Al-Anbar University

College of Sciences

Biology department

Subject name: Microbial Identification

Educational level: Master

Lecture title: Staining of Bacteria

Subject teacher

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Chapter 3

Staining Bacteria

Introduction

Staining bacterial cells for microscopic examination makes it possible to study their unique characteristics, including cell size, shape, arrangement, chemical properties, and structures. You can use these characteristics for bacterial identification. This chapter covers the morphological, differential, and structural stains routinely used in the microbiology laboratory.

Lab Procedure

PREPARING CELLS FOR STAINING

Purpose and Procedure Summary

Bacteria from environmental and clinical sources are cultured using either **agar** or **broth media** (see Chapter 4). After cultivation, bacteria are transferred to a glass slide for staining. However, you must prepare a bacterial **smear** before staining.

A smear is prepared by transferring bacterial cells with a sterile loop from an agar plate or broth tube to a glass slide. If transferred from an agar plate, the cells must be mixed thoroughly into a small drop of water on the slide to create a suspension that is slightly milky in color. The suspension is air dried, and then the cells are adhered to the slide by passing the slide several times over the flame of a Bunsen burner in a process called **heat-fixing.**

Tips for Success

- *Center* the smear on the slide. Central placement will help you locate the smear during staining and microscopic examination.
- Spread the smear over a *dime-sized* area. Such a small area is easier to cover with stain.
- Prepare a smear that consists of a *thin layer* of cells *without clumps*. Stains are unable to penetrate clumps, which also prevent the observation of individual cells.

Expected Results

A good smear preparation, once stained, looks similar to Figure 3.1. Notice that the smear thins out toward the edges. For this reason, microscopic examination should begin in the center of the smear and proceed outward to where there is a thin layer of cells.

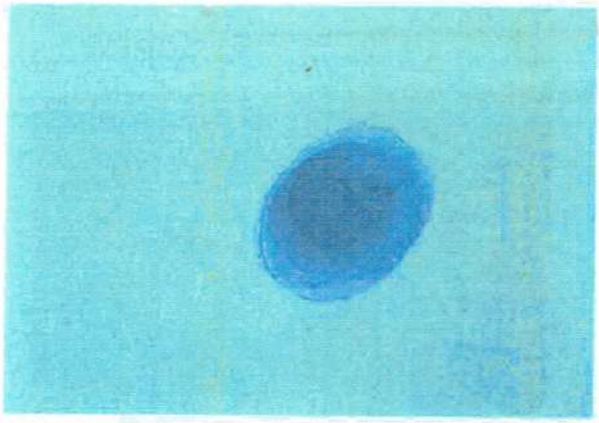

FIGURE 3.1 A smear on a glass slide, after fixing and staining with crystal violet. Notice that the smear thins toward the edge. Microscopic observation should begin in the center of the smear and proceed outward until a thin layer of cells is found.

MORPHOLOGICAL STAINS

Morphological stains color bacterial cells or their background to provide information about cell size, shape, and arrangement. Stains are classified as basic if they carry a positive charge and acidic if they carry a negative charge. **Basic stains**, because they are attracted to the opposite charge, color negatively charged bacterial cells. Basic stains include crystal violet, methylene blue, and safranin. **Acidic stains**, because they are repelled by a like charge, color the background surrounding negatively charged bacterial cells, so you can see the cells in outline. Acidic stains include congo red, nigrosin, and india ink. Both simple and negative stains are used to provide information about the morphology of cells.

Lab Procedure

SIMPLE STAIN

Purpose and Procedure Summary

Simple stains use a single basic stain to color bacterial cells so that their size, shape, and arrangement can be observed. The stain is applied to a fixed smear and then rinsed off with water. After blotting, the stained smear is examined under the microscope.

Tips for Success

- Cover the *entire smear* with stain.
- When rinsing off the stain, direct the stream of water so that it runs *gently* over the smear.
- Rinse the slide until *all excess stain* is removed.
- *Blot* the slide, but *do not wipe.*

Expected Results

The results of staining cells with crystal violet and methylene blue are shown in Figures 3.2 through 3.4. The cells appear the color of the stain, so that their size, shape, and arrangement are apparent.

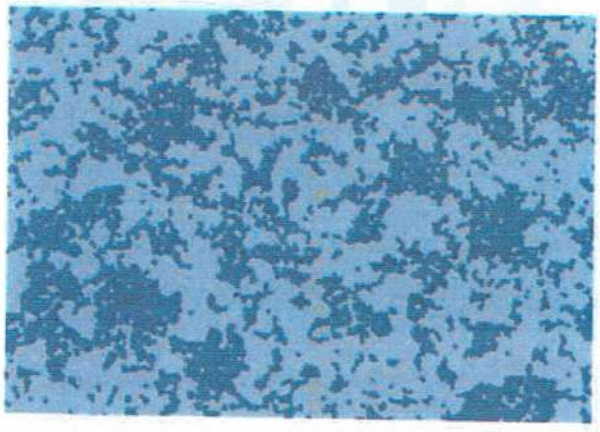

FIGURE 3.2 A crystal violet simple stain of the coccus *Staphylococcus aureus* (2500×).

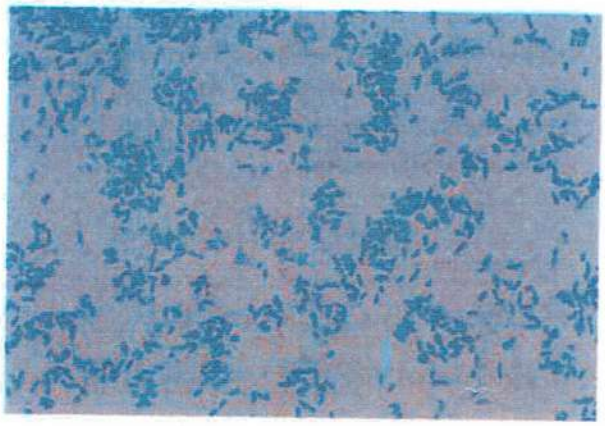

FIGURE 3.3 A methylene blue simple stain of the rod *Escherichia coli* (2500×).

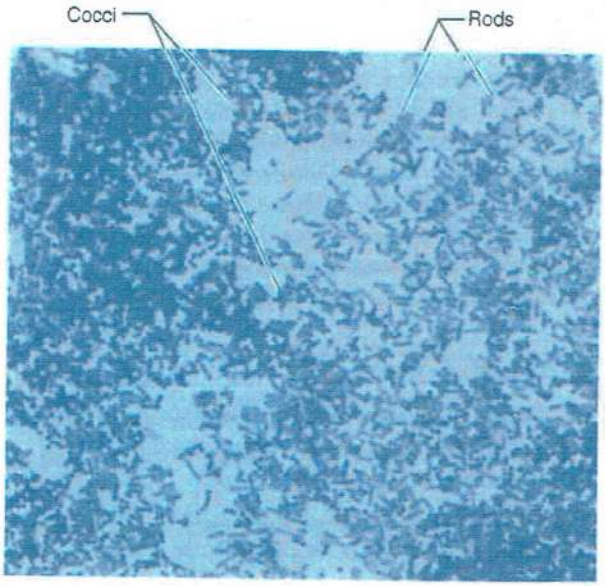

FIGURE 3.4 A methylene blue simple stain of a mixture of the coccus *Staphylococcus aureus* and the rod *Escherichia coli* (2500×).

Lab Procedure

NEGATIVE STAIN

Purpose and Procedure Summary

Negative stains require using a single acidic stain to color the background around cells, so that you can observe their size, shape, and arrangement. Because this procedure does not require heat-fixing or staining the cells, which can cause some cell shrinkage, it provides a more accurate determination of the size and shape of cells. It also allows the microscopic observation of cells that do not readily stain, such as spirilli and spirochetes.

Bacterial cells are transferred to a drop of nigrosin (or other acidic stain) on a glass slide and then mixed into the drop. A second slide is used to spread the drop into a thin film, which is allowed to air-dry before microscopic examination.

Tips for Success

- Use a *clean glass slide.*
- Use only a *small drop* of nigrosin.
- *Pull* the drop across the slide, instead of pushing. Pulling will form a more uniform film.
- Allow the film to *air-dry completely* before viewing.
- *Do not heat-fix* this preparation.
- View the *thinner* portions of the film.

Expected Results

The background of the slide is stained while the cells remain colorless. The result of a negative stain using nigrosin is shown in Figure 3.5.

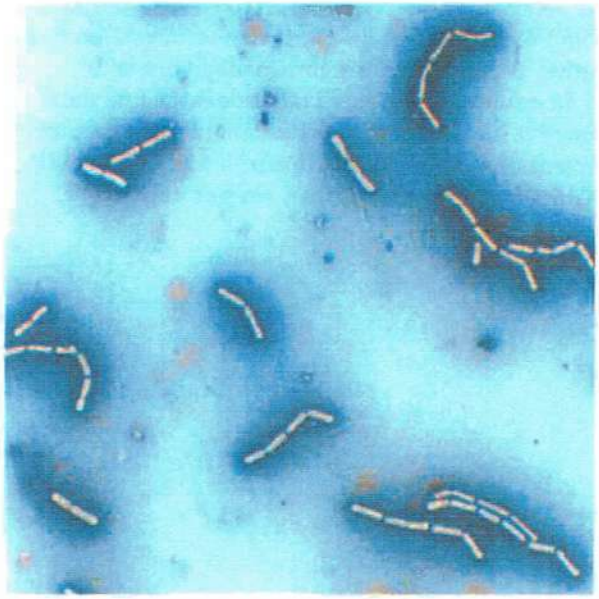

FIGURE 3.5 A negative stain of the rod *Bacillus* (3600×).

DIFFERENTIAL STAINS

Differential stains, such as the **Gram stain** and the **acid-fast stain,** differentiate bacteria based on the chemical composition of their cell wall. Differential stains use two stains instead of one. The first stain is called the **primary stain,** and the second is called the **counterstain.** A decolorization step occurs between application of the primary stain and the counterstain. Depending on the composition of the cell wall, bacteria will either retain the primary stain during decolorization or lose the primary stain and take up the counterstain.

Lab Procedure

GRAM STAIN

Purpose and Procedure Summary

Most bacteria possess a cell wall that contains either a thick **peptidoglycan layer** or a thin peptidoglycan layer with an additional **lipopolysaccharide layer.** This chemical difference is distinguished with the Gram stain. The Gram stain is helpful for distinguishing different bacterial types in a sample and determining their predominance. This stain is used

most often, however, to help identify unknown bacterial isolates from clinical and environmental sources.

The primary stain in the Gram stain is crystal violet, which is applied to the fixed smear and then rinsed off with water. Gram's iodine is then added to the smear and rinsed off with water. The smear is decolorized with 95% ethanol and counterstained with safranin. Bacteria with a thick peptidoglycan layer retain the primary stain, crystal violet, during alcohol decolorization and are referred to as **Gram positive.** Bacteria with a thin peptidoglycan layer and an additional lipopolysaccharide layer lose the primary stain during decolorization and take up the counterstain safranin. These bacteria are referred to as **Gram negative.** The Gram stain not only provides information about Gram reaction, but also about cell size, shape, and arrangement.

Tips for Success

- *Stop* the application of alcohol when it runs clear.
- Run known cultures, or **controls,** with unknowns until consistent results are obtained. Smears of known cultures are available commercially (Figure 3.6) or can be prepared in the laboratory.
- Perform Gram stains only on *24-hour cultures,* because older cultures often yield variable results. For example, cells in a 24-hour culture

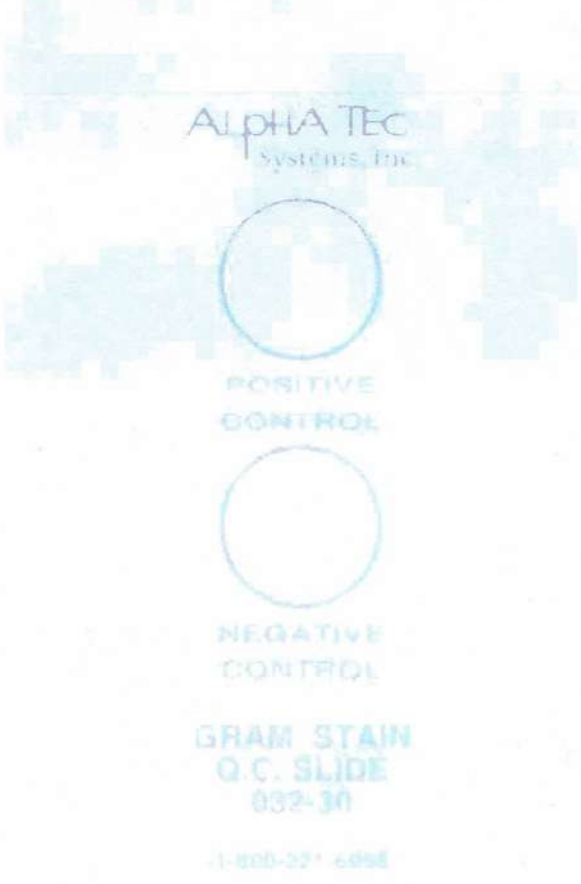

FIGURE 3.6 A Gram stain control slide, with a known Gram-positive organism above and a known Gram-negative organism below.

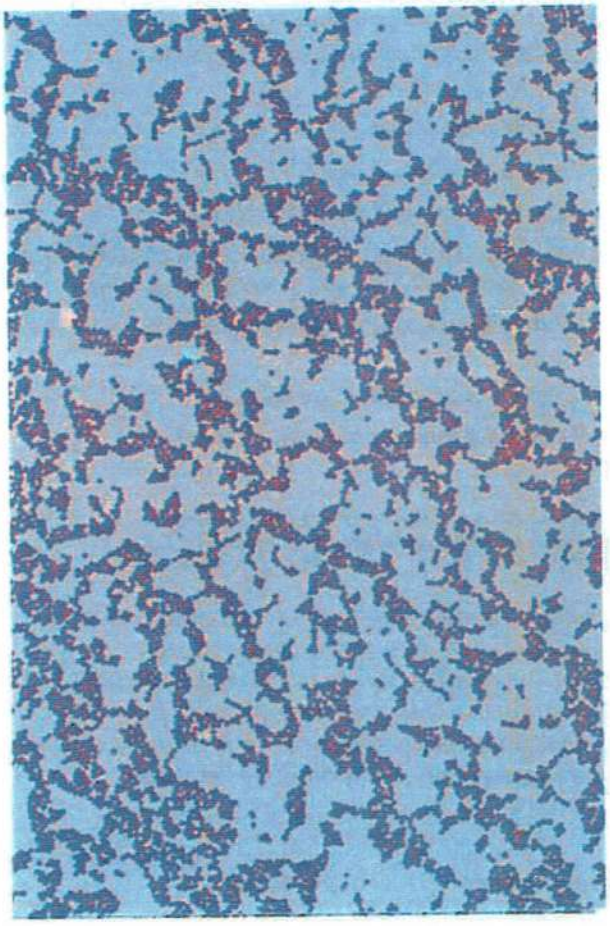

FIGURE 3.7 A Gram stain of a 24-hour culture of *Staphylococcus aureus,* yielding uniform results. All cells are purple, a Gram-positive reaction (3600×).

of *Staphylococcus aureus* uniformly stain Gram positive (Figure 3.7), but some cells in a 48-hour culture stain Gram negative (Figure 3.8). The number of cells that incorrectly stain Gram negative increases in a 72-hour culture (Figure 3.9). Similarly, cells in a 24-hour culture of *Escherichia coli* uniformly stain Gram negative (Figure 3.10), but 48- and 72-hour cultures contain cells that do not pick up enough stain to be seen clearly (Figures 3.11 and 3.12).

Expected Results

Gram-positive bacteria appear purple when viewed under the microscope. Notice in Figure 3.13 that a Gram stain can distinguish Gram-positive bacteria

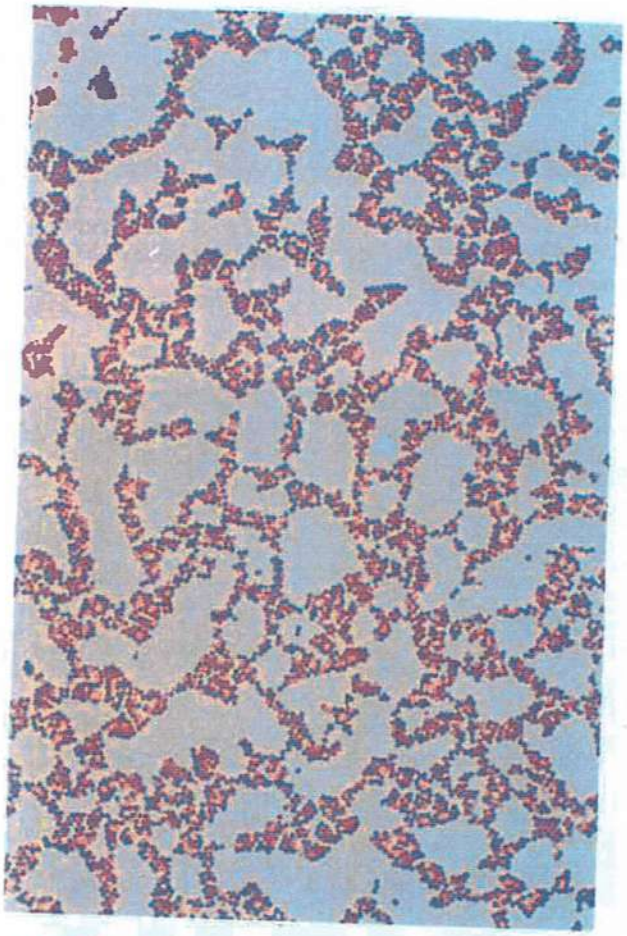

FIGURE 3.8 A Gram stain of a 48-hour culture of *Staphylococcus aureus*. A 48-hour culture does not yield uniform results. Even though most cells are purple, a Gram-positive reaction, some cells are red, a Gram-negative reaction (3600×).

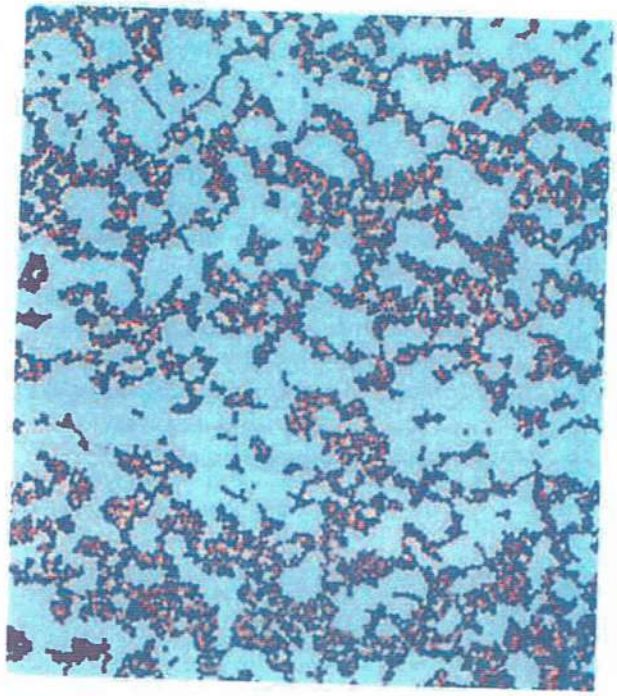

FIGURE 3.9 A Gram stain of a 72-hour culture of *Staphylococcus aureus*. Such older cultures yield highly variable results, with many cells showing a Gram-positive reaction and many a Gram-negative reaction (3600×).

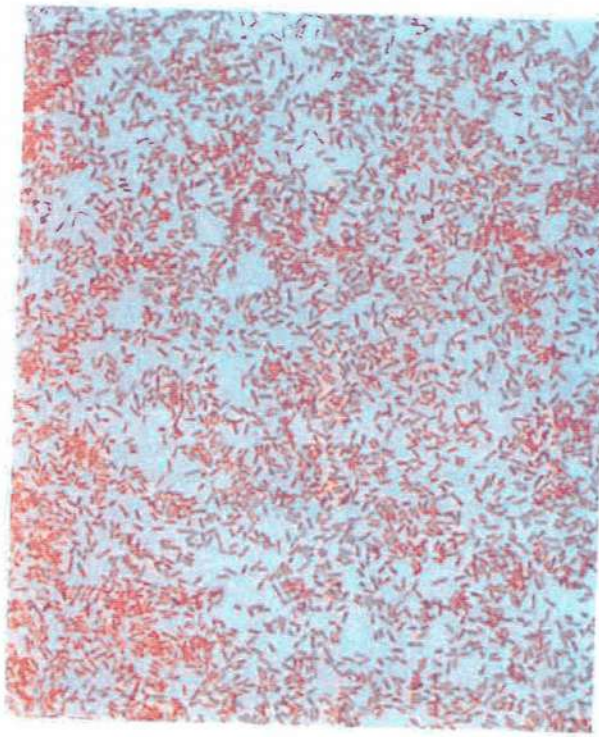

FIGURE 3.10 A Gram stain of a 24-hour culture of *Escherichia coli*, yielding uniform results. All cells are red, a Gram-negative reaction (3600×).

in a mixture of bacterial types. The Gram stain can also detect the predominance of these bacteria in a sample (Figure 3.14). As stated previously, the Gram stain is used most often in bacterial identification. In addition to *Staphylococcus aureus*, other Gram-positive bacteria include *Bacillus cereus* (Figure 3.15), *Enterococcus faecalis* (Figure 3.16), *Micrococcus luteus* (Figure 3.17), and *Staphylococcus epidermidis* (Figure 3.18).

Gram-negative bacteria appear red when viewed under the microscope. In addition to *Escherichia coli*, other Gram-negative bacteria include *Alcaligenes faecalis* (Figure 3.19), *Enterobacter aerogenes* (Figure 3.20), *Pseudomonas aeruginosa* (Figure 3.21), and *Serratia marcescens* (Figure 3.22).

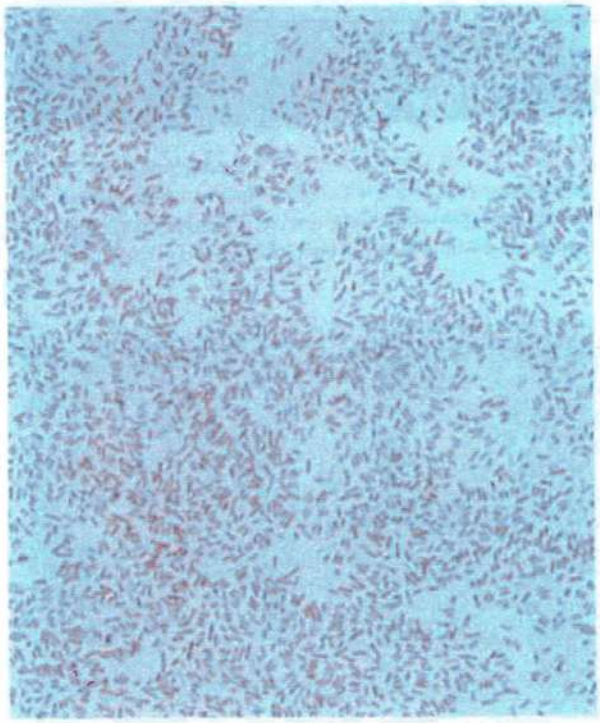

FIGURE 3.11 A Gram stain of a 48-hour culture of *Escherichia coli*. Most cells are red, a Gram-negative reaction, but some cells stain only lightly (3600×).

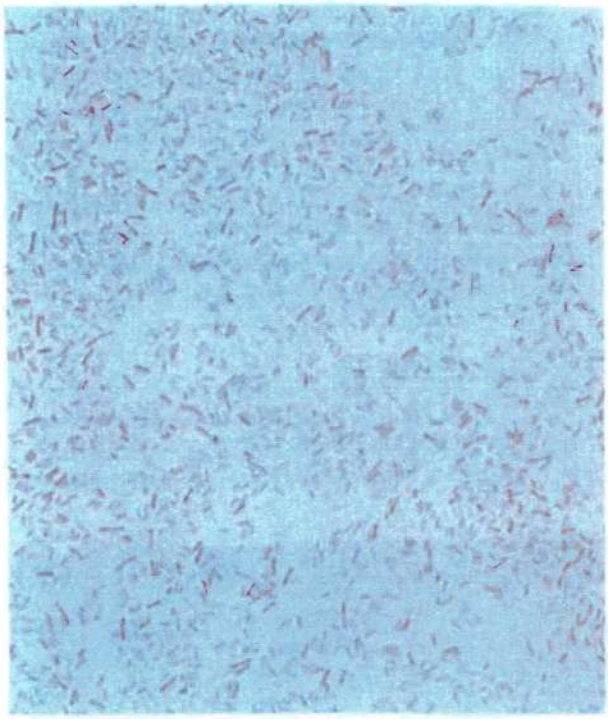

FIGURE 3.12 A Gram stain of a 72-hour culture of *Escherichia coli*. Only a few cells are red, and most cells stain only lightly (3600×).

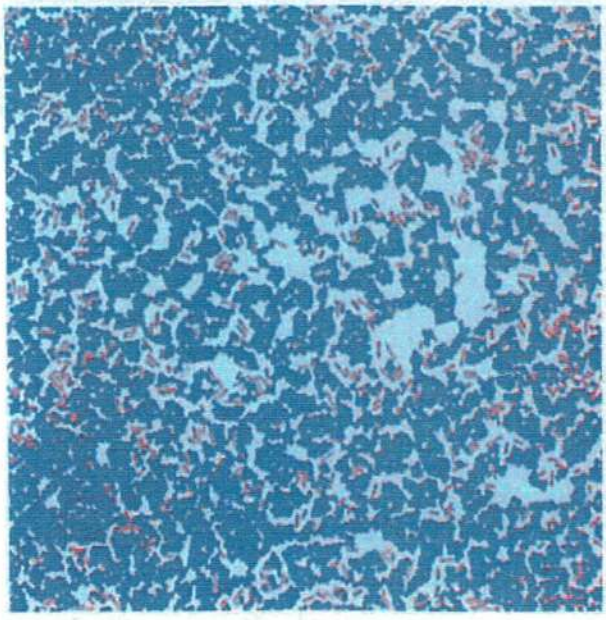

FIGURE 3.13 A Gram stain of a mixture of the Gram-positive coccus *Staphylococcus aureus* and the Gram-negative rod *Escherichia coli* (3600×).

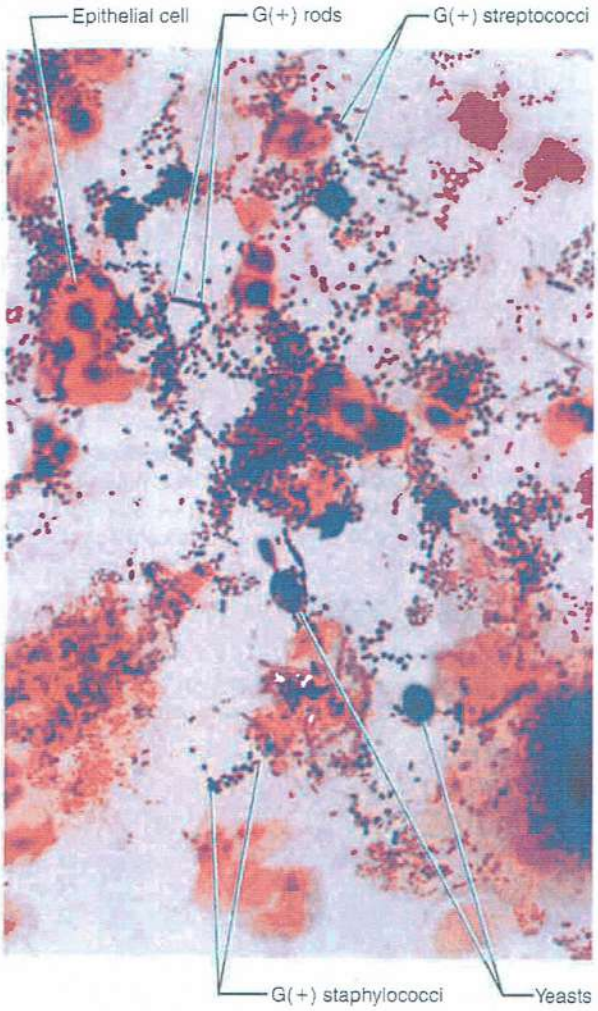

FIGURE 3.14 A Gram stain of teeth scrapings that reveals the predominance of Gram-positive bacteria (3600×).

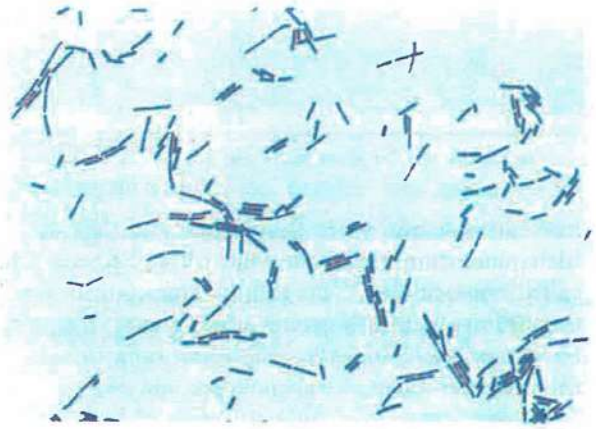

FIGURE 3.15 A Gram stain of *Bacillus cereus*, a Gram-positive rod (2500×).

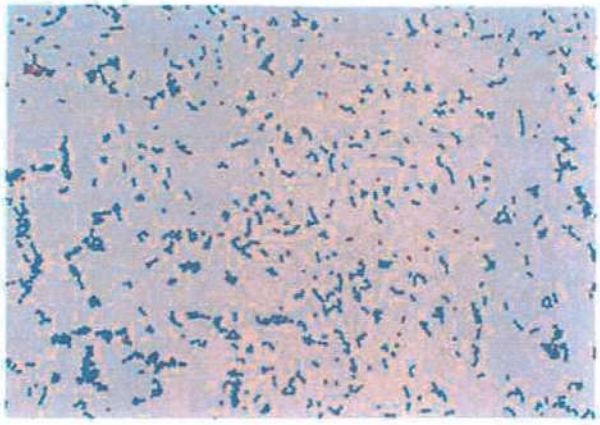

FIGURE 3.16 A Gram stain of *Enterococcus faecalis*, a Gram-positive coccus. Notice the chains of cells, a characteristic of the streptococci (2500×).

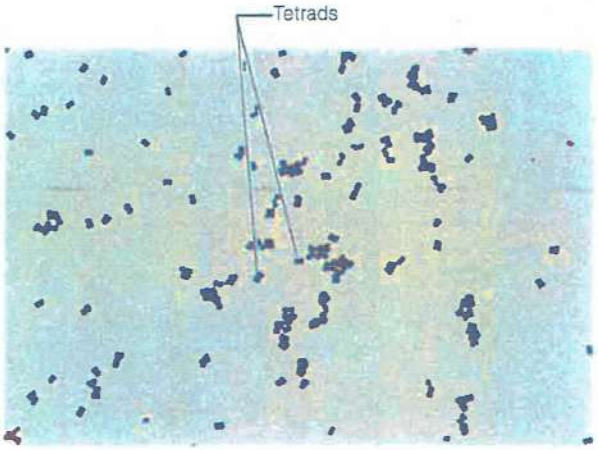

FIGURE 3.17 A Gram stain of *Micrococcus luteus*, a Gram-positive coccus. Notice the tetrads that are characteristic of this organism (2500×).

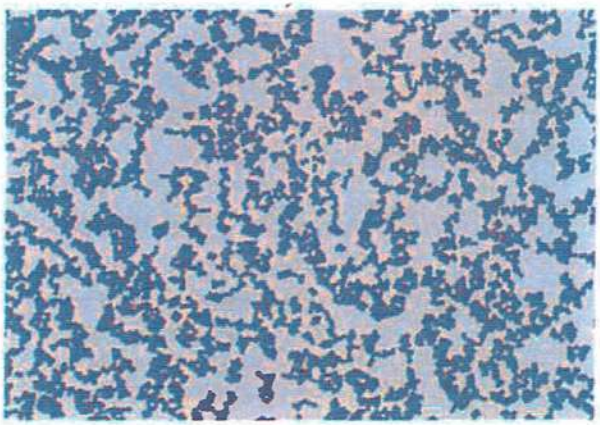

FIGURE 3.18 A Gram stain of *Staphylococcus epidermidis*, a Gram-positive coccus. Notice the clusters of cocci, a characteristic of the staphylococci (2500×).

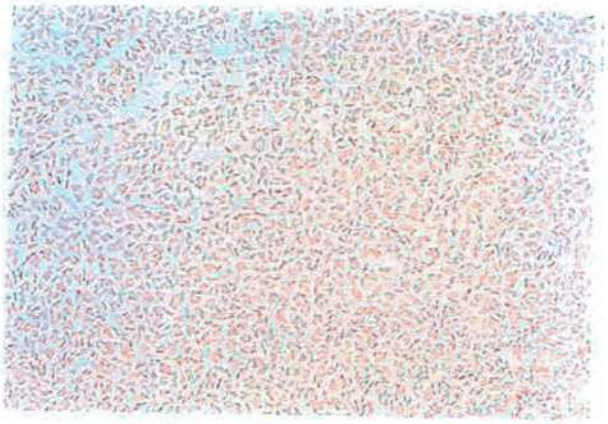

FIGURE 3.19 A Gram stain of *Alcaligenes faecalis*, a Gram-negative rod. Notice that rods are short and of approximately the same length (2500×).

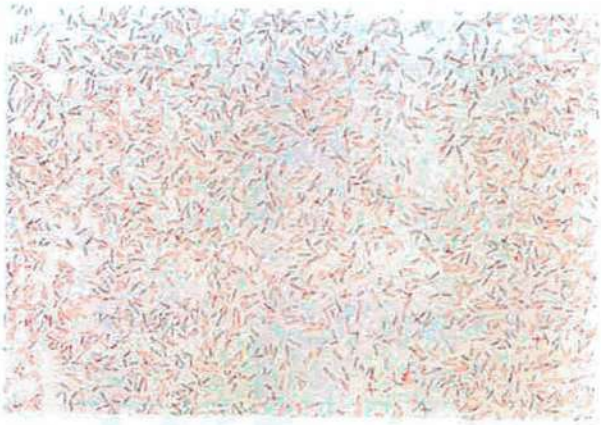

FIGURE 3.21 A Gram stain of *Pseudomonas aeruginosa*, a Gram-negative rod. Notice that most rods are approximately the same length and are 1.5 to 2 times as long as *Alcaligenes faecalis* (see Figure 3.19) (2500×).

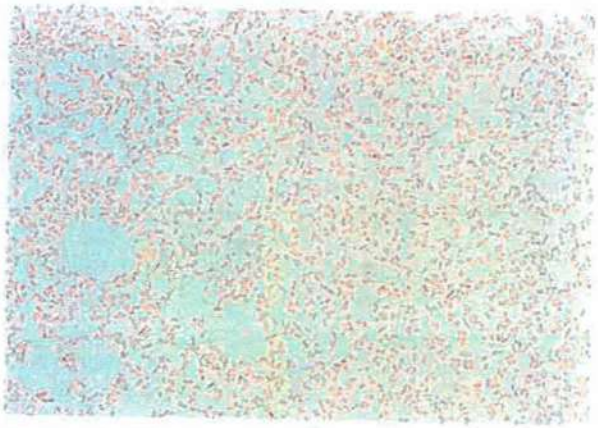

FIGURE 3.20 A Gram stain of *Enterobacter aerogenes*, a Gram-negative rod. Notice that some rods are shorter than others. These short rods should not be mistaken for cocci (2500×).

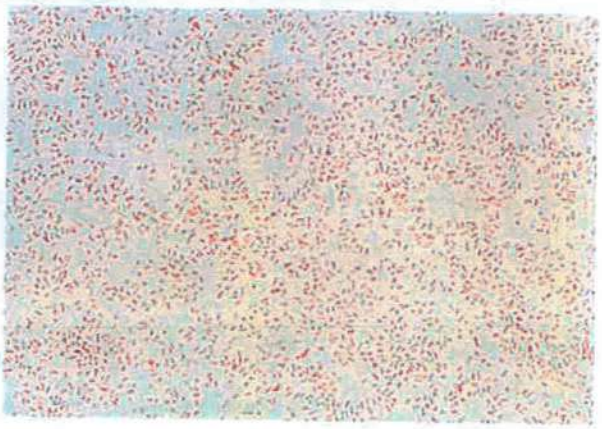

FIGURE 3.22 A Gram stain of *Serratia marcescens*, a Gram-negative rod. These rods are coccobacillus shaped and should not be mistaken for cocci (2500×).

Lab Procedure

ACID-FAST STAIN

Purpose and Procedure Summary

The acid-fast stain distinguishes different types of bacteria based on the wax content of their cell wall. Bacteria with a high wax content, which do not readily stain with the Gram stain, retain the primary stain carbolfuchsin when decolorized with acid-alcohol. These bacteria are called **acid-fast.** Bacteria with a low wax content in their cell wall lose carbolfuchsin when decolorized and take up the counterstain methylene blue. These bacteria are called **non-acid-fast.** This stain is important in distinguishing acid-fast bacteria of the genus *Mycobacterium,* including *Mycobacterium tuberculosis,* the causative agent of tuberculosis, and *Mycobacterium leprae,* the causative agent of leprosy.

The Ziehl-Neelsen acid-fast stain, a widely used version of this procedure, is performed by covering the smear with a piece of blotting paper, which is then soaked with carbolfuchsin. The smear is gently heated over a hot water bath and allowed to cool before removing the paper and rinsing the smear with water. The smear is decolorized with acid-alcohol and counterstained with methylene blue.

Tips for Success

- Mix cells with **sheep serum** or **egg albumin** during smear preparation. This will help cells adhere to the slide.
- *Add more carbolfuchsin* to the smear during heating to prevent drying.
- During decolorization, *stop* the flow of acid-alcohol when it runs clear.

Expected Results

Acid-fast bacteria, such as *Mycobacterium tuberculosis*, appear red when viewed under the microscope (Figure 3.23), while non-acid-fast bacteria appear blue (Figure 3.24).

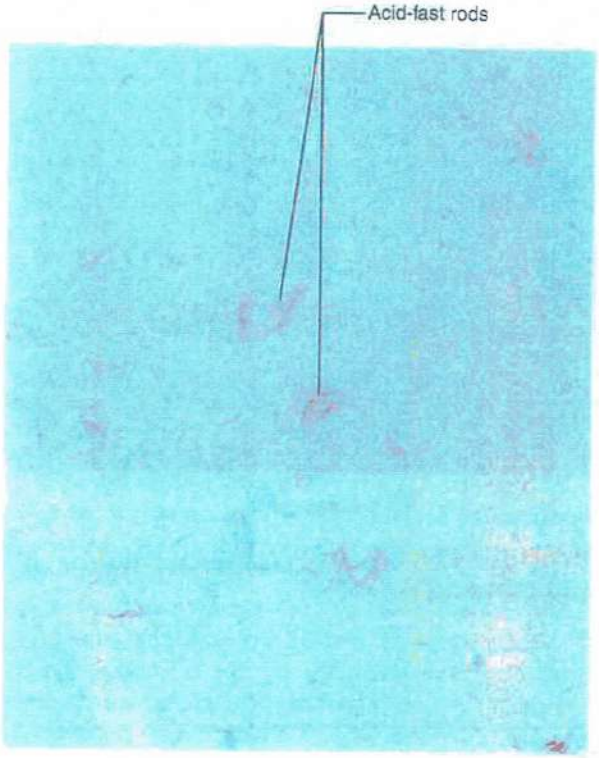

FIGURE 3.23 An acid-fast stain of *Mycobacterium tuberculosis*, an acid-fast rod. Acid-fast rods appear red (3600×).

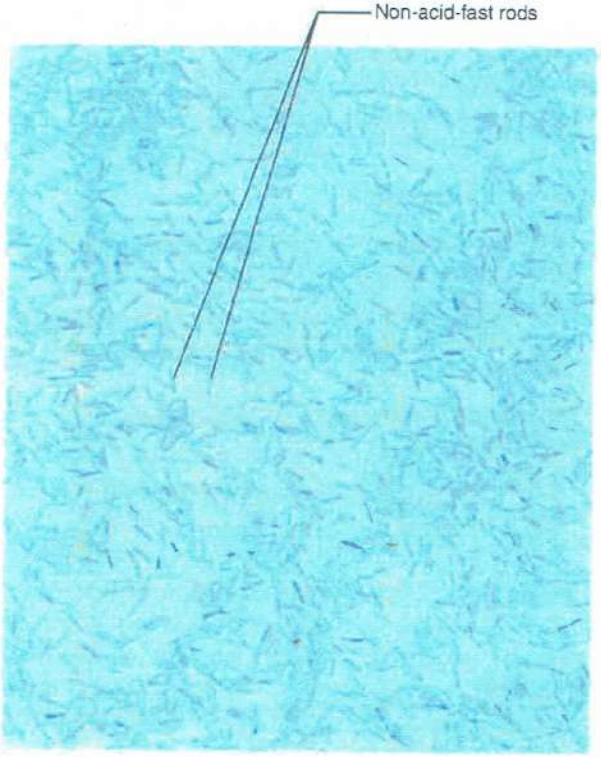

FIGURE 3.24 An acid-fast stain of *Pseudomonas aeruginosa*, a non-acid-fast rod. Non-acid-fast rods appear blue (3600×).

STRUCTURAL STAINS

Some stains are used to observe cell structures: capsules, endospores, and flagella. Detecting these structures in bacteria is an important step in their identification.

Lab Procedure

CAPSULE STAIN

Purpose and Procedure Summary

Some bacteria release polysaccharides and polypeptides during growth. These substances accumulate around the cell to form a structure called a capsule. Some clinically important bacteria form capsules, including *Klebsiella pneumoniae* and *Streptococcus pneumoniae,* causative agents of bacterial pneumonia. In these bacteria, the capsule is considered a virulence factor because it protects the cell from phagocytosis.

Because capsules are resistant to staining, the cell and the background must be stained to view a capsule. This is accomplished by using a combination of negative and simple stains. An acidic stain, such as congo red, nigrosin, or india ink, is used to stain the background, while a basic stain, such as crystal violet or carbolfuchsin, is used to color the cells.

Tips for Success

- Grow the organism on skim milk agar to promote capsule formation.
- *Do not heat* the slide, because heating can cause cell shrinkage and the false appearance of a capsule.
- Rinse *gently* with water so that cells do not wash off the slide.
- *Do not rinse too long,* because capsules are water soluble.

Expected Results

The capsule appears as a halo between the simple-stained cells and the negative-stained background. The capsule of *Klebsiella pneumoniae* is shown in Figure 3.25.

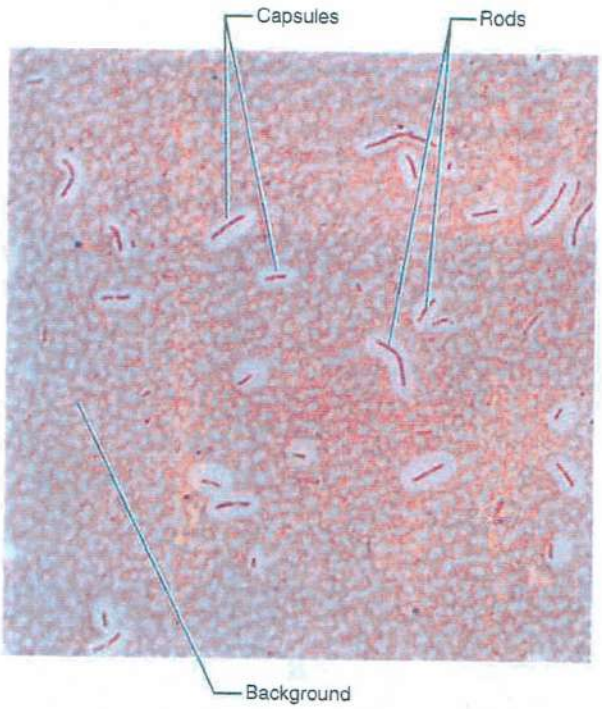

FIGURE 3.25 A capsule stain of *Klebsiella pneumoniae,* an encapsulated rod (3600×).

Lab Procedure

ENDOSPORE STAIN

Purpose and Procedure Summary

During growth, some bacteria form a highly resistant structure called an endospore (Figure 3.26). The endospore is formed within a vegetative cell by a process called sporogenesis and is released upon death of the vegetative cell. A free spore is a highly resistant structure that can survive in environments that are unfavorable for growth. In favorable conditions, a free spore can undergo germination to yield a vegetative cell.

Endospore formation is a useful characteristic in the identification of some bacteria, such as *Bacillus* and *Clostridium.* In these bacteria, spore shape and location vary. For example, the causative agent of anthrax, *Bacillus anthracis,* produces oval-shaped

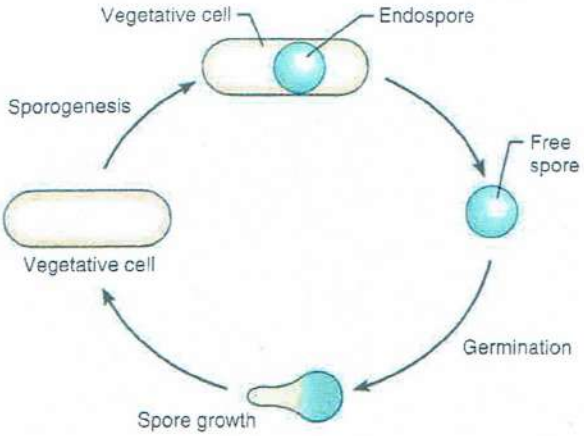

FIGURE 3.26 The life cycle of endospore-forming bacteria.

spores located in the center of the cell, while *Clostridium botulinum*, the causative agent of botulism, produces oval-shaped spores located between the center and the end of the cell. *Clostridium tetani*, the causative agent of tetanus, produces spherical endospores located at the end of the cell.

Due to the unique physical and chemical nature of the spore coat, spores do not pick up basic stains. The outline of unstained spores can be seen within the cells after vegetative cells are stained (Figure 3.27). Spores are best observed, however, after they are colored using a spore stain.

The Schaeffer-Fulton spore stain, a widely used version of this procedure, is performed by covering the smear with blotting paper, which is then soaked with malachite green. The smear is gently heated over a hot water bath and allowed to cool before removing the paper and rinsing the smear with water. Heating drives the malachite green into the endospores, where it is retained even after rinsing with water. The smear is then stained with safranin.

Tips for Success

- *Add more malachite green* to the smear during heating to prevent drying.
- Rinse the smear *thoroughly* with water before adding safranin. This is necessary to wash malachite green out of the vegetative cells, so that they can take up the safranin.

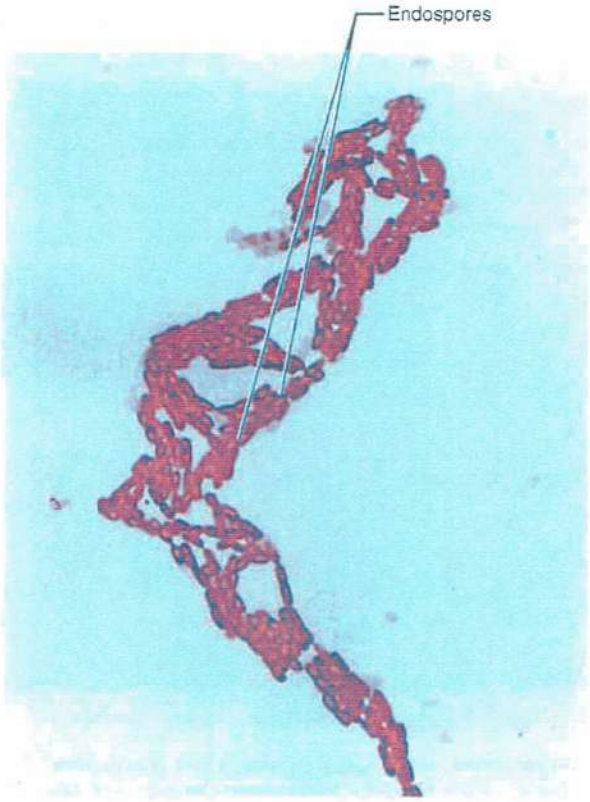

FIGURE 3.27 Endospores in cells of *Bacillus* appear as unstained ovals in the center of stained cells (3600×).

Expected Results

Spores are recognized as spherical or oval-shaped green objects, and their presence indicates endospore-forming bacteria (Figures 3.28 through 3.30). Notice that spores are more prevalent in nutrient-depleted older cultures. For example, compare a 1–2 day culture of *Bacillus cereus* (Figure 3.28) with a 3–4 day culture (Figure 3.29) and a 5–7 day culture (Figure 3.30). The absence of spherical or oval-shaped green objects indicates non-spore-forming bacteria. A spore stain of these bacteria will contain only red vegetative rods (Figure 3.31).

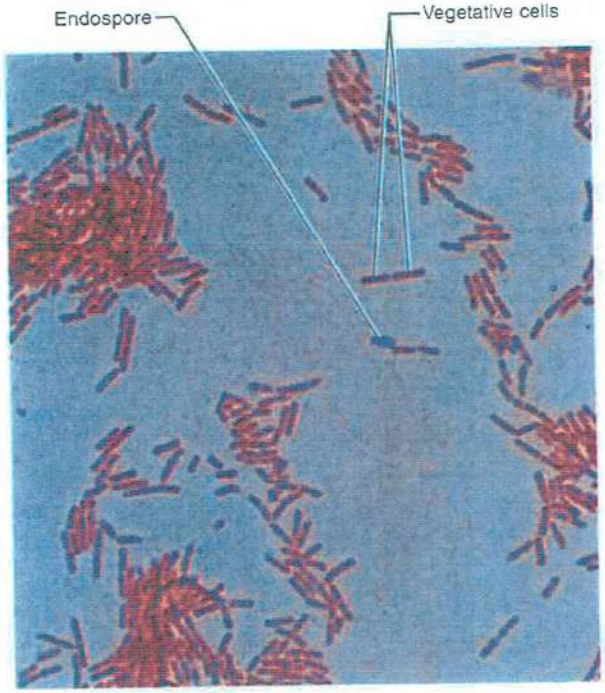

FIGURE 3.28 A spore stain of a 1–2 day culture of *Bacillus cereus,* an endospore-forming bacterium. This young culture contains many vegetative cells, but few endospores (4250×).

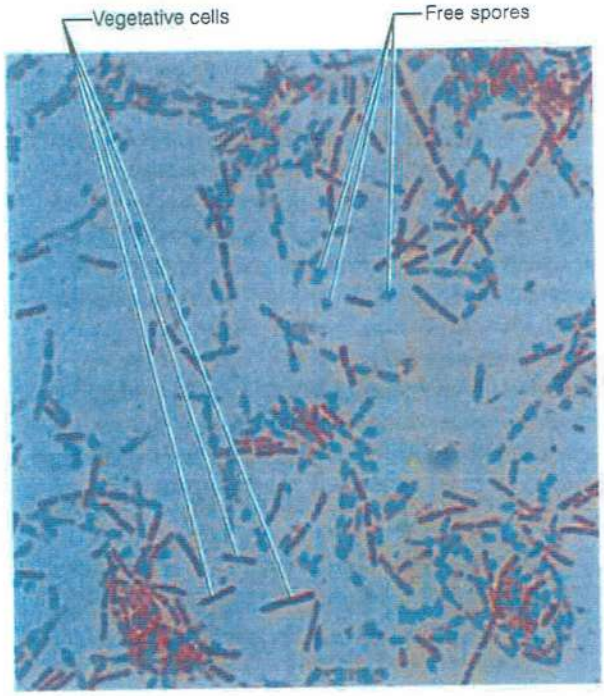

FIGURE 3.29 A spore stain of a 3–4 day culture of *Bacillus cereus.* This older culture still contains many vegetative cells, but many free spores are now present (4250×).

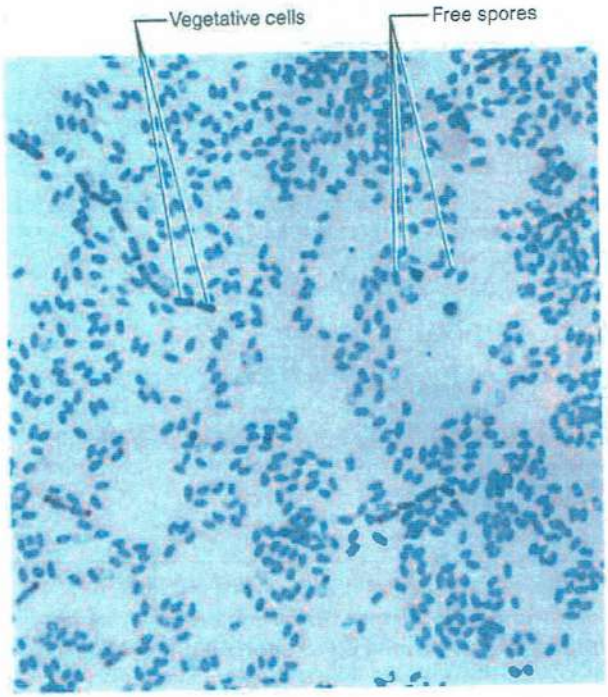

FIGURE 3.30 A spore stain of a 5–7 day culture of *Bacillus cereus.* This old culture contains mostly free spores and few vegetative cells (4250×).

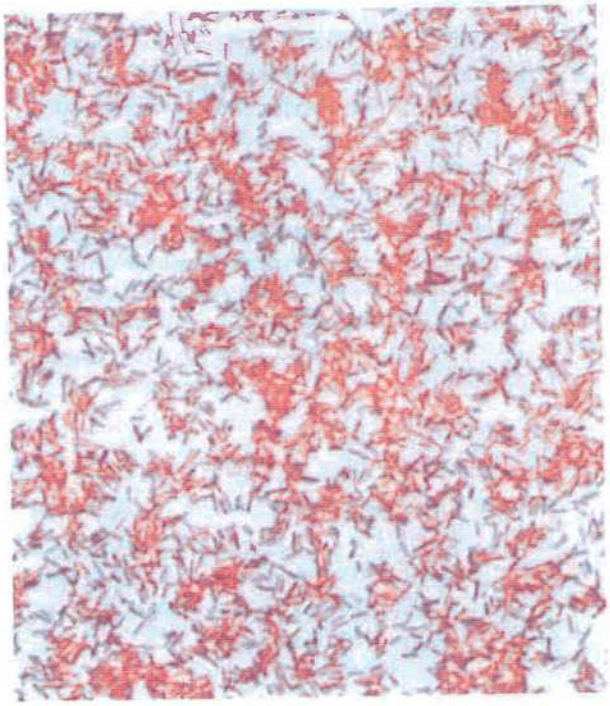

FIGURE 3.31 A spore stain of a culture of *Escherichia coli,* a non-spore-forming bacteria. Non-spore-forming bacteria appear as red rods that lack green spherical or oval-shaped spores (4250×).

Lab Procedure

FLAGELLA STAIN

Purpose and Procedure Summary

Some bacteria possess external whiplike structures called **flagella.** These thin structures can be seen under the light microscope only after a stain clumps around them to increase their diameter.

After bacteria are grown on an agar slant, a broth medium is added to the slant to collect motile cells. The broth is decanted into a centrifuge tube for centrifugation at a slow speed until a visible pellet forms at the bottom. The pellet is resuspended in 10% formalin, and a loopful is transferred to a clean glass slide that is tilted to allow the drop to run down the slide. The suspension is allowed to air-dry before it is covered with pararosaniline stain. After the surface develops a gold film and a precipitate appears throughout, the stain is rinsed off with water.

Tips for Success

- Use *clean* glass slides.
- Perform all steps as *gently* as possible, because flagella are very fragile and easily break off bacterial cells.
- *Search thoroughly* to find cells with suitable flagella. Such cells are more prevalent at the *edges* of the stained area.

Expected Results

Bacterial flagella have four possible arrangements (Figure 3.32) that can be useful for identification. The bacterium *Pseudomonas aeruginosa* has **monotrichous** flagellation (Figure 3.33), *Spirillum volutans* has **amphitrichous** flagella (Figure 3.34), *Pseudomonas marginalis* has **lophotrichous** flagella (Figure 3.35), and *Proteus vulgaris* has **peritrichous** flagella (Figure 3.36).

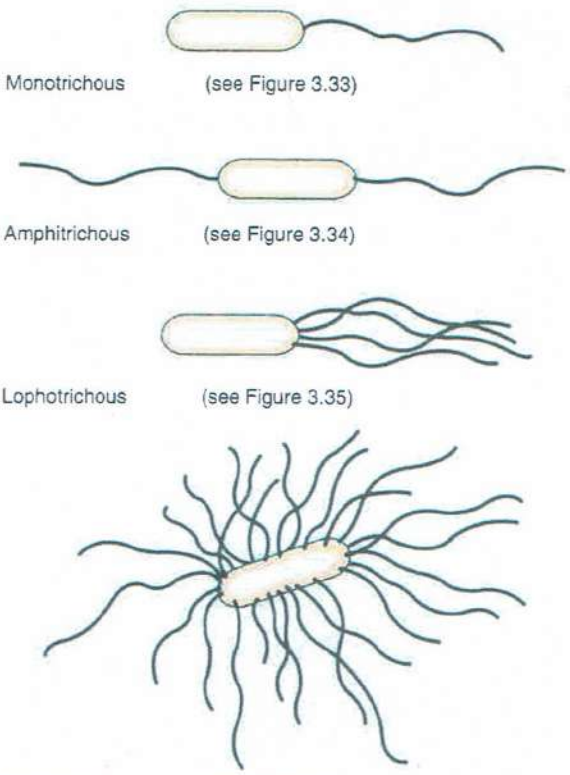

FIGURE 3.32 Flagella arrangements in bacteria. In *monotrichous* flagellation, a single flagellum is located at one end of the cell. In *amphitrichous* flagellation, a single flagellum is located at both ends of the cell. In *lophotrichous* flagellation, many flagella are grouped at one end of the cell. *Peritrichous* flagella are located all around the cell.

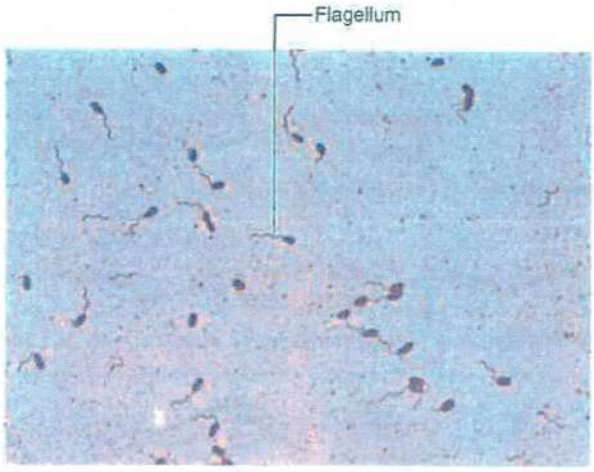

FIGURE 3.33 Monotrichous flagellation in *Pseudomonas aeruginosa* (3600×).

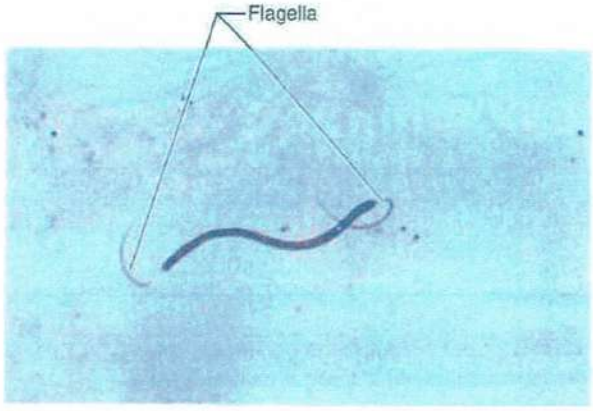

FIGURE 3.34 Amphitrichous flagella of *Spirillum volutans* (3600×).

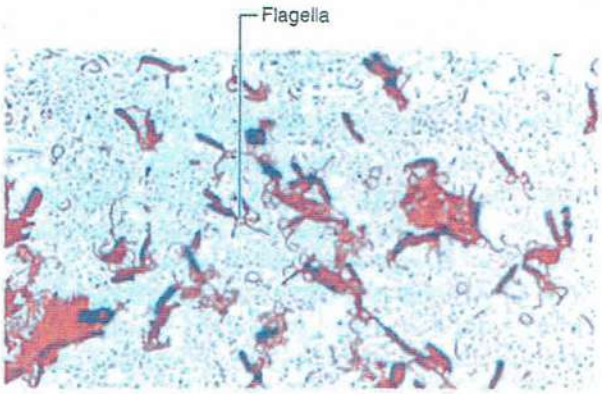

FIGURE 3.35 Lophotrichous flagella of *Pseudomonas marginalis* (3600×).

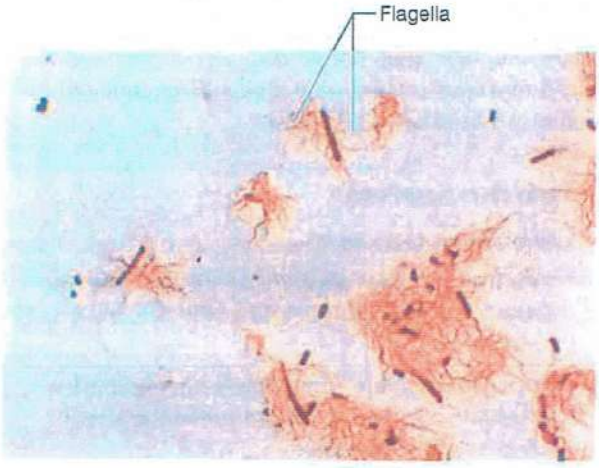

FIGURE 3.36 Peritrichous flagella of *Proteus vulgaris* (3600×).

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(1) Michael J. Leboffe. Burton E. Pierce. (2016) A photographic Atlas for the Microbiology Laboratory. 5th Edition.

(2) FAIRBROTHER, R.W. (2013). **A Textbook of Bacteriology, Fourth Edition.**