

Lab 7: Bacteria

Introduction

As outlined in the lab manual No. 6, eubacteria are the most common and important bacteria in marine systems, both pelagic and benthic. These bacteria can occur in different morphologies, cocci, rods, curved bacteria and spirillae (see fig. 3 in lab manual no. 6). Since bacteria, besides their gross morphology, lack fine structure visible to the light microscope, the classic bacteria taxonomy used cellular characteristics visualized by specific histochemical staining.

The most prominent of these stains is the **Gram stain** developed by the Danish physician Christian Gram in the late 1800's. Differences in the chemical composition of the cell wall cause the cells to be stained differentially when treated with a combination of the dye safranin and an iodine solution. Cells retaining a violet color are called gram-positive and those with pinkish-red color are called gram-negative (Fig. 7).

Some bacteria can be motile, mostly by one to multiple **flagella** (Fig. 1). The flagella are thin proteinaceous structures that originate in the cytoplasm and project out from the cell wall. They are very fragile and not visible with a light microscope. For light microscopic visualization, bacteria flagella are stained by special protocols. The stain coats the flagella, thereby increasing their diameter. The presence and location of flagella are helpful in the identification and classification of bacteria. Flagella are of two main types: **peritrichous** (all around the bacterium) and **polar** (at one or both ends of the cell). Motility of bacteria can be determined by observing wet mounts of live, unstained bacteria.

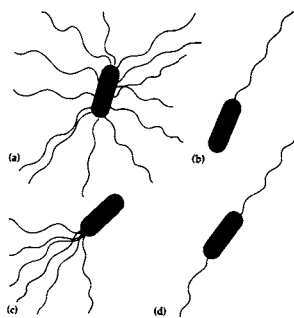


Fig. 1: Flagellar arrangements in bacteria. (a) peritrichous; (b) monotrichous; (c) lophotrichous; (d) amphitrichous.

Several genera of bacteria form **endospores**, the genus *Bacillus* being the most prominent of them. Endospores are called “resting bodies” because they do not metabolize and are resistant to heat, various chemicals, and many harsh environmental conditions. They are formed when essential nutrients or water are not available. Once an endospore forms in a cell, the cell will disintegrate and the endospores can remain dormant for a long period of time to germinate into a new vegetative cell when environmental conditions turn favorable again. The presence and position of an endospore is taxonomically helpful. Endospores are impermeable to most stains, so heat is usually used to drive the stain into the endospore.

Many bacteria secrete chemicals that adhere to their surfaces, forming a viscous coat. This structure is called **capsule** when it is round or oval in shape, and a **slime layer** when it is irregularly shaped and loosely bound to the bacterium. The ability to form a capsule is genetically determined, but the size of the capsule is influenced by the medium on which the bacterium is growing. Most capsules are composed of polysaccharides. Most capsule-staining techniques stain the bacteria and the background, leaving the capsules unstained.

Cocci (singular *coccus*): Almost all of the marine cocci are gram-positive and usually found in irregularly shaped clusters or in groups of eight. In other environments they may form long chains or groups of two or four. None of the cocci are capable of locomotion.

Rods: Rods, or **Bacilli** (singular *bacillus*), occur in both gram-positive and gram-negative forms. Some move by flagella. Some gram-positive rods produce heat-resistant spores. The most common marine bacteria, members of the genus *Pseudomonas*, are flagellated, gram-negative rods. Members of the genus *Photobacterium* are luminescent, exclusively marine gram-negative rods; some are free-swimming, and many occur symbiotically with fish.

Spirillae (singular *spirillum*) are all gram-negative, flagellated bacteria.

Spirochaeta are very long, slender helical cells with thin cell walls. They are quite common in marine mud, where their rapid spiral movements are easily seen under a microscope. They apparently swim by contracting bundles of fibers, known as axial filaments, made of protein threads. Most spirochaetes are anaerobic (live in regions devoid of O₂), either free-living or parasitic. The best-known marine member of this group is the genus *Cristispira* found in the digestive tract of clams and other mollusks.

Lab Work

1. Quantification of bacteria in the field samples

1.a. Total abundance of heterotrophic bacteria by flow cytometry

The total abundance of heterotrophic bacteria in the field samples has been established by flow cytometry by your instructor prior to this class. Bacteria were fixed with formaldehyde (1% final conc.) and stored at 4°C until analysis. For analysis, RNA in the bacteria was digested with RNase for 30 min at 37°C, and bacteria DNA stained by Sybr Green I[®] (Molecular Probes, Eugene, OR). The DNA stain produces a green fluorescence under the blue-light (488 nm) excitation of the flow cytometer, which allows detection of bacteria cells. Sample processing time for each sample and an average count of ca. 10,000 cells is ca. 2 min, as compared to ca. 20 min and 200-500 counted cells by an experienced worker on the epifluorescence microscope. Since the number of counted cells is so much higher on the flow cytometer, the counting error is much lower on the flow cytometer as compared to the microscope.

Your instructor will provide you with a printout of the cytometric analyses showing a typical cytometry two-parameter histogram and the counting results right to it. Include this printout in your final lab report. Discuss the abundance of bacteria and the differences among the stations in relation to factor governing the distribution of bacteria: nutrient concentration and phytoplankton biomass. Does the bacteria abundance follow the abundance of phytoplankton among the four sampled stations? Does the bacteria abundance at the different stations explain the differences in dissolved oxygen (% saturation) that you measured during the field trip (refer to your lab journal for oxygen saturation values)?

1.b. Abundance of cultivable heterotrophic bacteria – plate counts



Fig. 2: Colony counter

The general bacteria nutrient agar plates (yellow) you inoculated in the last class (refer to lab manual no. 6 for details) have been incubated at 37°C for 24 hrs and then stored at 4°C. Take your petri dishes and place them on the colony counter, bottom side up (so you look “through” the bottom). The colony counter (Fig. 2) has a magnifying glass to facilitate recognition of small colonies. Count the colonies on your plate. Use a permanent marker to mark all the colonies you have counted to avoid confusion as to which colonies are counted and which are not yet counted. Convert your colony counts to bacterial abundance. Remember that each colony is assumed to have originated from one single bacterial cell. Thus, the number of colonies on your plate represents the number of cultivable bacteria in 100 μl of your field sample. Multiply your colony count by 10 to convert to bacteria cells ml^{-1} .

Report your final abundance of cultivable bacteria to the spreadsheet on the central lab computer or the blackboard. For your final lab report, discuss the differences in cultivable bacteria among the sampled station. How do the plate counts relate to the total number of bacteria estimated by flow cytometry? Which percentage of the total bacteria is cultivable on the provided general bacteria growth medium?

1.c. Abundance of coliform bacteria – plate counts

The coliform bacteria plates (Hektoen Enteric Agar, Levine Eosin Methylene Blue Agar) have been incubated at 37°C for 48 hrs and then stored at 4°C. Coliforms growing on EMB Agar will appear as shiny dark-green colonies, those growing on Hektoen Enteric Agar will appear bright orange.

Count your colonies using the colony counter, and convert your coliform bacteria numbers to cells ml^{-1} by multiplying by 10. Report your coliform bacteria abundance (average of the two plates) for each medium to the spreadsheet on the central lab computer or blackboard.

Did you grow any coliform bacteria on your plates? What does the number of coliforms (cells ml^{-1}) tell about the water quality at the different field stations? Refer to the Introduction section of lab manual no. 6 for coliform cell numbers in relation to recreational and mussel harvesting waters. Does the distribution of coliform bacteria concur with your expectations? Discuss these questions in your final lab report.

2. Differential and cytological staining of bacteria

2.a. General bacteria staining

To practice the bacteria smear preparations, we will start with a general bacteria stain by methylene blue and crystal violet. We will use the colonies on your general nutrient agar plates as material for this and the subsequent staining preparations.

From your plate, select four different, distinct colonies of larger size. Each group member will make the preparations for one of these four colonies. Before you start any preparation, inspect your colony and describe its shape (Fig. 3), size, and color. Note your observations in your lab journal.

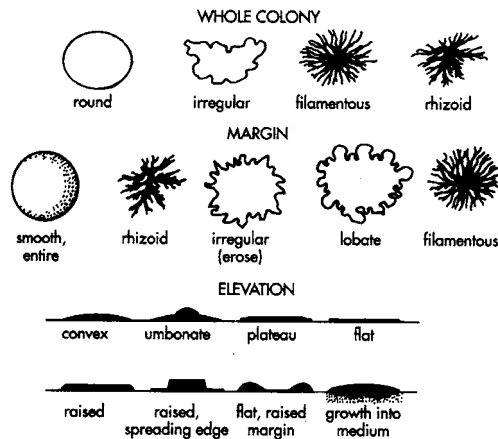


Fig. 3: Bacterial colony features. Use these terms to describe colonial morphology. Color, surface characteristics (dull, shiny), consistency (dry, buttery, moist) and optical properties (opaque, translucent) can be included.

To prepare a **bacteria smear**, follow the below protocol (Fig. 4):

1. Get a microscope slide from the supply box and clean it with detergent, rinse with water and dry it
2. With a fresh toothpick, apply a small droplet of sterile seawater to the center of the slide; keep the droplet small, otherwise air drying will take too long
3. From your plate, pick one colony with a toothpick and smear bacteria into the water droplet
4. Allow the smear to air dry (Never use an air jet; this will make an aerosol and distribute bacteria into the lab air, which can be hazardous when working with pathogenic bacteria; the air from air jets are also contaminated with other bacteria)
5. Pass the dry slide slowly through the Bunsen burner flame three times. The cells should be heat-killed, but not incinerated. So don't leave the slide within the flame too long. The temperature should rise to 70°C – the slide should be hot, but still touchable

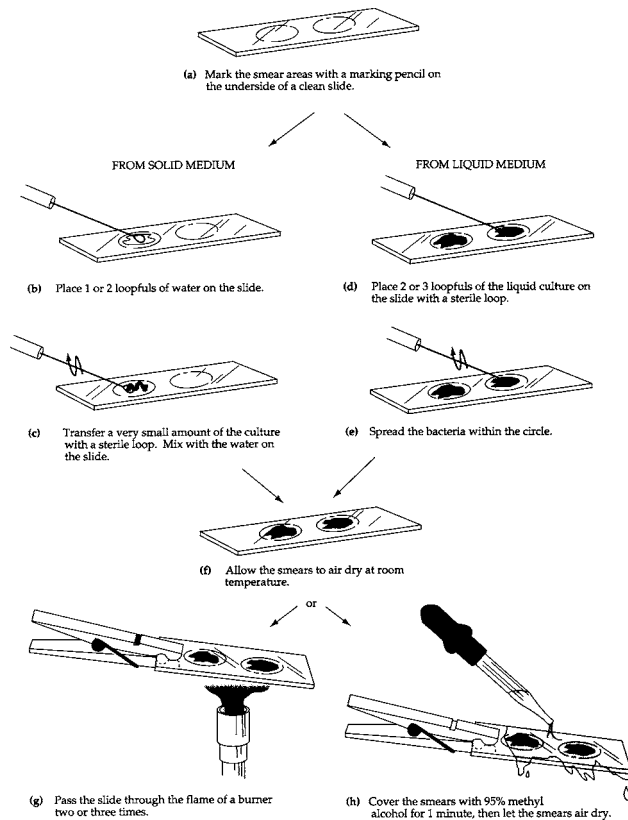


Fig. 4: Preparing a bacterial smear

We will then prepare a general bacteria stain with methylene blue and crystal violet. Half of your group members will use methylene blue, the other half crystal violet. Follow the protocol below (Fig. 5):

1. Cover the smear with stain; crystal violet for 10 sec, methylene blue for 30 sec; hold slide over a big beaker to let all dye (and water) run into the beaker
2. Gently wash of the dye with distilled water. Do **not** squirt the water directly over the bacteria smear but let it run from above over the smear
3. Blot dry the preparation with tissue but

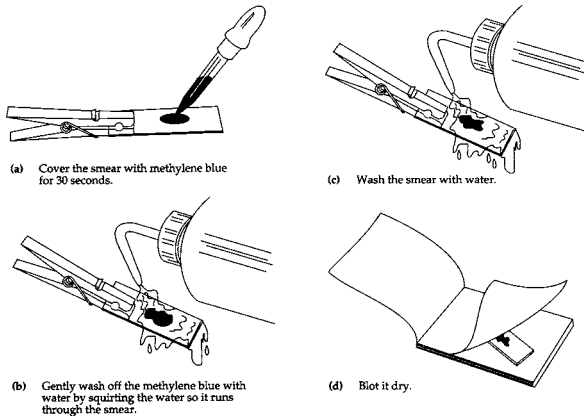


Fig. 5: Simple bacteria staining by methylene blue

sample from. Do you have only one type of bacteria in your preparation? Remember that all colonies are supposed to have arisen from one cell; if this assumption holds true, your preparation should, in fact, display only one type of bacteria.

2.b. Gram stain

Each group will examine a minimum of four colonies for the general nutrient agar plates of their field station on gram-positive and gram-negative bacteria. This will leave each group member to select one colony and prepare a Gram stain preparation. Select a colony from your plate and note the physical colony description in your lab journal. Then prepare a bacteria smear as described above (Fig. 4). Perform the Gram stain as follows (Fig. 6):

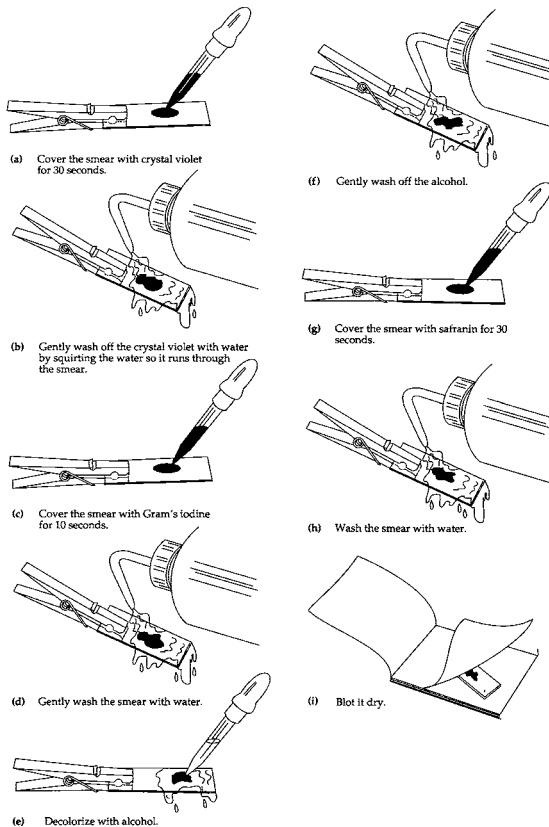


Fig. 6: Bacteria Gram Stain

- do not** rub the preparation
- 4. Air dry the preparation. The slide must be completely dry before putting it on your microscope
- 5. Cover the preparation with immersion oil. Observe first with low power (20× objective) to locate a good view field. Add a drop of immersion oil and swing the 100× oil immersion objective lens into the oil. Use only **fine focus** to bring image into focus.

Describe the bacteria that you see in your preparation (form, size, single vs. aggregates) and add your description to the macroscopic description of the plate colony you took the

1. Stain the smear with crystal violet for 30 sec to 1 min
2. Rinse with water; do not squirt water directly on the smear but let water run over smear
3. Stain with Gram's iodine solution for 10 sec to 1 min
4. Rinse with water
5. Decolorize with 95% ethyl alcohol by letting alcohol run over smear until no large amounts of purple wash out (usually 10-20 sec). *Do not over-decolorize!*
6. Immediately wash with distilled water
7. Stain with safranin for 30 sec
8. Wash with water
9. Blot dry preparation, then air dry to total dryness before putting under the microscope
10. Cover the preparation with immersion oil. Observe first with low power (20× objective) to locate a good view field. Add a drop of immersion oil and swing the 100× oil immersion objective lens into the oil. Use only **fine focus** to bring image into focus.

Describe the bacteria that you see in your preparation (form, size, single vs. aggregates) and add your description to the macroscopic

description of the plate colony you took the sample from. Are your bacteria gram-positive or negative (refer to Fig. 7). Collect the information from your co-workers in your group and report how many of your studied colonies are gram-positive or gram-negative and include the morphology of these cells in your report.

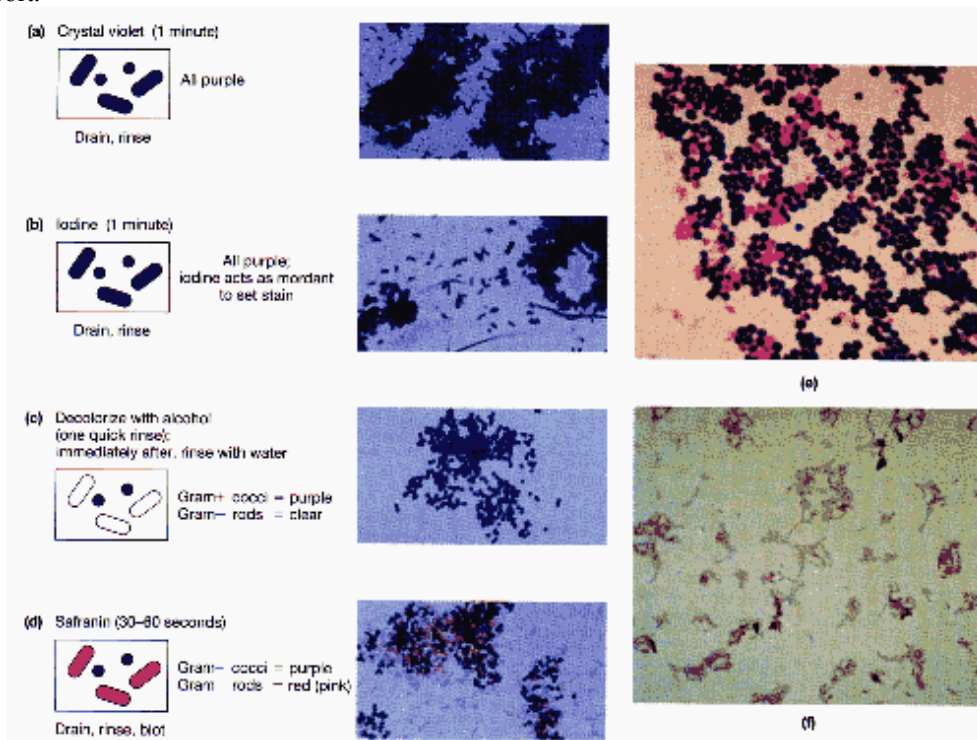
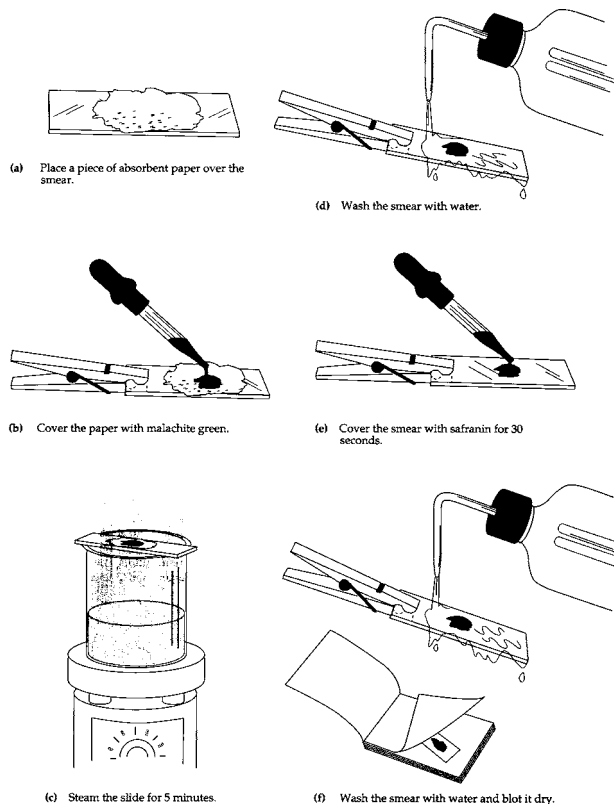


Fig. 7: The Gram Stain. (a) – (d) Steps in the Gram staining. (e) Gram-positive cells retain the purple color of crystal violet, whereas (f) gram-negative cells are decolorized by alcohol and subsequently pick up the red color of the safranin counterstain (from JG Black: Microbiology, 4th ed. 1999; John Wiley & Sons, Inc.).



2.c. Endospore stain

If you discover colonies on your plates that are formed by *Bacillus*-type, rod-shaped cells, you can further study them by the endospore staining. In good Gram stain preparations you can get a first impression on whether your rods may have endospores. Endospores are refractive to most staining procedures and appear as clear areas in the Gram stain. Poly-beta hydroxybutyrate (bacterial fat) and other intracellular inclusions can also not stain in the Gram stain and appear as clear areas, though. The spore stain allows you to clearly demonstrate the presence of endospores. The malachite green stain is driven into the endospores by heat in this procedure (Fig. 8).

1. Prepare your heat-fixed bacteria smear
2. Cut a piece of paper towel that is smaller than the microscope slide
3. Cover the bacteria smear with the cut paper towel

Fig. 8: Endospore staining

4. Cover the piece of paper towel with malachite green
5. Steam the slide for 5 min over a beaker with boiling water. **Do not** let the stain dry on the slide; to prevent, add stain when it gets close to drying out
6. Allow slide to cool; remove the paper towel and put it in the waste bucket (not the sink!)
7. Rinse smear with water
8. Counterstain with safranin for 30 sec
9. Rinse with water
10. Blot dry, then air dry preparation to total dryness before putting under the microscope
11. Cover the preparation with immersion oil. Observe first with low power (20× objective) to locate a good view field. Add a drop of immersion oil and swing the 100× oil immersion objective lens into the oil. Use only **fine focus** to bring image into focus.

2.d. Flagella stain

Scan your colonies on your general nutrient agar plates for motile forms by putting small amounts of unstained colony into a small drop of sterile seawater using a toothpick. Cover with a cover slip and put under the microscope. Observe first with low power (20× objective) to locate a good view field. Add a drop of immersion oil and swing the 100× oil immersion objective lens into the oil. Use only **fine focus** to bring image into focus. Motile bacteria possessing flagella should be easily detectable by their movement through the preparation. Do not confuse this active motion by the small, undirected movement (“wobbling”) of bacteria and other small particles in suspension by Brownian molecular motion. If you find colonies that host motile bacteria, attempt the flagella staining according to the below protocol. Flagella staining is the most difficult histochemical staining and usually requires some amount of practice. Also note that no heat-fixation is used when staining bacteria flagella!

1. Prepare a thoroughly cleaned and well dried microscope slide
2. With forceps and a scalpel, cut out the colony in which you detected motile bacteria
3. Gently touch the culture side of the agar piece to a clean microscope slide
4. Allow the imprint to air-dry. **Do not heat-fix!**
5. Flood the dried bacteria film with pararosaniline (basic fuchsin) stain until a golden film with precipitate forms. This may take up to 15 min.
6. Rinse very gently with water
7. Air dry preparation, do not blot dry!
8. Cover dry preparation with immersion oil. Observe first with low power (20× objective) to locate a good view field. Add a drop of immersion oil and swing the 100× oil immersion objective lens into the oil. Use only **fine focus** to bring image into focus.

Describe the cell form and number of flagella that you observe in your preparation. Prepare a drawing of your cells to document staining results in your lab report.

2.e. Capsule stain

The capsule staining is a negative staining in which the background, not the cells, are stained. In some protocols, the bacteria cells can be counterstained, leaving a colored background, stained bacteria cells, and an uncolored area around the cells, which represents the capsule.

Capsule preparations are not heat-fixed and no bacteria smear is produced. Instead, the stain and cell suspension is pulled across a glass slide (Fig. 9). The result is a (perfectly) one cell thick layer. Note that by pulling one slide over the other as depicted in fig. 9, the suspension is not pushed but pulled.



Fig. 9: Preparing a thin layer of cell suspension by two microscope slides for capsule staining.

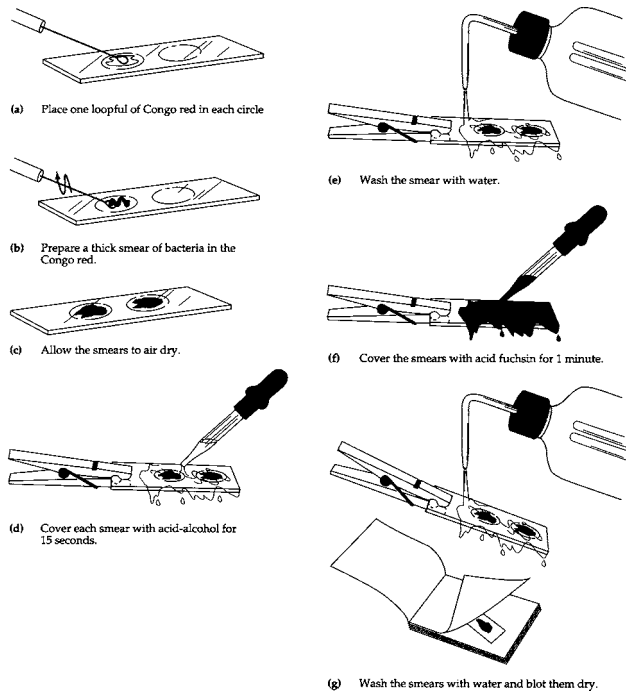


Fig. 10: Negative capsule staining

1. Bring a small drop of congo red to one side of the microscope slide
2. With a toothpick, pick part of the colony from your agar plate (use the colonies that you already used part of for Gram- and endospore staining) and dispense in the congo red solution
3. Touch the congo red cell suspension with the short side of a second microscope slide as depicted in fig. 9. Draw the suspension across the slide in one clean motion
4. Let smear air dry to total dryness
5. Cover dry smear with acid alcohol for 15 sec
6. Wash gently with water
7. Stain with acid fuchsin for 1 min
8. Wash with water
9. Blot dry, then air dry preparation
10. Cover dry preparation with immersion oil. Observe first with low power (20× objective) to locate a good view field. Add a drop of immersion oil and swing the 100× oil immersion objective lens into the oil. Use only **fine focus** to bring image into focus.

Describe the cell form of your bacteria. In which of your colonies are capsules present? What shape do the capsules have, and what is their dimension relative to the size of the bacterial cells?

2.f. Staining conclusions

For your final lab report, document what kind of bacteria you have found on your general nutrient agar plates for your field station. For your studied colonies, for each colony include a description of the colony morphology and appearance; if this colony contained gram-positive or gram-negative bacteria; what form the bacteria in this colony had (cocci, rods, spirillae); were they in aggregates or single; did they possess endospores, flagella, or a capsule? Gather and discuss the results of your working group team members. Which bacteria were the most common in your field station? Discuss your findings in your final lab report.