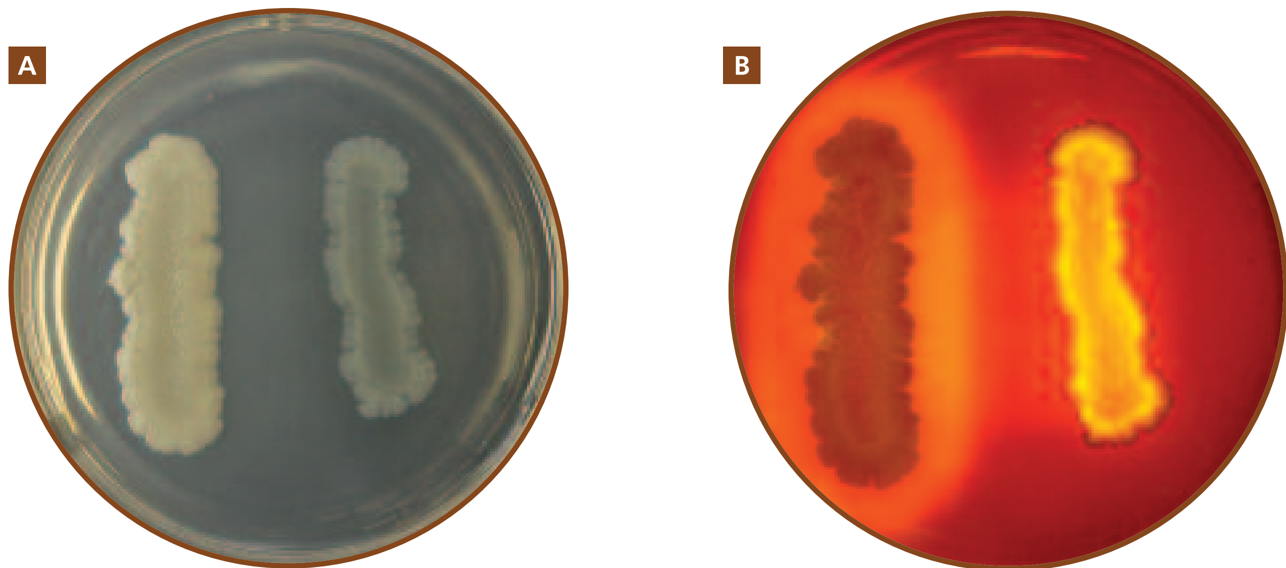
α-D-Glucose
(many)

7-85 Starch Hydrolysis by α-Amylase and oligo-1,6-Glucosidase

to approximately every 30th glucose in the main chain. These side chains are identical to the main chain except that the number 1 carbon of the first glucose in the side chain is bonded to carbon number 6 of the main chain glucose. The bond, therefore, is a 1,6-α-glycosidic linkage.

Starch is too large to pass through the bacterial cell membrane. Therefore, to be of metabolic value to the bacteria it must first be split into smaller fragments or individual glucose molecules. Organisms that produce and secrete the extracellular enzymes **α-amylase** and **oligo-1,6-glucosidase** are able to hydrolyze starch by breaking the glycosidic linkages between the sugar subunits. Although there usually are intermediate steps and additional enzymes utilized, the overall reaction is the complete hydrolysis of the polysaccharide to its individual α-glucose subunits (Figure 7-86).

Starch agar is a simple plated medium of beef extract, soluble starch, and agar. When organisms that produce α-amylase and oligo-1,6-glucosidase are cultivated on starch agar they hydrolyze the starch in the area surrounding their growth. Because both the starch and its sugar subunits are soluble and virtually invisible in the medium, the reagent iodine is used to detect the presence or absence of starch in the vicinity around the bacterial growth. Iodine reacts with starch and produces a blue or dark brown color; therefore, any microbial starch hydrolysis will be revealed as a clear zone surrounding the growth (Figure 7-86).



7-86 STARCH AGAR **A** *Bacillus subtilis* is on the left. *Escherichia coli* is on the right. Notice the wavy margin of *E. coli*. **B** After iodine has been added, the clearing in the medium around *B. subtilis* demonstrates a positive result for starch hydrolysis. *E. coli*, with no clearing, is negative. Note that the wavy margin of *E. coli* produced a lighter region around the growth that might be misinterpreted as clearing. To prevent reading a false positive, it is a good idea to establish the margin of growth for each tested organism prior to adding iodine.

Sulfur Reduction (SIM Medium)

Purpose

The Sulfur Reduction Test is used to differentiate members of *Enterobacteriaceae*, especially the sulfur-reducing *Salmonella*, *Francisella*, and *Proteus* from the non-reducing *Morganella morganii* and *Providencia rettgeri*.

Principle

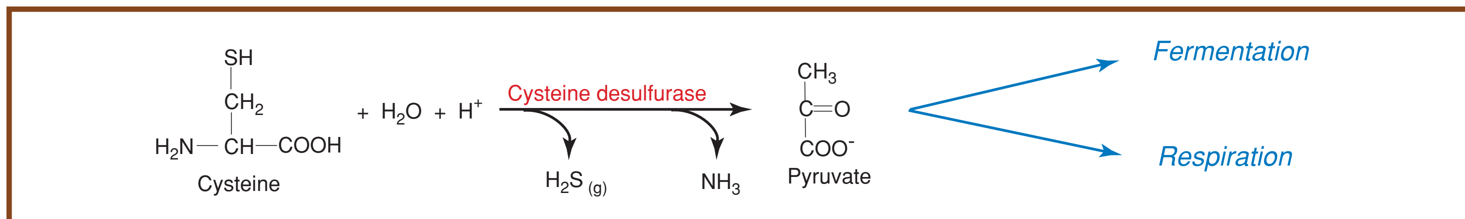
The Sulfur Reduction Test, as it appears in this manual, is performed using SIM medium. SIM medium also tests for indole production (page 74) and motility (page 82). It is a semi-solid medium that is formulated with casein and animal tissue as sources of amino acids, an iron-containing compound, and sulfur in the form of sodium thiosulfate.

Sulfur reduction to H_2S is an anaerobic activity and can be accomplished by bacteria in two different ways, depending on the enzymes present.

1. The enzyme **cysteine desulfurase** catalyzes the putrefaction of the amino acid cysteine to pyruvate (Figure 7-87).
2. The enzyme **thiosulfate reductase** catalyzes the reduction of sulfur (in the form of sulfate) at the end of the

anaerobic respiratory electron transport chain (Figure 7-88).

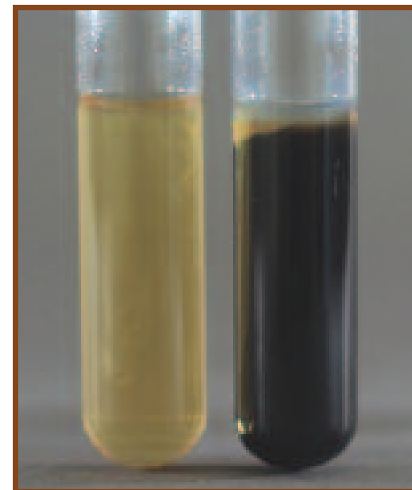
Both systems produce hydrogen sulfide gas (H_2S). When either reaction occurs in SIM medium, the H_2S produced combines with iron (ferrous ammonium sulfate in the medium) to form ferric sulfide (FeS), a black precipitate (Figure 7-90). Any blackening of the medium is an indication of sulfur reduction and a positive test. Absence of blackening in the medium indicates no sulfur reduction and a negative reaction (Figure 7-90).



7-87 Putrefaction of Cysteine Putrefaction involving cysteine desulfurase produces H_2S. The reaction is a mechanism for getting energy out of the amino acid cysteine.

7-88 Reduction of Thiosulfate Anaerobic respiration with thiosulfate as the final electron acceptor also produces H_2S.

7-89 Indicator Reaction Hydrogen sulfide, a colorless gas, can be detected when it reacts with ferrous ammonium sulfate in the medium to produce the black precipitate ferric sulfide.



7-90 Sulfur Reduction in SIM Medium On the left is *Escherichia coli* (H_2S-negative); on the right is *Proteus mirabilis* (H_2S-positive).

SXT Susceptibility Test

Purpose

The SXT (Sulfamethoxazole-Trimethoprim) Susceptibility Test is used to differentiate Groups A and B streptococci (SXT resistant) from other β-hemolytic streptococci (SXT susceptible). Used in conjunction with the Bacitracin Susceptibility Test (as in this example) it also differentiates Groups A and B streptococci from each other.

Principle

When combined, Sulfamethoxazole and Trimethoprim act synergistically to disrupt bacterial folic acid metabolism. SXT disks typically contain 23.75 μg of Sulfamethoxazole and 1.25 μg of Trimethoprim. When a disk is placed on the surface of a Sheep Blood Agar plate inoculated to produce confluent growth, a clearing will appear around the disk if the organism is susceptible (S) to the antibiotic mixture. Growth up to the edge of the disk indicates resistance (R).

The combination SXT and Bacitracin Susceptibility Test (page 58) is performed by placing one of each disk on the plate at least four centimeters apart (Figure 7-91). Any clearing around either disk is interpreted as susceptibility. Table 7-5 summarizes Bacitracin SXT susceptibilities of various streptococci.

7-91 Bacitracin-SXT Test This is a Bacitracin–SXT Susceptibility Test on a Sheep Blood Agar plate containing *Streptococcus pyogenes* (Group A). Bacitracin is on the left (S) and SXT (R) is on the right.

TABLE 7-5 Reactions of β-Hemolytic Streptococci to Bacitracin and SXT

TABLE OF RESULTS		
Organism	Bacitracin	SXT
Group A	S	R
Group B	R	R
Groups C, F, and G	S or R	S

Triple Sugar Iron Agar

Purpose

Triple Sugar Iron Agar (TSIA) is primarily used to differentiate members of *Enterobacteriaceae* and to differentiate them from other Gram-negative rods such as *Pseudomonas*.

Principle

TSIA is a rich medium designed to differentiate bacteria on the basis of glucose fermentation, lactose fermentation, sucrose fermentation, and sulfur reduction. In addition to the three carbohydrates, it includes beef extract, yeast extract, and peptone as carbon and nitrogen sources, and sodium thiosulfate as a source of reducible sulfur. Phenol red is the pH indicator and the iron in ferrous sulfate is the hydrogen sulfide indicator.

The medium is prepared as a shallow agar slant with a deep butt, thereby providing both aerobic and anaerobic growth environments. It is inoculated by a stab in the agar butt followed by a fishtail streak of the slant. The incubation period is 18 to 24 hours for carbohydrate fermentation and up to 48 hours for hydrogen sulfide reactions. Many reactions in various combinations are possible (Figure 7-92 and Table 7-6).

When TSIA is inoculated with a glucose-only fermenter, acid products lower the pH and turn the entire medium yellow within a few hours. Because glucose is in short supply (0.1%), it will be exhausted within about 12 hours. As the glucose is used up, the organisms located in the aerobic region (slant) will begin to break down available amino acids, producing NH_3 and raising the pH. This process, which takes 18 to 24 hours to complete, is called a **reversion** and only occurs in the slant because of the anaerobic conditions in the butt. Thus, a TSIA with a red slant and yellow butt after a 24-hour incubation period indicates that the organism ferments glucose but not lactose.

Organisms that are able to ferment glucose *and* lactose *and/or* sucrose also turn the medium yellow throughout. However, because the lactose and sucrose concentrations are ten times higher than that of glucose, greater acid production results and both slant and butt will remain yellow after 24 hours. Therefore, a TSIA with a yellow slant and butt at 24 hours indicates that the organism ferments glucose

7-92 TSI Agar Slants From left to right: *Pseudomonas aeruginosa* (K/NC), uninoculated control, *Morganella morganii* (K/A, atypically not producing gas), *Escherichia coli*, (A/A, G) and *Proteus mirabilis* (K/A, H_2S).

TABLE 7-6 TSI Test Results and Interpretations

TABLE OF RESULTS		
Result	**Interpretation**	**Symbol**
Yellow slant/yellow butt	Glucose and lactose and/or sucrose fermentation with acid accumulation in slant and butt.	A/A
Red slant/yellow butt	Glucose fermentation with acid production. Proteins catabolized aerobically (in the slant) with alkaline products (reversion).	K/A
Red slant/red butt	No fermentation. Peptone catabolized aerobically and anaerobically with alkaline products. Not from *Enterobacteriaceae*.	K/K
Red slant/no change in butt	No fermentation. Peptone catabolized aerobically with alkaline products. Not from *Enterobacteriaceae*.	K/NC
No change in slant / no change in butt	Organism is growing slowly or not at all. Not from *Enterobacteriaceae*.	NC/NC
Black precipitate in the agar	Sulfur reduction. (An acid condition, from fermentation of glucose or lactose and/or sucrose, exists in the butt even if the yellow color is obscured by the black precipitate.)	H_2S
Cracks in or lifting of agar	Gas production.	G

and one or both of the other sugars. Gas produced by carbohydrate fermentation will appear as fissures in the medium or will lift the agar off the bottom of the tube.

Hydrogen sulfide (H_2S) may be produced by the reduction of thiosulfate in the medium or by the breakdown of cysteine in the peptone. Ferrous sulfate in the medium reacts with the H_2S to form a black precipitate, usually seen in the butt. Acid conditions must exist for thiosulfate reduction; therefore, black precipitate in the medium is an indication of sulfur reduction *and* fermentation. If the black precipitate obscures the color of the butt, the color of the slant determines which carbohydrates have been fermented (*i.e.*, red slant = glucose fermentation, yellow slant = glucose and lactose and/or sucrose fermentation).

An organism that does not ferment any of the carbohydrates but utilizes peptone and amino acids will alkalinize the medium and turn it red. If the organism can use the peptone aerobically and anaerobically, both the slant and butt will appear red. An obligate aerobe will turn only the slant red.

Timing is critical in reading TSIA results. An early reading could reveal yellow throughout the medium, leading one to conclude that the organism is a lactose or sucrose fermenter when it simply may not yet have exhausted the glucose. A reading after the lactose and sucrose have been depleted could reveal a yellow butt and red slant leading one to falsely conclude the organism is a glucose-only fermenter. Tubes that have been interpreted for carbohydrate fermentation and are negative for sulfur reduction can be re-incubated for 24 hours before H_2S determination. Refer to Table 7-6 for information on the correct symbols and method of reporting the various reactions.

Urease Tests

Purpose

The Urease Test is used to differentiate organisms based on their ability to hydrolyze urea with the enzyme **urease**. Urinary tract pathogens from the genus *Proteus* may be distinguished from other enteric bacteria by their rapid urease activity.

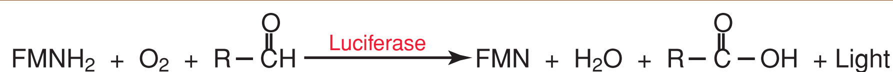
Principle

Urea is a product of decarboxylation of certain amino acids. It can be hydrolyzed to ammonia and carbon dioxide by bacteria containing the enzyme urease. Many enteric bacteria (and a few others) possess the ability to metabolize urea, but

Bioluminescence

Principle

A few marine bacteria from genera *Vibrio* and *Photobacterium* are able to emit light by a process known as **bioluminescence.** Many of these organisms maintain mutualistic relationships with other marine life. For example, *Photobacterium* species living in the Flashlight Fish receive nutrients from the fish and in return provide a unique device for frightening would-be predators.



19-20 CHEMISTRY OF BIOLUMINESCENT BACTERIA

Bioluminescent bacteria are given the ability to emit light because of an enzyme called **luciferase** (Figure 19-20). In the presence of oxygen and a long-chain aldehyde (*e.g.*, glyceraldehyde), luciferase catalyzes the oxidation of reduced flavin mononucleotide ($FMNH_2$). In the process, outer electrons surrounding FMN become excited. Light is emitted when the electronically excited FMN returns to its ground state (Figure 19-21).

It is estimated that a single *Vibrio* cell burns between 6000 and 60000 molecules of ATP per second emitting light (ATP hydrolysis occurs in conjunction with synthesis of the aldehyde). It also is known that their luminescence only occurs when a certain threshold population size is reached in a phenomenon called **quorem sensing.** This system is controlled by a genetically produced **autoinducer** that must be in sufficient concentration to trigger the reaction.

19-21 BIOLUMINESCENCE ON AN AGAR PLATE This is an unknown bioluminescent bacteria growing on Seawater Complete (SWC) Agar.

Winogradsky Column

Purpose

The Winogradsky column is a method for growing a variety of microbes with uniquely microbial metabolic abilities. Bacterial photoautotrophs, chemolithotrophs, and photoheterotrophs may be found in a mature column. And more "typical" chemoheterotrophs and photoautotrophs also are likely to be found. A mature Winogradsky column is a good source for studying these organisms in the laboratory.

Principle

The Winogradsky column bears the name of its developer, Sergei Winogradsky (1856–1953), a Russian microbiologist and pioneer in microbial ecology. He studied sulfur bacteria because of their ease of handling and cultivation, and then moved on to bacteria associated with the nitrogen cycle. One of his major discoveries was finding microorganisms (*Beggiatoa*) capable of the unheard of type of metabolism that came to be known as chemolithotrophic autotrophy (see below). Until he made his discovery, only photoautotrophs—those performing photosynthesis—were known to be autotrophs.

As a result of his work and the work of others, metabolic categories of microorganisms have been identified based on their carbon, energy, and electron sources. These are listed below. Note that in practice, terms are combined to describe the organism more fully.

Autotroph: an organism capable of obtaining all of its carbon from CO_2.

Heterotroph: an organism that can only get its carbon from organic molecules.

Chemotroph: an organism that gets its energy from the oxidation of chemicals.

Phototroph: an organism that gets its energy from light ($h\nu$).

Organotroph: an organism that gets its electrons from an organic molecule.

Lithotroph: an organism that gets its electrons from an inorganic molecule.

Winogradsky pioneered this method of growing microbes in the late 19th century. It was (and is) used as a convenient laboratory source to supply for study a variety of **anaerobic, microaerophilic,** and **aerobic** bacteria, including purple

APPENDIX

So much of what is done in microbiology relies on an understanding of basic biochemical pathways. It's not as important to memorize them (although, with exposure they will become second nature) as it is to understand their importance in metabolism and to interpret diagrams of them when available. The following discussion is provided so you can see how the various biochemical tests presented in this manual fit into the overall scheme of cellular chemistry.

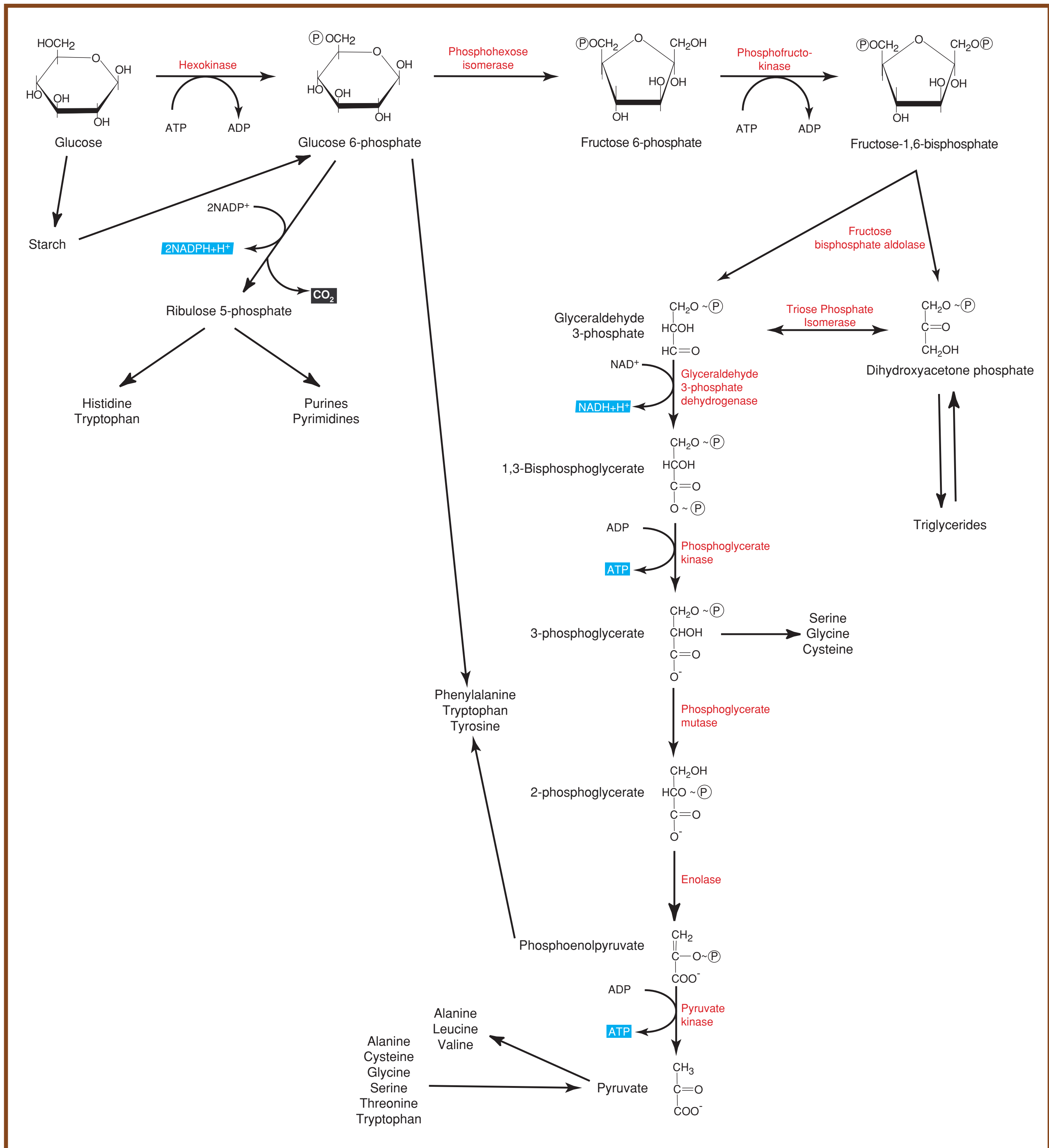
Oxidation of Glucose: Glycolysis, Entner-Doudoroff, and Pentose-Phosphate Pathways

Most organisms use **glycolysis** (also known as the "Embden-Meyerhof-Parnas pathway, Figure A-1) in energy metabolism. It performs the stepwise disassembly of glucose into two pyruvates, releasing some of its energy and electrons in the process. The exergonic (energy-releasing) reactions are associated with ATP synthesis by a process called **substrate phosphorylation**. Although a total of four ATPs are produced per glucose in glycolysis, two ATPs are hydrolyzed early in the pathway, leaving a net production of two ATPs per glucose. In one glycolytic reaction, the loss of an electron pair (oxidation) from a three-carbon intermediate occurs simultaneously with the reduction of NAD^+ to $NADH + H^+$. The $NADH + H^+$ then may be oxidized in an electron transport chain or a fermentation pathway, depending on the organism and the environmental conditions. The former yields ATP, and the latter generally does not. In summary, each glucose oxidized in glycolysis yields two pyruvates, 2 NADH + 2 H^+, and a net of 2 ATPs (Table A-1).

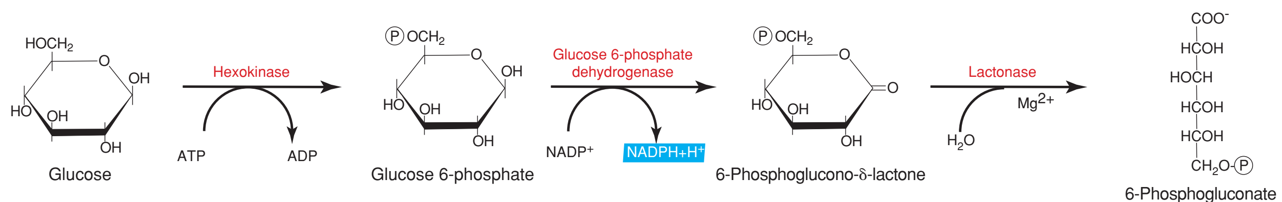
Although the intermediates of glycolysis are carbohydrates, many are entry points for amino acid, lipid, and nucleotide catabolism. Many glycolytic intermediates also are a source of carbon skeletons for the synthesis of these other biochemicals. Some of these are shown in Figure A-1. ***Note***: For clarity, many details have been omitted from these other pathways in Figure A-1. Single arrows may represent several reactions, and other carbon compounds not illustrated may be required to complete a particular reaction.

The **Entner-Doudoroff pathway** (Figure A-2) is an alternative means of degrading glucose into two pyruvates. This pathway is found exclusively among prokaryotes (*e.g., Pseudomonas* and *E. coli*, as well as other Gram-negatives and certain *Archaea*). It allows utilization of a different category of sugars (aldonic acids) than glycolysis and therefore improves the range of resources available to the organism. It is less efficient than glycolysis because only one ATP is phosphorylated and only one NADH is produced. Table A-2 summarizes this pathway.

The **pentose-phosphate pathway** is a complex set of cyclic reactions that provides a mechanism for producing five-carbon sugars (**pentoses**) from six-carbon sugars (**hexoses**). Pentose sugars are used in ribonucleotides and deoxyribonucleotides, as well as being precursors to aromatic amino acids. Further, this pathway produces NADPH, which is used as an electron donor in anabolic pathways. Unlike NADH, produced in glycolysis and Entner-Doudoroff, NADPH is not used as an electron donor in an electron transport chain for oxidative phosphorylation of ADP.



A-1 Glycolysis and Associated Pathways The names of glycolytic intermediates are printed in black ink; the enzyme names are in red. Reducing power (in the form of NADH + H+) and ATP are highlighted in blue. The major key to getting product yields correct is to recognize that *both* C_3 compounds (Glyceraldehyde 3-phosphate and Dihydroxyacetone phosphate) produced from splitting Fructose 1,6-bisphosphate can pass through the remainder of the pathway because of the triose phosphate isomerase reaction. The conversion of each into pyruvate results in the formation of 2 ATPs and 1 NADH + H+ (Table A-1).



A-2 Entner-Doudoroff Pathway

Notice the similarities between this pathway and glycolysis (Figure A-1). The main difference is in the six-carbon compound that is split into two three-carbon compounds. The result of this split is pyruvate and glyceraldehydes-3-phosphate, which is oxidized as in glycolysis to pyruvate. Because only one three-carbon compound goes through the sequence of reactions leading to pyruvate, the ATP and NADH yield is one-half that of glycolysis. But one NADPH is produced that is not made in glycolysis.

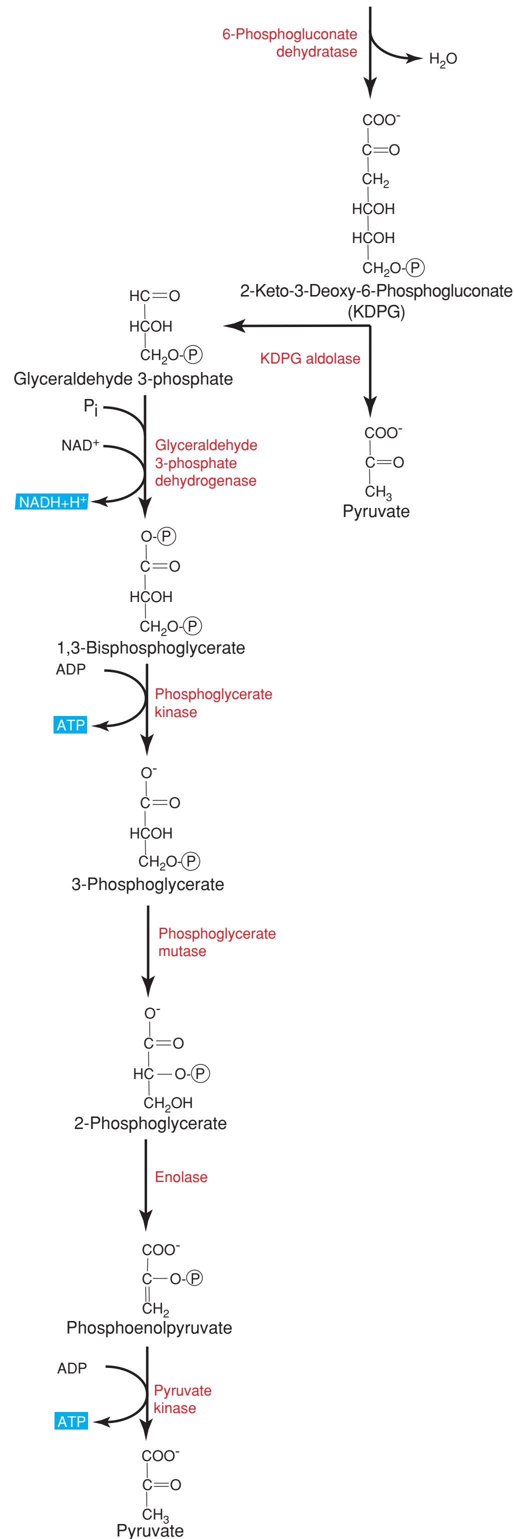


TABLE A-1 Summary of Glycolytic Reactants and Products per Glucose

TABLE OF RESULTS

Reactant	Product
Glucose ($C_6H_{12}O_6$)	2 Pyruvates ($C_3H_3O_3$)
2 ATP	2 ADP
4 ADP	4 ATP
NET: 2 ADP	NET: 2 ATP
2 NAD^+	2 NADH + 2 H^+

TABLE A-2 Summary of Entner-Doudoroff Reactants and Products per Glucose

TABLE OF RESULTS

Reactant	Product
Glucose ($C_6H_{12}O_6$)	2 Pyruvates ($C_3H_3O_3$)
1 ATP	1 ADP
2 ADP	2 ATP
NET: 1 ADP	NET: 1 ATP
1 NAD^+	1 NADH + 1 H^+
1 $NADP^+$	1 NADPH + 1 H^+

The pentose-phosphate reactants and products are listed in Table A-3, and the overall path is shown in Figure A-3. To completely oxidize one hexose to $6CO_2$, a total of six hexoses must enter the cycle as glucose-6-phosphate and follow one of three different routes (notice the symmetry of pathways as drawn). Notice in Figure A-3 that each hexose loses a CO_2 upon entry into the cycle, but at the end, five hexoses are produced. Thus, the net reaction is one hexose being oxidized to $6CO_2$. Notice also the reactions that transfer two-carbon and three-carbon fragments between the five-carbon intermediates. **Transketolase** catalyzes the two-carbon transfer, whereas **transaldolase** catalyzes the three-carbon transfer. Alternatively, the five-carbon intermediates can be redirected into pathways for synthesis of aromatic amino acids and nucleotides (not shown).

TABLE A-3 Summary of Pentose Phosphate Reactants and Products per Glucose-6-phosphate

TABLE OF RESULTS	
Reactant	**Product**
Glucose-6-phosphate (C_6)	6 CO_2 + 1 P_i
12 $NADP^+$	12 NADPH + 12 H^+

Oxidation of Pyruvate: The Krebs Cycle and Fermentation

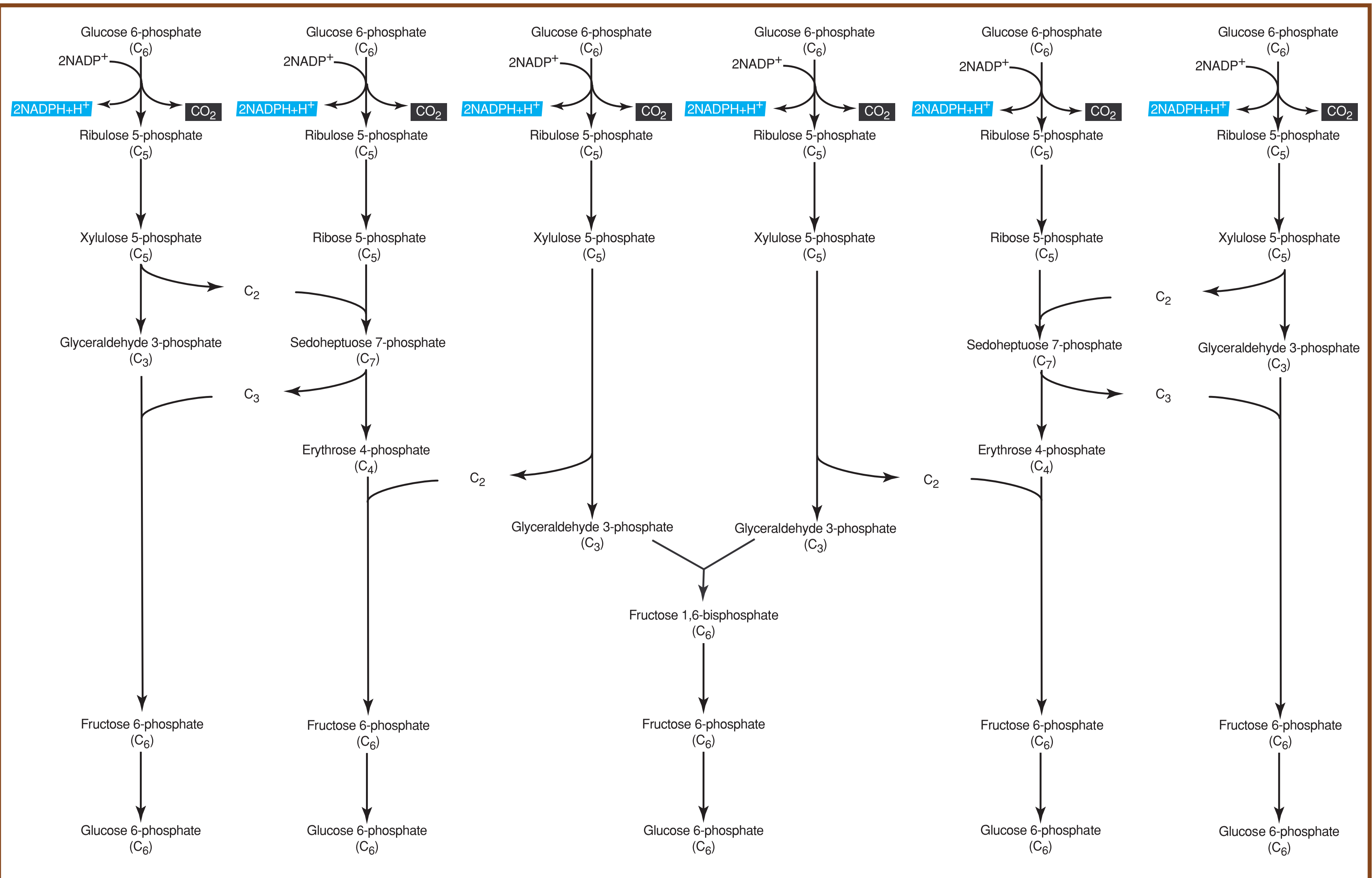
Pyruvate represents a major crossroads in metabolism. Some organisms are able to further disassemble the pyruvates produced in glycolysis and Entner-Doudoroff and make more ATP and NADH + H^+ in the **Krebs cycle**. Other organisms simply reduce the pyruvates with electrons from NADH + H^+ without further energy production in **fermentation**.

The Krebs cycle is a major metabolic pathway used in energy production by organisms that respire aerobically or anaerobically (Figure A-4). Pyruvate produced in glycolysis or other pathways is first converted to acetyl-coenzyme A during the **entry step** (also known as the **intermediate** or **gateway step**). Acetyl-CoA enters the Krebs cycle through a condensation reaction with oxaloacetate. Products for each pyruvate that enters the cycle via the entry step are: 3 CO_2, 4 NADH + H^+, 1 $FADH_2$, and 1 GTP. (Because two pyruvates are made per glucose, these numbers are doubled in Table A-4). The energy released from oxidation of reduced coenzymes (NADH + H^+ and $FADH_2$) in an electron transport chain is then used to make ATP. ATP yields are summarized in Table A-5.

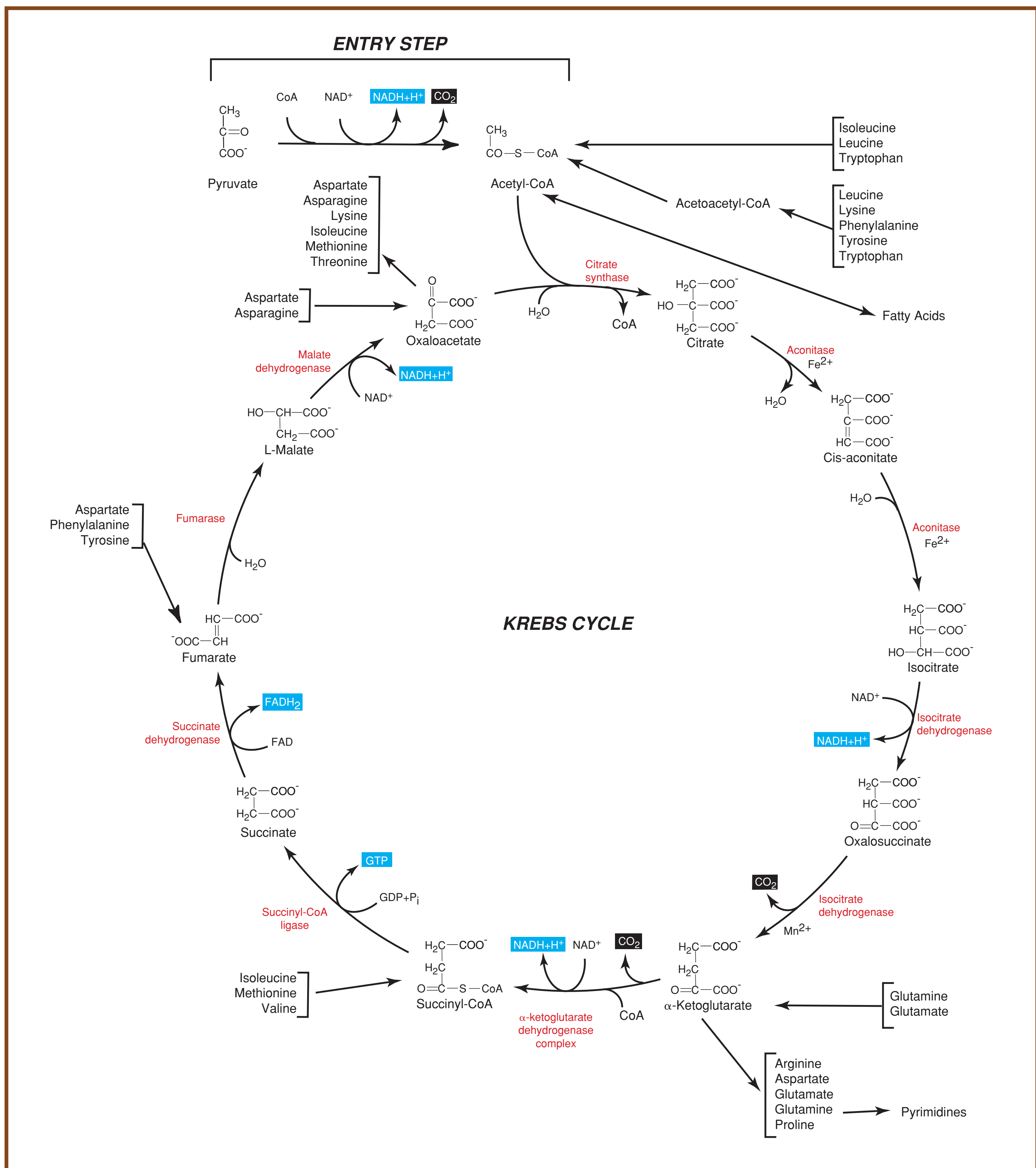
Like glycolysis, many of the Krebs cycle's intermediates are entry points for amino acid, nucleotide and lipid catabolism, as well as a source of carbon skeletons for synthesis of the same compounds. These pathways are shown, but details have been omitted. Single arrows may represent several reactions, and other carbon compounds, not illustrated, may be required to complete a given reaction.

Figure A-5 illustrates some major fermentation pathways exhibited by microbes (though no single organism is capable of all of them). Pyruvate (shown in the blue box) is typically the starting point for each. End products of fermentation are shown in red. Fermentation allows a cell living under anaerobic conditions to oxidize reduced coenzymes (such as NADH + H^+ and shown in blue) generated during glycolysis or other pathways. Some bacteria (aerotolerant anaerobes) rely solely on fermentation and do not use oxygen even if it is available. Table A-6 summarizes major fermentations and some representative organisms that perform each.

Notice that fermentation end products typically fall into three categories: acid, gas, or an organic solvent (an alcohol or a ketone). The specific fermentation performed is the result of the enzymes present in a species and often is used as a basis of classification.



A-3 Pentose-Phosphate Cycle For every six glucose-6-phosphates that enter and complete the cycle, 6CO_2 and 12 NADPH + H^+ are produced. Some of the five-carbon intermediates, however, may be redirected into synthesis of aromatic amino acids and nucleotides. If the cycle is performed as shown, 36 carbons enter as six glucose 6-phosphate ($6 \times C_6 = 36C$). Six CO_2 are immediately lost, leaving a total of 30C to get shuffled around by the remaining reactions to form five glucose 6-phosphates ($5 \times C_6 = 30C$).



A-4 The Entry Step and Krebs Cycle The names of intermediates are printed in black ink; enzymes are in red. Reducing power (in the form of NADH + H^+ and $FADH_2$) and GTP are highlighted in blue. CO_2 produced from the oxidation of carbon is highlighted in black.

TABLE A-4 Summary of Reactants and Products per Glucose in the Entry Step and the Krebs Cycle

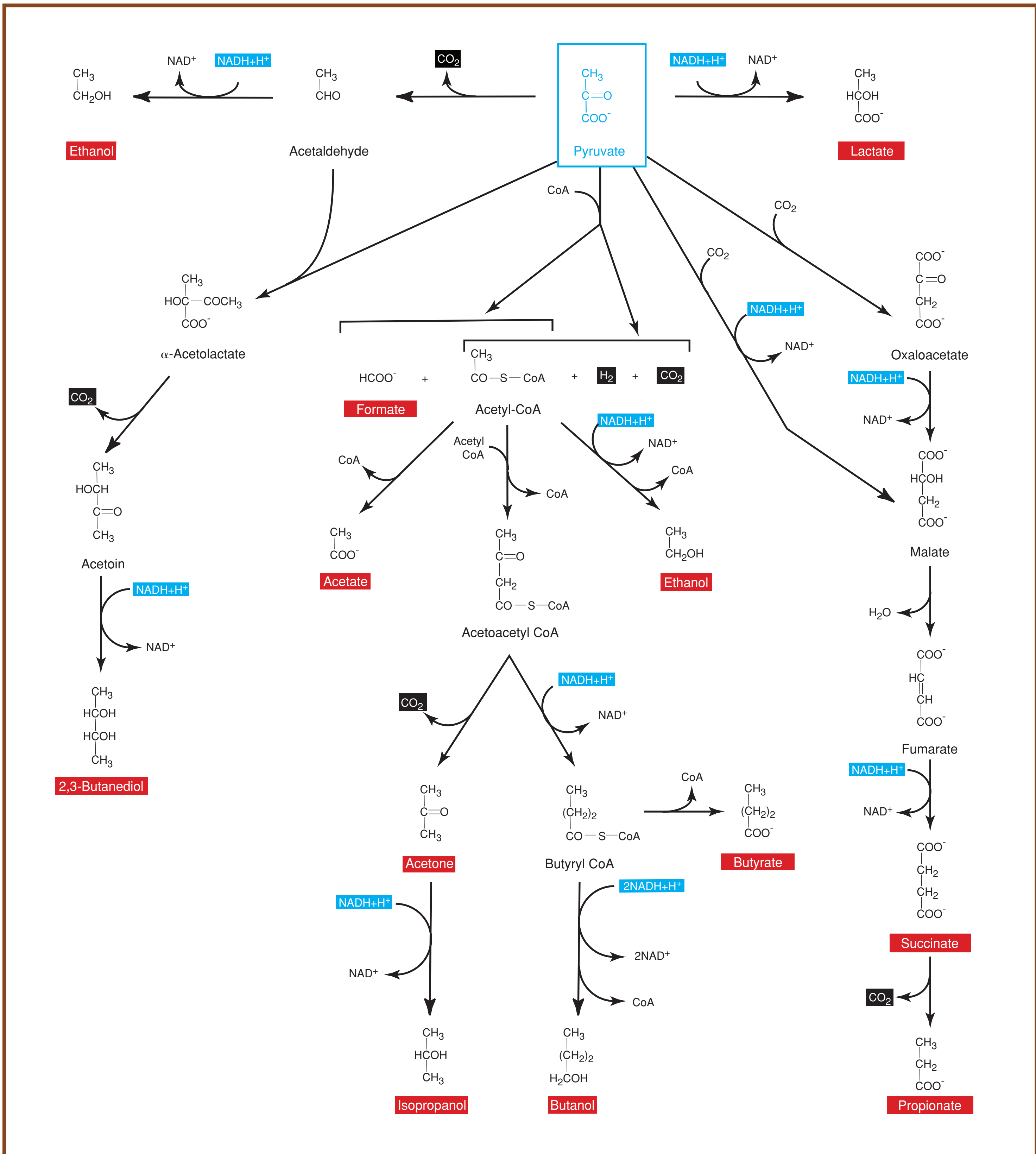
Entry Step		Krebs Cycle	
Reactant	Product	Reactant	Product
2 Pyruvates	2 Acetyl CoA + 2 CO_2	2 Acetyl CoA	4 CO_2
2 Coenzyme A			2 Coenzyme A
2 NAD^+	2 NADH + 2H^+	6 NAD^+	6 NADH + 6H^+
		2 GDP + 2 P_i (= 2 ADP + 2 P_i)	2 GTP (= 2 ATP)

TABLE A-5 ATP Yields from Complete Oxidation of Glucose to CO_2 by a Prokaryote Using Glycolysis, Entry Step, and the Krebs Cycle with O_2 as the Final Electron Acceptor

Compound	Number Produced	ATP Value in the Aerobic ETC	Total ATPs per Glucose
NADH + H^+	10	3	30
$FADH_2$	2	2	4
ATP (by substrate phosphorylation)	4		4

TABLE A-6 Major Fermentations, Their End-Products, and Some Organisms That Performed Them

Fermentation	Major End Products	Representative Organisms
Alcoholic fermentation	Ethanol and CO_2	*Saccharomyces cerevisiae*
Homofermentation	Lactate	*Streptococcus* and some *Lactobacillus*
Heterofermentation	Lactate, ethanol, and acetate	*Streptococcus*, *Leuconostoc*, and *Lactobacillus*
Mixed acid fermentation	Acetate, formate, succinate, CO_2, H_2, and ethanol	*Escherichia*, *Salmonella*, *Klebsiella*, and *Shigella*
2,3-Butanediol fermentation	2,3-Butanediol	*Enterobacter*, *Serratia*, and *Erwinia*
Butyrate/butanol fermentation	Butanol, butyrate, acetone, and isopropanol	*Clostridium*, *Butyrivibrio*, and some *Bacillus*
Propionic acid fermentation	Propionate, acetate and CO_2	*Propionibacterium*, *Veillonella*, and some *Clostridium*



A-5 A Sampling of Fermentation Pathways Note that all pathways start with pyruvate, have a step(s) where NADH+H+ (in blue) is oxidized to NAD+, and produce end-products falling into one of three categories: acid, gas, or alcohol.