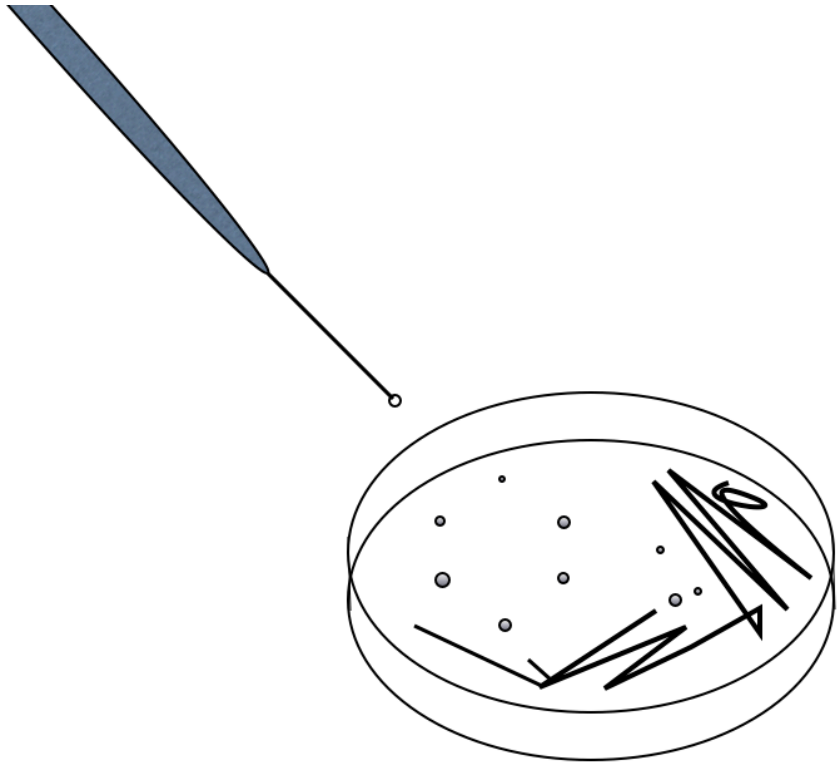
General Microbiology Laboratory Manual

Biology 490



Δ

Second Edition

Sam R. Zwenger, Ph.D.

Fort Hays State University
Department of Biological Sciences

Note to Reader:

Many of the lab excercises in this manual are adaptations from existing lab manuals. Hence, diagrams within this manual are diagrams that have been redrawn with modifications from existing lab manuals or textbooks. The excercises form a core set of skills and a body of knowledge that many university-level microbiology courses include, thus many excercises can also be found in nearly any university-level lab manual. Each image has been modified from one of the sources listed below.

Willey, Joanne, Linda Sherwood, Chris Woolverton. *Prescott's Microbiology*, 8th edition. New York: McGraw Hill, 2011. Print.

Willey, Joanne, Linda Sherwood, Chris Woolverton. *Lab Excercises in Microbiology*, 8th edition. New York: McGraw Hill, 2011. Print.

James G. Cappuccino and Natalie Sherman. *Microbiology: A Laboratory Manual*, 7th edition. Benjamin Cummings, 2004. Print.

Contents

Appendices

Exercise 1: Microbiological Growth Media

Introduction:

Several types of media are available to grow bacteria. No matter what microbe a person is trying to culture, microbes (like all other organisms) require nutrients and energy for growth.

Media for bacteria differ by

1. Chemical composition

    a.) defined (synthetic) media contains known chemical constituents to permit knowing exactly what is being metabolized

    b.) complex media has some unknown chemical constituents, which is sometimes necessary since the exact requirements are unknown, restricting the proper preparation of a defined medium; often complex media contains digested proteins (peptones) isolated from meat, gelatin, or soy to serve as a carbon, nitrogen, & energy source for the microbe.

2. Physical nature

    a.) liquid (broth); often prepared in Erlenmeyer flasks or test tubes

    b.) semisolid; contains a lower amount of agar (see below) than solid media

    c.) solid; great for bacterial isolation from a mixture of organisms, this type of media requires agar, which has been extracted from red algae. Agar has useful properties such as melting at 90°C. Once it has melted it can be held in suspension at temperatures around 45°C (cool to the touch). This allows pouring media into Petri dishes and letting the media cool and solidify. Perhaps two other qualities that are important are the inability of microbes to utilize the carbohydrate as an energy source and the flexibility that it offers in regards to the wide range of temperatures at which it can be incubated.

3.) Function

    a.) general-purpose media (supportive media)- includes tryptic soy agar/broth (TSA/TSB) and can support a wide range of microbes

b.) selective media- favors growth of one microbe over another microbe; includes eosin methylene blue (EMB), which inhibits Gram positive organisms

c.) differential media- differentiates among various groups of microbes; includes phenol red broth (PRB), which (after addition of specific sugars) differentiates among microbes that can ferment particular sugars

d.) enriched media- supportive media with added nutrients; blood agar is a great example of enriched media

Materials:

- TSA or other microbiological media (dry powder)
- Clean 500 ml Erlenmeyer flask
- Deionized (DI) water (H_2O)
- Aluminum foil and autoclave (indicator) tape
- Autoclave

Methods:

- Properly and accurately weigh the necessary amount of media. Add water until the total volume (media + water) equals the target amount of media. Gently mix by swirling.
- Cover the nonsterile media with aluminum foil and a small piece of autoclave tape. Place in the autoclave to sterilize. The instructor will provide proper instruction on how to operate the autoclave.
- When the media is finished (the autoclave is finished) remove it using the orange autoclave gloves. Place the media on the bench top and allow it to cool until it is comfortable enough touch (~45°C) with bare hands.
- Carefully pour the media into Petri dishes, allowing the lid to partially cover the media. When the media has solidified, cover the Petri dish.

Results:

Exercise 2: Culturing Bacteria & Aseptic Technique

Introduction:

Bacteria are ubiquitous and can be found growing on almost any substrate available. Although only ~1% of bacteria are able to be grown in a laboratory setting, several species of bacteria can easily be cultured. An important component of culturing bacteria involves isolating a single species from a mixed culture, where several species might be present.

Agar plates with general purpose media offer a great way to sample bacteria from the environment. Care must be taken to provide as direct method as possible when transferring a swab sample to media for culturing so as not to introduce bacteria from unwanted sources. For example, a person needs a sterile (wet) swab to sample a (dry) surface. Exposure time of air to that of the inside of the Petri dish should also be minimized.

When maintaining cell lines, microbiologists often work in a laminar flow hood. This apparatus is designed to minimize the introduction of contamination into a pure culture or to assist in isolating one species from a mixture of species. It relies on a high efficiency particulate air (HEPA) filter and airflow.

If the goal is to obtain a pure culture, a streak plate is made. Streaking for isolation often uses agar plates or slant tubes. Agar plates are a great way for isolating bacteria but they are also often used for calculating the number of bacteria per volume of substance. Slant tubes have the benefit of minimizing workspace and can be stored longer than Petri plates, since they often minimize the rate of dehydration.

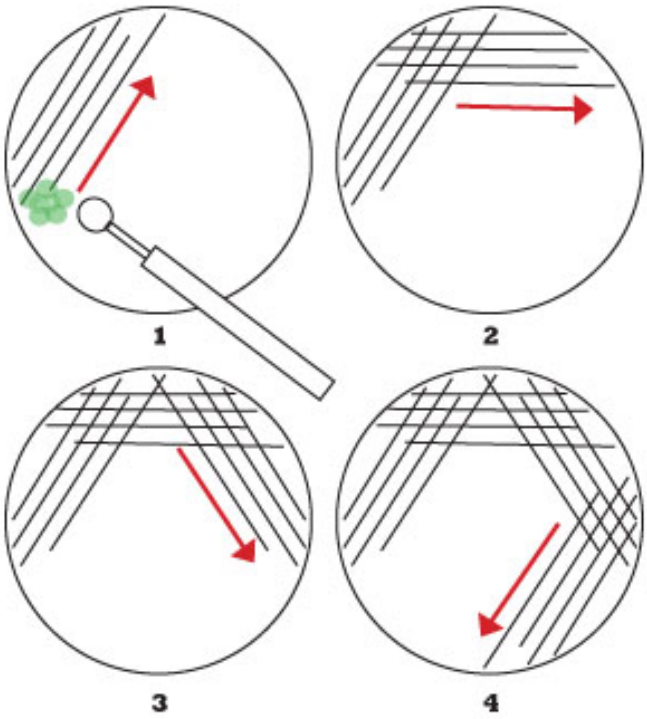


Figure 1. Quadrant streak method for isolating microbes (Modified after Cappuccino and Sherman, 2004).

Streaking for isolation relies on a quadrant streak (Fig. 1), but this can depend on cell density. Regardless, for our purposes, it is highly important to practice the quadrant streak when streaking for isolation.

Materials:

- 2 Petri dishes with general purpose media (e.g. TSA)
- Sterile swab
- Sterile water
- Overnight culture in broth tube

Methods:

- Swab something of your choice and streak the sample onto the media. If it is a dry surface, moisten the swab before streaking the media. It might help to slowly rotate the swab.

- Use a quadrant streak (Fig. 1) to obtain a pure culture of the bacteria in the test tube.

- Begin by first sterilizing a loop in the bacticinerator. Remove the loop and let cool briefly. Remove a loop of bacteria from the test tube and place this sample on a small sector of the Petri dish.

- Transfer the bacteria aseptically, that is, without contaminating the original culture.

- Sterilize the loop again to kill everything. Streak the end of the previous sample on the plate in a manner that drags some of the microbes to a new sector of the Petri dish.

- Again, sterilize the loop and return to the plate to streak again. After performing the quadrant streak, incubate both plates at 37°C.

Results:

Exercise 3: Colony Morphology

Introduction:

When grown on solid media (and even some liquid media), bacteria can take on distinct shapes and growth patterns. Colony morphology describes the overall appearance of the colony and varies according to

1. Whole colony (form)

    a.) punctiform- small dots

    b.) round- like a circle

    c.) filamentous- with stringy extensions

    d.) rhizoid- appear like tree roots

2. Elevation

    a.) convex

    b.) flat

    c.) raised

    d.) growth into medium

    e.) pulvinate

3. Margin (edges)

    a.) smooth entire

    b.) irregular

    c.) rhizoid

    d.) lobate

    e.) filamentous

    f.) curled

To better understand colony morphology, a pictorial guide is provided in Figure 2.

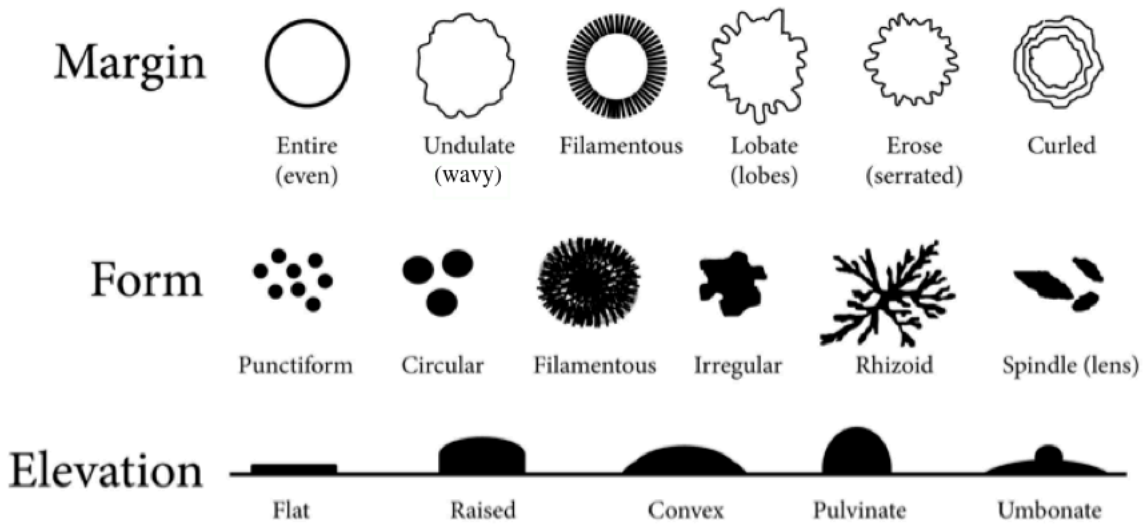


Figure 2. A sample of colony morphologies based on margin, form, and elevation (Modified after Willey et al., 2011).

Materials:

- Petri dish with different species of bacterial (and/or fungal) colonies with different colony morphologies

Methods:

- Observe the variety of colony morphologies.
- Draw the different morphologies and take careful note of details.
- Use a dissecting microscope to assist in determining differences (serrations, filaments, colors, etc.) in morphology.

Results:

Exercise 4: Aerotolerance

Introduction:

Microbes require a certain concentration of oxygen in order to carry out their metabolic reactions. Often, the amount of oxygen required correlates with their energy supply.

Depending on their O_2 requirements, organisms can be one of the following

1. Obligate aerobe

   completely dependent on O_2 for growth

2. Microaerophile

   often damaged by normal levels of atmospheric O_2 levels (~20%) and require O_2 concentration of 2-10%

3. Aerotolerant anaerobe

   organisms that grow equally well in the presence or in the absence of O_2; O_2 is not required and they are not harmed by it

4. Facultative anaerobes

   do not require O_2 for growth, but do better in the presence of O_2; in other words, O_2 is not required, but it is used when available

5. Obligate anaerobe

   obligated to live anaerobically and are killed in the presence of O_2

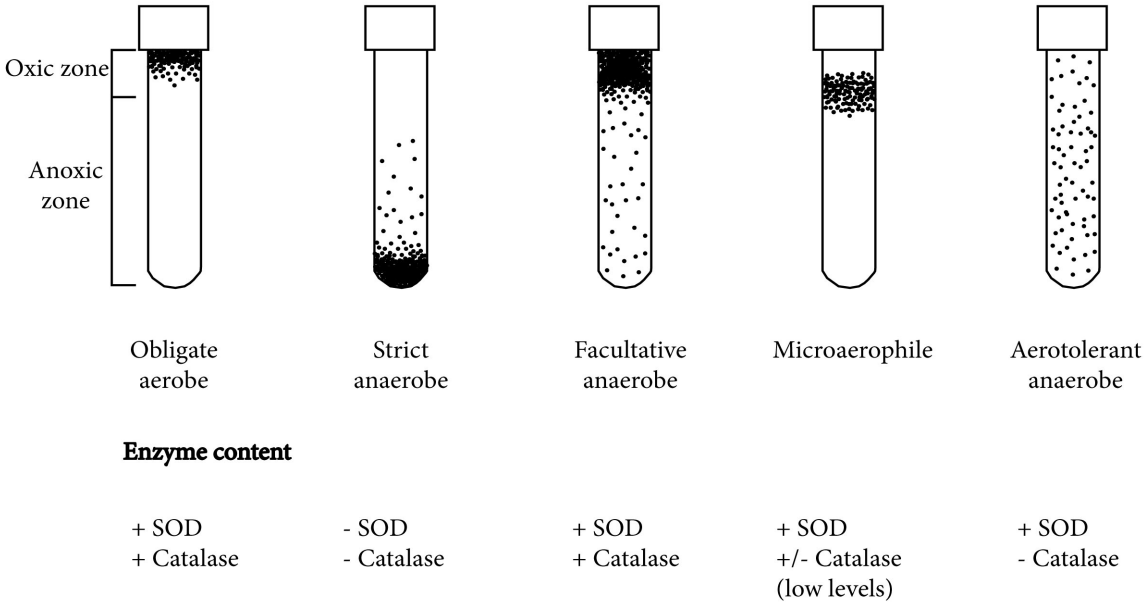


Figure 3. Aerotolerance and the enzymes responsible for inactivating ROS (Modified after Willey et al., 2011).

The various relationships with O_2 can be attributed to several things such as inactivation of O_2-sensitive proteins and the damaging effects of O_2 derivatives. For example, nitrogenase, which is an enzyme responsible for fixing atmospheric nitrogen gas, is very sensitive to O_2. Additionally, enzymes can become inactivated when sulfhydryl groups are oxidized. Unpaired electrons (e^-) in the outer shell of the oxygen atom lend to its instability. Several types of O_2 derivatives can form when O_2 is reduced. The different molecular species are called reactive oxygen species (ROS). Some examples are

$$O_2 + e^- \text{ ----------> } O_2^-\bullet \qquad \text{(superoxide radical)}$$

$$O_2^-\bullet + e^- \text{ ----------> } H_2O_2 \qquad \text{(hydrogen peroxide)}$$

$$H+ H_2O_2 + e^- \text{ ----------> } H_2O + \bullet OH \qquad \text{(hydroxyl radical)}$$

Many reactive oxygen species will damage macromolecules, which influences their capability to function properly. Therefore, it is important that microbes protect themselves and their macromolecular structures to avoid death.

A key difference from other microbes is that obligate aerobes and facultative aerobes have the enzymes superoxide dismutase (SOD) and catalase. These enzymes convert harmful ROS to water.

$$2O_2 + 2H^+ \text{ -------SOD-------> } H_2O_2 + 2H_2O$$

$$2H_2O_2 \text{ -------catalase-------> } 2H_20 + O_2$$

When organisms oxidize substances (food), a small percentage of the time (1-2%) reactive oxygen species are generated. Interestingly, many immune cells delibertately produce ROS to kill organims invading the body.

Free monosaccharides and some disachharides are readily available to be acted upon by enzymes in the cell. Therefore, foods with high sugar content are thought to cause more oxidative damage.

Environments devoid of O_2 or that have a low amont of O_2 can be obtained. An anaerobic jar relies on a palladium catalyst to combine O_2 and H_2 to form water. Water condenses on the sides of the anaerobic jar after incubation. The present experiment investigates the effect of various O_2 concentrations on different microbial species.

Materials:

- Anaerobic jar with "gas pack"

  $$O_2 + H_2 \text{ ------palladium--------> } H_2O$$

- 5 TSB tubes
- 5 thioglycollate tubes
- *Staphylococcus aureus*
- *Clostridium sporogenes*
- *Enterococcus faecalis*
- *Pseudomonas aeruginosa*
- *Escherichia coli*

Methods:

- Carefully inoculate one TSB tube and one thioglycollate broth tube with a bacterial species (one species per tube)
- Place inoculated TSB tubes into the anaerobic jar with palladium catalyst
- Place the thioglycollate directly in the incubator. Be sure that the anaerobic jar is also placed in the incubator as well
- Incubate at 37°C for ~20 hrs

Results:

Exercise 5: Getting to know your Microscope

Introduction:

Microscopy is an excellent tool to help determine the anatomical differences between cells, basic morphology, and can even help determine where a protein product might be translocated. Indeed, the microscopic biological world is difficult to study if we only use macroscopic methods, such as describing colony morphologies or oxygen tolerance. Much more information on microbes was able to be gained after people started using microscopes. The first widely known microscope used was by Anton von Leuwenhoek, who was (at the time) best known for his sales and repairs in the clothing business! His microscopes were simple compared to today's microscopes but nonetheless, allowed observations that were the best for that historical time period.

Several types of microscopes now exist. The dissecting microscope often magnifies a specimen up to 30X and is used for larger organisms but can be used to help describe colony characteristics. The compound light microscope uses two lenses (objective and ocular) to magnify cells; for bacterial cells, the oil-immersion objective lens is used to magnify a specimen 1,000X.

The ocular lens has an ocular micrometer, which is a series of scale bars for estimating the size of objects or specimens. Basically, lenses function by focusing the incoming light rays at a particular plane referred to as the focal point. Lenses help manipulate the incoming light by changing the focal point and making the image appear larger. Parfocality refers to the ability of a microscope to remain in focus when the objectives are changed. Therefore the coarse focus should not be needed as objectives are changed.

Another type of microscope, the phase-contrast microscope, uses a phase ring to advance light waves 1/4 wavelength, which interfere with other wavelengths that have been slowed 1/4 wavelength. There are several other types of microscopes including fluorescent, confocal, & scanning electron microscopes. Each differs in how a specimen is viewed and thus, each provides different pieces of information.

One of the ways to understand how well a compound light microscope helps increase the resolution is to first understand what is meant by resolution. Resolution can be best explained using the following equation

$$d = \frac{0.5\lambda}{n \sin \theta}$$

where

d = minimal distance between two objects that allow viewing of the two objects (this is resolution)

λ = wavelength of light (nm) that is being used for specimen illumination

n sin θ = the ability of the lens to collect light, which is known as the numerical aperture (often 1.25, but can vary depending on the medium)

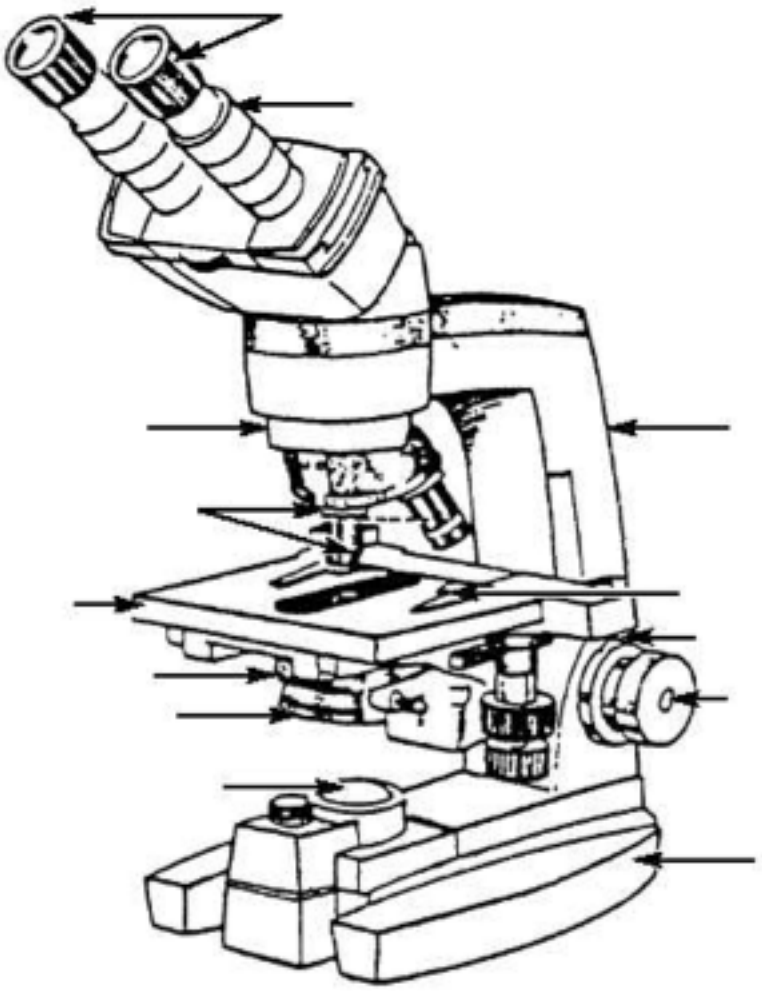
n = refractive index of the air or medium (oil) near the lens

θ = 1/2 the angle of the cone of light that is entering an objective lens

The refractive index of different media influence the curvature (or capturing) of light and direct the light into the objective. In the case of the 100X objective, oil must be used to provide a lower refractive index. Better resolution is given because the oil increases the numerical aperture.

Using this information, what is the resolution of the compound light microscope? Why can you not see proteins and DNA?

To become more familiar with the parts of the microscope, label the parts on the following diagram



Knowing a little more about the compound light microscope, a student can easily learn to estimate size of the bacteria and other microorganisms being viewed. However, the microscope must first be calibrated using the stage micrometer. The stage micrometers are *very* expensive, so be careful when using them.

On average a bacterial cell is ~2 μm in length, although there are several variations depending on the species. The following table will help in understanding cell length.

Unit	Abbreviation	Value
meter	m	1
centimeter	cm	10^{-2} meter
millimeter	mm	10^{-3} meter
micrometer	μm	10^{-6} meter
nanometer	nm	10^{-9} meter

The microscope has an ocular micrometer, which looks like a miniature ruler. The ocular micrometer must be calibrated using a stage micrometer, which, unlike the ocular micrometer, has hash marks with a known distance between each hash mark. Each of the objective lenses will change the value of the ocular micrometer and so calibration must be done for the different magnifications. Most stage micrometers have major divisions that are 100 μm apart and have the farthest left divisions divided into 10 μm increments. To calibrate the microscope and to determine magnification size, ratios are determined.

Materials:

- Compound light microscope
- Stage micrometer
- Prepared slide with specimen of choice (several specimens per table)
- Calculator

Methods:

- On the lowest power objective, superimpose the ocular micrometer onto the stage micrometer so that the furthest divisions to the left are aligned.
- Determine the number of ocular micrometer units that perfectly align with the stage micrometer.
- Find the ratio of the stage micrometer (in micrometers) to the number of ocular units to determine how many micrometers per ocular unit. This is the distance between each ocular unit in micrometers.
- Use the ocular micrometer as a ruler to measure specimens by multiplying the number of ocular units times the calibration (μm/ocular unit). This will give the estimated cell dimension(s).

- On the highest magnification, draw the specimen. Use a ruler to determine how large the drawing is by taking the ratio of the size of the drawing by the estimated size of the object.

Results:

Objective Lens	Calibration (µm/ocular unit)
Scanning	
Low power	
High dry power	
Oil immersion	

Exercise 6: Simple Stain

Introduction:

Stains attach to something because of charge differences between the object and the stain. Different stains can appear as a different color because they contain different chromophore groups, which vary in the wavelength of light they absorb. In general there are two main stain types. Positively charged stains have a positive chromophore. The second type, negatively charged stains, has a chromophore that carries a negative charge. Positively charged stains are excellent in binding negatively charged structures such as bacterial cell walls and, if they can enter the cell, many macromolecular structures such as DNA and proteins.

Cationic (basic) stains have a positive charge associated with them while anionic (acidic) stains carry a negative charge. Examples of cationic stains include crystal violet, safranin, basic fuschin, & methylene blue. Examples of anionic stains include eosin, nigrosin, & congo red. Acid dyes are often used to stain the slide background, which leaves the microbe transparent. Thus, in the field of view the microbe will appear as clear dots against an opaque background.

Stains require a short exposure time to their target followed by a brief, light rinse with deionized (DI) water. This removes any excess stain and allows better viewing of the cells that carry the stain.

Materials:

- Safranin & crystal violet
- Overnight bacterial cultures of *S. aureus* & *B. megaterium*
- Glass slide
- Nichrome loop

Methods:

Place a loop-full of bacteria from an overnight culture onto the center of a glass slide

Heat fix cells by placing the slide on top of the bacticinerator until it appears dry-use a forceps to handle the slide (it might be hot!)

Results:

Exercise 7: Gram Stain

Introduction:

One of the most important stains performed by both the fledgling microbiologist and professional microbiologist is the Gram stain. This stain is named after Hans Christian Gram who was the first to implement the technique. Although Hans did not know it at the time, the Gram stain allows differentiation between Gram negatives and Gram positives. This is largely due to the structure of the cell wall and the presence or absence of an outer membrane that occurs in Gram-negative bacteria.

After heat-fixing a loop-full of overnight culture, the cells are ready to be stained. First a primary stain, called crystal violet, is used as a primary stain. This cationic stain will adhere to all organisms, since cells carry an overall negative charge. After rinsing, a mordant is applied called Gram's iodine, which promotes retention of the primary stain.

The second rinse is performed with EtOH. The EtOH functions to do several things one of which is to shrink the pores in the peptidoglycan layer. This traps the crystal violet-iodine complex in the Gram-positive cell. The larger pores and thinner peptidoglycan in Gram-negative organisms is not changed to such an extent compared to the Gram-positive organisms. However, the EtOH also removes the outer membrane of the Gram-negative organisms. In essence, the EtOH rinse effectively leaves the Gram-negative cells colorless.

While the Gram positives are stained purple, a counterstain is applied to stain the colorless Gram negatives. These will appear reddish in color.

What would a simple drawing of a Gram-positive and Gram-negative cell look like? What are the different parts (cell wall, outer membrane, periplasmic space, etc.) and their relative sizes?

Materials:

- Overnight cultures of *S. aureus*, *E. coli,* & an unknown
- Crystal violet
- Gram's iodine
- Safranin
- DI H_2O

- EtOH

Methods:

- On three different areas of the glass slide place *S. aureus*, the unknown, and *E. coli*. Do not add too many cells.
- Heat fix cells to glass slide.
- Add enough crystal violet to cover the specimens and wait ~1 minute.
- Wash with DI H_2O
- Add Gram's Iodine and wait ~1 minute.
- Add a few drops of EtOH and tilt the slide onto the collection pan. Rinse.
- Counterstain with safranin for ~1 minute, rinse, and blot dry

Results:

Exercise 8: Negative Stain and Capsule Stain

Introduction:

Several types of stains can be performed on bacteria to determine their cellular makeup. The negative stain relies on using nigrosin, which leaves nonheat-fixed cells colorless against a dark background. There are several advantages to using a negative stain compared to a simple stain where cells are heat-fixed.

One of the major advantages is that cells are more easily viewed in their "natural" state. This sometimes gives a better view of cell size and their morphological arrangement. For example, spirochetes will often lose their shape when heat-fixed. Additionally, when cells are heat-fixed, they can be susceptible to lysing or damage, which leaves them difficult to view. For this reason, cells that are very delicate can be viewed much better using a negative stain.

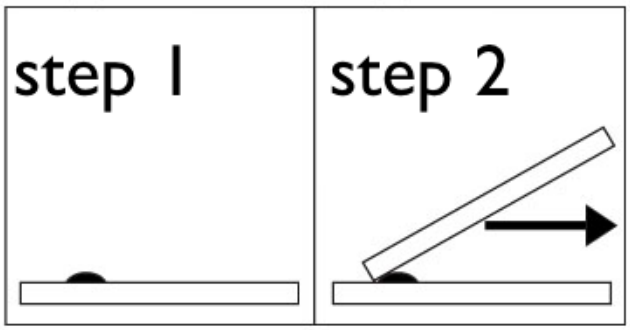
The acidic stain nigrosin is used to leave the cells transparent, since cells are surrounded by negative charges (following the old adage, "opposites attract"). In the negative stain, the acidic dye is repelled by the cellular charges and leaves the cell unstained.

Materials:

- Overnight cultures of *Serratia marcescens*
- Two glass slides
- Nigrosin

Methods:

- Place a drop of nigrosin to the left side of the slide.
- Place a loop-full of bacteria and mix with the nigrosin.
- Using the second slide, touch and drag the nigrosin across the first slide as shown.



(Modified after Willey et al., 2011)

- Allow the slide to air dry.

Capsule Stain

Introduction:

Some microbes produce an outer, sticky matrix consisting primarily polysaccharide. Bacterial capsules confer an advantage in attachment to surfaces and evading immune systems. For microbiologists wanting to view the capsule under a compound light microscope, a simple stain cannot be used since much of the time stains will not adhere to the capsule due to its chemical nature. Additionally, some stains will shrink away from the bacterial cells when drying, giving the false impression that a capsule is present.

To properly stain a cell with a capsule, two stains are used. The first stain, crystal violet, targets the cell. However, the capsule itself is not stained due to its nonionic nature. A 20% copper sulfate solution can then be used to rinse the crystal violet off and will leave the bacteria looking purple with a faint but noticeable halo.

Alternatively, a smear can be made using a loopfull of bacteria and small amount of India ink. The smear is air dried and rinsed. Next, crystal violet is added and allowed to sit for about one minute. This stains the cells purple. The slide can be rinsed and blotted with bibulous paper.

What colors will the background, cells, and capsule appear?

Materials:

- Crystal violet
- India ink
- Overnight cultures of *Klebsiella pneumoniae* & *Micrococcus luteus*

Methods:

- Put a drop on India ink on one side of the slide
- Using sterile technique, place a loopfull of bacteria with the India ink and smear
- Air dry
- Add crystal violet and wait ~1 minute
- Rinse and blot dry using bibulous paper

Results:

Exercise 9: Flagellar Stain

Introduction:

Some bacteria display locomotion (movement). A very important structure that functions in giving the bacteria motility is the flagellum (p. flagella). Motile bacteria can seek out nutrients or direct their movement towards or away from certain chemicals or signaling molecules released by other microbes (chemotaxis). Flagellar arrangement varies among taxa and some of the basic arrangements you might observe are presented in Figure 4.

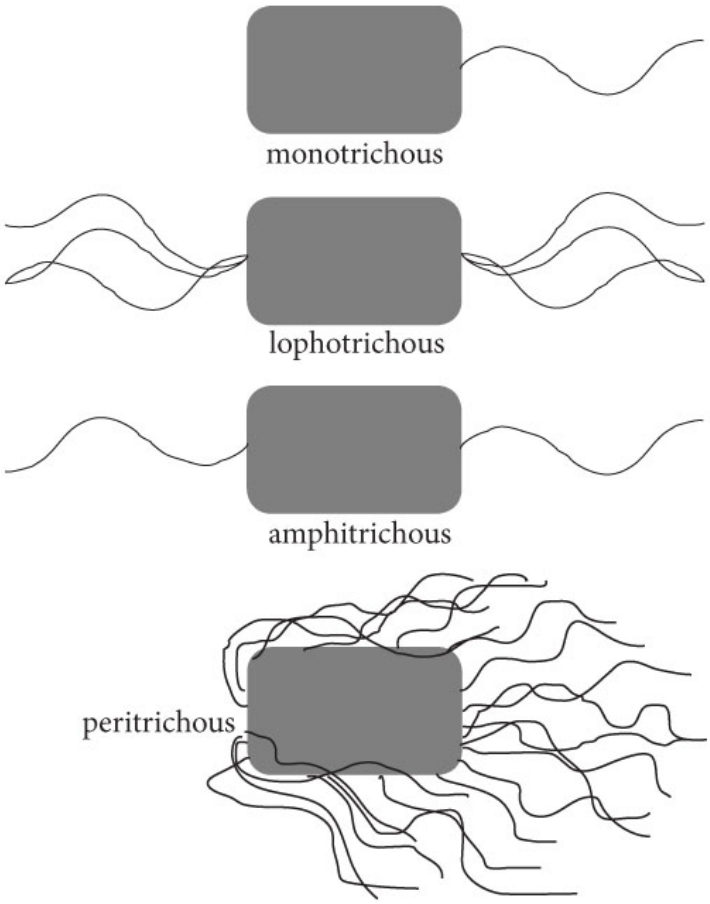


Figure 4. Flagellar arrangements for bacterial cells. Different species can display different arrangements (Modified after Willey et al., 2011).

Flagella are fairly thin structures with a diameter of about 20 nanometers. Thinking back to the microscope lab. There is a lower limit of resolution. What is the lower size limit that is able to be viewed with a compound light microscope?

One method of viewing the flagella with a light microscope is to increase the diameter of the flagella, which permits viewing of the flagella if present. First, a few loopfulls of bacteria are placed onto the slide and allowed to air dry. Rapid heating might destroy the flagella structure, so it needs to dry slowly. Next, a mordant such as tannic acid is to interact with the flagella for several minutes. An ethanol solution of crystal violet is added. As the ethanol slowly evaporates, the crystal violet is left behind. The crystal violet precipitate increases the thickness of the flagella and allows the individual to determine the presence and the arrangement of the flagella.

Materials:

- Overnight cultures of *Alcaligenes faecalis* or *Pseudomonas areuginosa.* Handle the cultures carefully since vigorous shaking can disrupt the flagella.
- Mordant (tannic acid and aluminum potassium sulfate).
- Alcoholic solution of crystal violet.

Methods:

- Place 3 drops of water onto a slide
- *Gently* place a loopfull of bacteria in the water
- Air dry for at least 15 minutes
- Cover the smear with the mordant for 4 minutes and rinse with DI water
- Place the slide over a boiling water bath and cover with a small piece of paper towel
- Add enough crystal violet to soak the smear
- Remove the slide and flood with distilled water for 1 minute
- Remove the water by gently shaking the slide and then let air-dry

Results:

Exercise 10: Endospore Stain

Introduction:

Bacteria are ubiquitous; however, conditions that are conducive for rapid growth are not always present. Under harsh conditions, such as extreme temperatures, some bacteria can produce a spore within their cells, aptly called an endospore. The endospore is a survival mechanism that can persist under extreme conditions. Several interesting studies have been done to determine the endospore wall composition.

Equally interesting are the different phases of endospore development. The vegetative cell expresses distinct sets of genes to produce the endospore in a process called sporogenesis. Upon completion of the endospore the vegetative cell can die, leaving a free spore (Figure 5.). Free spores are extremely hardy and can survive boiling and intense radiation. When conditions conducive for growth return, the spore will germinate (germination) and a new vegetative cell results. The vegetative cell can undergo binary fission and result in several more bacteria. If harsh growing conditions return, an endospore is generated again. The cycle can continue over and over.

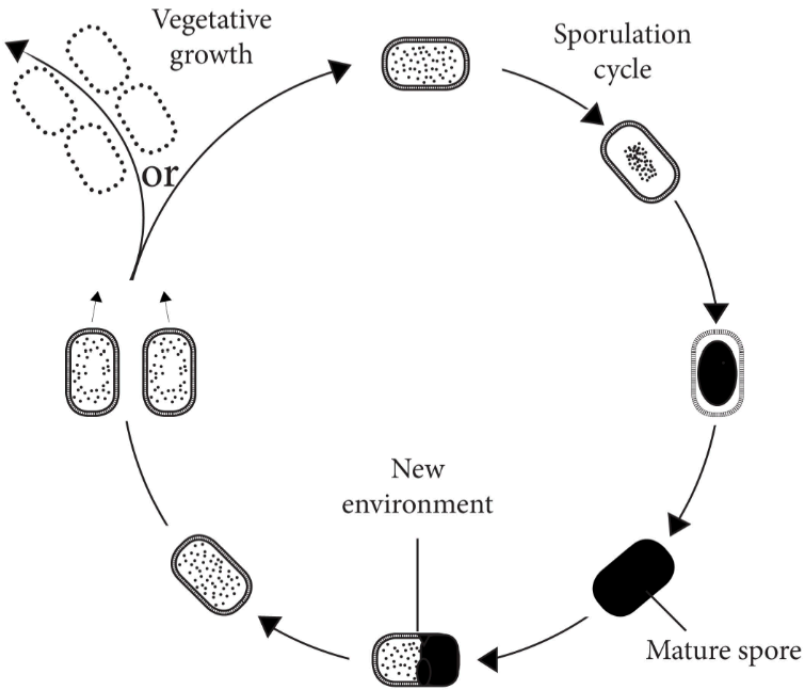


Figure 5. Sporulation cycle. Endospores exposed to environmental conditions conducive for growth lead to upregulation of genes responsible for metabolic pathways and vegetative growth (Modified after Willey et al., 2011).

Two genera of bacteria that have medical importance are *Clostridium* and *Bacillus*. *Clostridium tetani* produces an endotoxin that causes tetanus. *C. perfringens* and *C. defficile* can cause gas gangrene and pseudomembranous colitis, respectively.

Materials:

- Overnight culture of *Bacillus megaterium*
- Hot water bath
- Malachite green
- Safranin

Methods:

- Place a loopfull of *B. megaterium* on a glass slide.
- Heat-fix the specimen.
- Place the slide over the boiling water bath.
- Cover the specimen with a small piece of paper towel.
- Apply malachite green.
- Allow the heat to carry the malachite green into the endospore for 6-7 minutes. Be sure to reapply malachite green if it the towel becomes dry.
- Rinse the slide with water and apply safranin as the counterstain.
- After 1 or 2 minutes, rinse and blot dry.

Results:

Exercise 11: Acid-Fast Stain

Introduction:

While there are definitive Gram-positive and Gram-negative bacteria, there are some bacteria that do not give good Gram stain results. These bacteria are sometimes *acid-fast bacteria*. This is primarily due to the presence of additional materials, called mycolic acids, in their cell walls that disrupt the expected function (and results) of the Gram stain reagents.

To properly identify acid-fast bacteria, a special staining procedure is used. First, a loop-full of bacteria is placed on the slide and heat fixed. The slide is then placed over a heat source and carbolfuschin is added. A small piece of paper towel is placed over the specimen as well. The heat helps drive the carbolfuschin into the cell while the paper towel helps retain moist conditions. The slide is removed, rinsed, and decolorized using acid-alcohol. Methylene blue is used as a counterstain. After the carbolfuschin step, all bacteria are purplish in color. After the acid-alcohol rinse, only acid-fast bacteria will retain the carbolfuschin. Non acid-fast bacteria will be colorless. However, this changes after the methylene blue is added.

Mycobacterium is a genus that has acid-fast members. Some *Mycobacterium* species that cause serious disease in humans include *M. leprae* and *M. tuberculosis*. Two relatively easy species to work with are *M. smegmatis* and *M. phlei*, which we will look at in this exercise.

A final word of caution should be given when viewing acid-fast specimens. Similar to the Gram-positive or negative species, some acid-fast species show inconsistent stain results. Think about why this might be the case.

Materials:

- Overnight cultures of *M. smegmatis* or *M. phlei*
- Steam bath (Hot! Be careful!)
- Carbolfuschin
- Acid-alcohol
- Methylene blue

Methods:

- Heat fix *E. coli and Mycobacterium* cells to glass slide
- Place the slide over a boiling water bath
- After 4-5 minutes, remove the slide
- Rinse with acid-alcohol until the slide remains light pink
- Rinse with water for a few seconds
- Use methylene blue to counterstain and wait about 2 minutes
- Rinse and blot dry

Results:

Exercise 12: Sulfur Cycle & the Winogradsky Column

Introduction:

Microbial process can be studied at various levels, from ecosystems to single-gene experiments. Before the advent of microbial ecology, microbes were too difficult to grow or poorly understood to be cultured on a large scale. A scientist who pioneered growing microbes to study their entire processes was Sergei Winogradsky (1856-1953). He was the first to conceptualize culturing microbes in jars and manipulating the chemistry in various ways in order to determine microbial ecological processes.

Winogradsky was particularly interested in the sulfur cycle and its importance to life's functions. Due to its abundance in the Earth's crust and various redox states, sulfur plays a major role in microbial processes.

Other microbial processes carried out by various genera are listed below. Keep in mind that O_2 is the e^- acceptor in *aerobic respiration*. Respiration is a general term and includes aerobic (involving O_2) and *anaerobic respiration* (some other electron acceptor). Respiration, therefore, is simply a set of metabolic reactions that occur in cells to convert biochemical energy. Molecules generated in respiration are used to generate ATP for use in catabolic reactions. These catabolic reactions include redox reactions that form sugars, amino acids, fatty acids, etc.

In anaerobic respiration, the e^- acceptor can be

Acceptor	Reduced Product	Example
CO_2	CH_4	Methanogens
NO_3^- (nitrate)	NO_2^- (nitrite)	nitrate reductase/ *Staphylococcus* spp.
S^0 (elemental sulfur)	H_2S	*Desulfuromonas*
Fe^{3+} (ferric iron)	Fe^{2+} (ferrous iron)	*Pseudomonas*, *Bacillus*
SO_4^{2-} (sulfate)	H_2S (hydrogen sulfide)	*Desulfovirbrio*
SO_3^{2-} (sulfite)	SO_4^{2-} (sulfate)	*Clostridium*

Aerobic respiration takes e- from organic molecules and "donates" them to NAD^+ and FAD, which in turn lose the e^- to the electron transport chain that uses O_2 as a final electron acceptor, generating water. Aerobic respiration yields the most energy due to oxygen's high redox potential (it *really* wants electrons).

Microbes use sulfur in its various forms as an e^- acceptor or e^- donor.

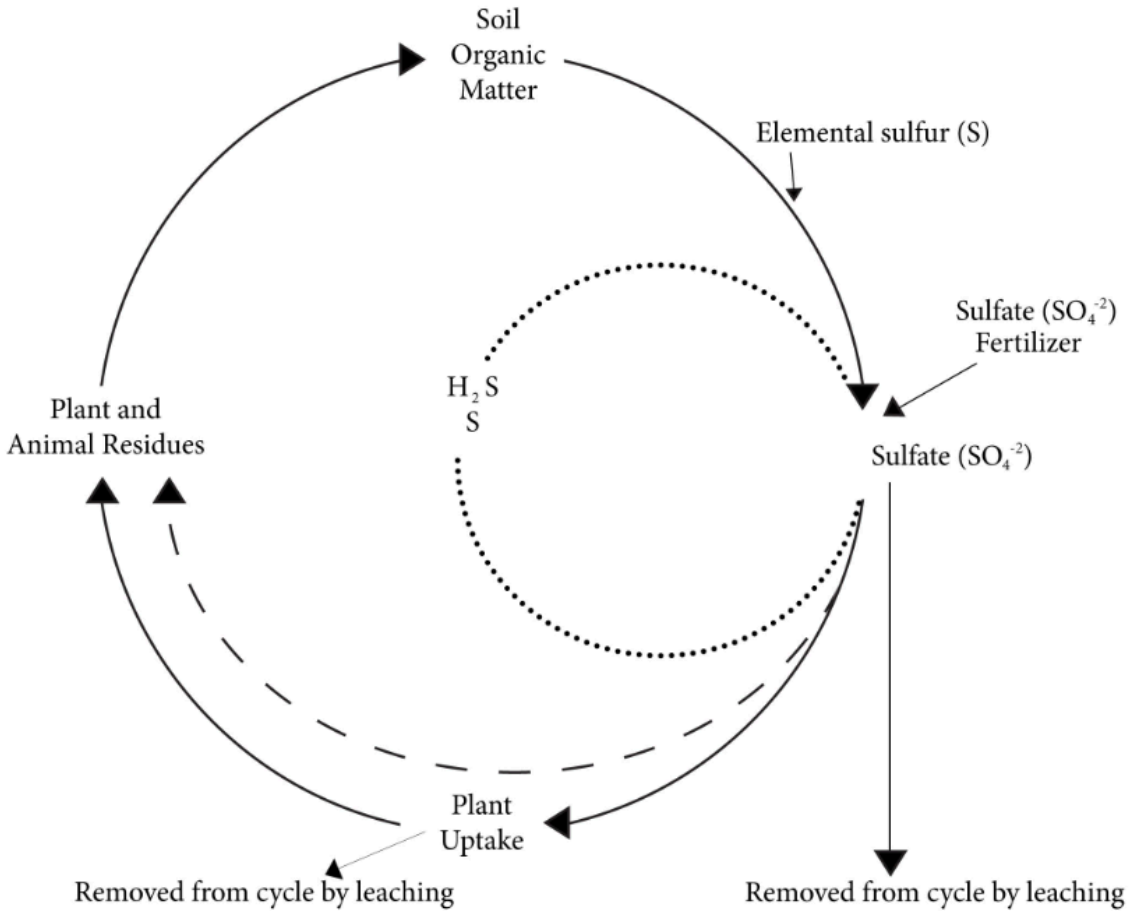


Figure 6. Simplified sulfur cycle. Bacteria and fungi can dissimilate soil organic matter that contains sulfur (Modified after Willey et al., 2011).

What changes are expected to be observed as the jar ages? What reactions are taking place?

Materials:

Soil sample (usually mud from a stream but can be from almost anywhere)

Empty glass jar; tall narrow jars are best and the changes will be most easily observed

Sulfur source

Newspaper

Nails or other iron source

Stir or tamping stick

Methods:

Fill the jar about 3/4 full with soil or mud. Add in nails, paper, sulfur source or other material as the jar fills. If more newspaper is at the bottom it will favor obligate anaerobes such as *Clostridium.*

Fill the remaining 1/4 of the jar with water.

Put the lid on but do not make it airtight.

Place the Winogradsky column by a light source and take note of the changes that occur.

Results:

Exercise 13: *Agrobacterium*-Mediated Plant Transformation

Introduction:

Agrobacterium can be found in soil environments. It is a Gram-negative rod that is known to have the capability to cause crown-gall disease in plants. The disease is so named due to the large galls (swelling) that typically occur near the crown (or base) of the plant. However, any wound site on a plant can get infected and thus, a gall can develop on higher regions of the plant. Often, *Agrobacterium* causes the nodules seen on tree branches.

The gall forms in response to *Agrobacterium* and its ability to essentially "hijack" the plant cellular machinery. The bacterium harbors a type IV secretion system (T4SS), similar to a molecular syringe. It injects the plant cell with DNA that encodes genes to produce plant hormones, which the plant cell responds to by making a gall. It is the gall where *Agrobacterium* then takes up residency and lives off of plant metabolites.

Scientists eventually learned, when studying the secretion abilities and nucleic acid transfer, that many of the gall-forming genes were found on a plasmid located between recognition sequences. Since tumors (galls) resulted, the plasmid was called a tumor inducing plasmid, or Ti plasmid (Fig. 1). Interestingly, it was later learned that gall-forming genes could be removed from the Ti plasmid, which "disarmed" *Agrobacterium*. However, the T4SS genes remained. Even more interesting was that recognition sequences (right and left border) that the T4SS recognized as a splice site, could be placed onto a separate plasmid. In this way the border sequences and a gene of interest (inserted between the left and right border) could be on a different plasmid. Since this was the second plasmid (vector) present in *Agrobacterium*, it was called a binary vector. The plasmid with the gene of interest (GOI) and antibiotic resistance genes (i.e. *nptII*) could then be manipulated, ligated into a plasmid, and then the plasmid could be introduced to *Agrobacterium*.

The marker and GOI could then be transferred to the plant instead of the gall-forming genes.

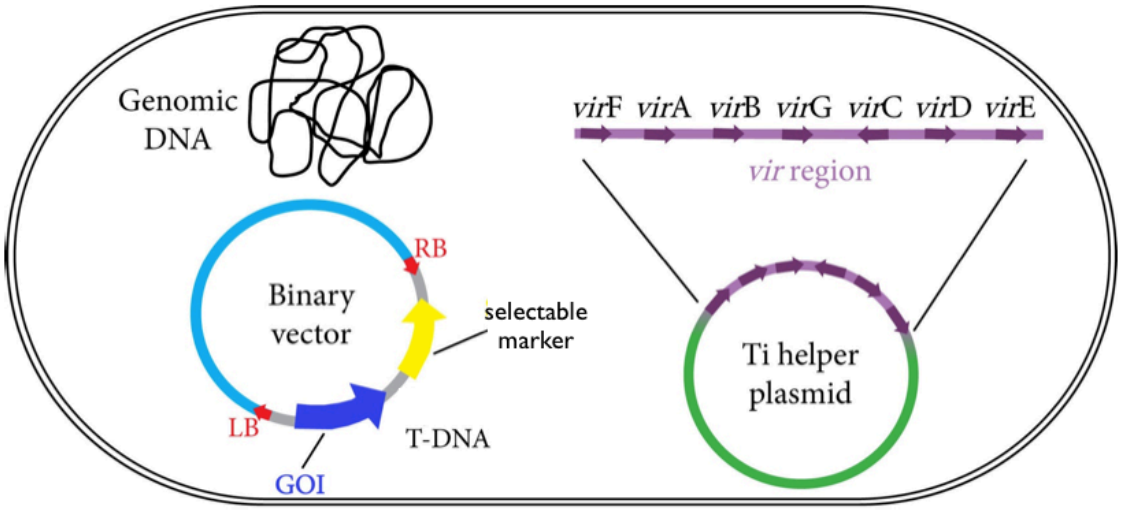


Figure 7. Mechanism of gene transfer and use of a Ti helper plasmid in *Agrobacterium*.

Within the last decade or so, it was realized that *Agrobacterium* could infect many plant tissue types such as ovules (immature plant seeds). A small percentage of the ovules exposed to *Agrobacterium* would be "transformed". That is, they take up foreign DNA. The plant originally used (and most commonly used) in *Agrobacterium*-mediated plant transformation is *Arabidopsis thaliana*. A popular selectable marker used in plant transformation studies is the *nptII* gene, which encodes a protein that transfers a phosphate to kanamycin, rendering the kanamycin ineffective. In its active (non-phosphorylated form), kanamycin is known to bind to the bacterial 30S ribosomal subunits and inhibit protein synthesis.

We will use *Agrobacterium* to transform *Arabidopsis thaliana* ovules and allow the ovules to mature into viable seeds. The seeds will be sterilized and plated onto plant growth media impregnated with kanamycin at 50 µg/ml. Transformed plants will grow, while nontransformants will die.

Why does kanamycin, which inhibits bacterial ribosomes, stop a plant (a eukaryote) from growing?

Materials:

- *Agrobacterium tumefaciens* with binary vector carrying the *nptII* gene
- Selective media with Kanamycin

- 75 ml centrifuge tubes
- Ultracentrifuge
- Flowering *Arabidopsis* plants
- Pipettes
- Humidifying bags
- Spectrophotometer

Methods:

- Prepare 100 ml of TSB with 50 µg/ml kanamycin.
- Use the stock solution of kanamycin (50 mg/ml).
- Culture *Agrobacterium* in TSB for 2 days at 28°C.
- After two days place the cells in a 75 ml centrifuge tube.
- Centrifuge for 7 minutes at 6,000 RPM.
- Decant supernatant and resuspend cells in a 5% sucrose and 1% Silwet solution.
- Use a spectrophotometer to obtain an optical density of 600 nm at 0.8.
- Dip the flowers to infect the ovules and place the entire plant into the plastic bag for ~20 hours, after which remove the plant and allow for recovery.
- Collect the seeds and axenically sow on 1/2 MS agar with 50 µg/ml kanamycin.
- Grow under constant light for 7-10 days.

Results:

Exercise 14: Selective Media

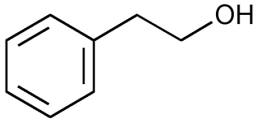
Introduction:

Selective media allows for some microorganisms to grow, while others are inhibited. Favoring the growth of specific microbes may be accomplished by using inhibiting reagents or dyes. There are several types of selective media and each has particular attributes that lend to its usefulness, depending upon the goal of the researcher. Formulation of selective media is such that they select some groups of bacteria, largely based on particular biochemical activities. These include but are not limited to

- ability to hydrolyze or ferment different sugars
- ability to alter the pH (often this is to an acid, but sometimes to more basic)
- ability to tolerate toxic substances such as sodium desoxycholate (a bile acid), dyes, high salts, and alcohols

It should be cautioned that selective media is one step towards identification of the organism and further tests might be needed.

Phenyl ethyl alcohol (PEA) agar is used to isolate *Staphylococcus* from mixtures of bacterial flora. It allows for the growth of Gram-positive organisms but restricts many Gram-negatives. This is based on the presence of phenyl ethyl alcohol interfering with DNA synthesis in Gram-negative organisms, although some argue that the cell membrane of gram negatives is a primary target. The phenyl ethyl molecule has the following structure



Mannitol salt agar (MSA) is used to help isolate and differentiate pathogenic *Staphylococcus* species; however, it is mainly used for *S. aureus*. Mannitol is a six carbon sugar alcohol and if fermented, the media turns yellow (pH 6.8 or lower). The media is typically red (pH 7.4-8.4) but will turn pink to light red at a high pH (>8.4) Pathogenic *Staphylococci* ferment mannitol and will appear to have yellow halos surrounding the colonies. Some strains of *S. epidermidis* are completely inhibited by MSA.

Hektoen enteric agar (HEA) is used to isolate and differentiate *Salmonella* and *Shigella* species from other Gram-negative enterics. Enterics are intestinal bacteria that are often harmless. HEA is based on the ability of some microbes to ferment lactose and sucrose. Bile salts, bromothymol blue, and fuschin (the latter two being dyes) are used to inhibit most Gram-positive cocci. *Salmonella*, *Shigella*, and *Proteus* do not produce acid from fermentation and so will appear blue-green. Other enterics will be yellow to pink in color. *Proteus* and *Salmonella* reduce sulfur to H_2S and this forms a black precipitate-the colonies will appear black. *Serratia* often appears yellow or salmon colored. If yellow, the organisms ferment lactose. *P. vulgaris* should appear as small blue or brown colonies.

MacConkey agar is used to isolate and differentiate members of the Enterobacteriaceae and is based on the ability to ferment lactose (galactose + glucose). The Enterobacteriaceae (order = Gammaproteobacteria) is a rather large family of Gram-negative (mostly rods) and are facultative anaerobes. It includes *Klebsiella, Shigella, Yersinia, Salmonella, and Escherichia*. Bile salts and dyes inhibit Gram positives. Neutral red dye is added as a pH indicator, which is colorless above 6.8. As acid accumulates from lactose fermentation, the pH drops.

Eosin methylene blue (EMB) agar is used to isolate fecal coliforms. To be designated as a coliform, a bacterium must be

- Gram-negative rod
- nonspore forming
- lactose fermenter
- produce acid and gas

EMB agar contains peptone, lactose, sucrose, and the dyes eosin and methylene blue (these inhibit Gram positives). EMB agar is not only selective, but also differentiates between strong fermenters such as *E. coli*, which appear darker, sometimes having a green metallic sheen.

Materials:

- Overnight cultures of *S. aureus, S. epidermidis, E. coli, Enterobacter aerogenes, Salmonella, Serratia,* and *P. vulgaris*
- Phenylethyl agar
- Mannitol salt agar

- Hektoen enteric agar
- MacConkey agar

Methods:

- Inoculate the PEA agar with *S. aureus* and *E. coli*
- Inoculate the MSA with *S. aureus, S. epidermidis, E. coli,* and *Enterobacter aerogenes*
- Inoculate HEA with *Salmonella, Proteus vulgaris, S. aureus* and *E. coli*
- Inoculate MacConkey agar with *E. coli, Enterobacter aerogenes, S. aureus* or *Serratia,* and *P. vulgaris*
- Inoculate the EMB agar with *E. coli, S. aureus,* and *Salmonella*
- Incubate all media at 37°C for 24 hours

Results:

Exercise 15: Differential Media

Introduction:

Differential media is used to differentiate between organisms based on their ability to produce gas, utilize certain metabolites, or produce different products. There are several types of differential media, not all of which are discussed here.

Triple sugar iron (TSI), often in a slant form, tests for fermentation gas production and sulfur reduction of Gram-negative enterics. Gas production is observed as fissures in the media. TSI is especially useful if it is used in conjunction with MacConkey, EMB, and SS agar.

The principle ingredients of TSI include

- a small amount of dextrose (0.1%)- stays red if only this is fermented
- lactose and sucrose (~1.0%)
- sodium thiosulfate
- phenol red (yellow if pH is below 7 and red at higher pH)

Reading out of a TSI slant should include

1. Slant
   Acid (A)
   Alkaline (K)

2. Butt
   Acid (A)
   Alkaline (K)

3. Sulfur reduction (+/-)

4 Gas production (+/-)

An alkaline slant, acid butt, sulfur reducer, non-gas producing organism would read K/A/+/-. The presence of ferrous sulfate will give a black color if the organism reduces thiosulfate to H_2S.

Another type of differential media is **phenol red broth (PRB)**. It contains a known sugar and will indicate if an organism can metabolize that sugar or not. There are various types of PRB and these include

- phenol red (PR) lactose (glucose + galactose)
- PR glucose
- PR sucrose (glucose + fructose)

Included in the PRB is a Durham tube to indicate gas production. If sugar metabolism occurs the acidity will increase (pH will drop) and the red will turn yellow (pH of 7.4). Phenol red is red at pH 7.4 or higher, although above 8.4 it gives a rather pinkish appearance.

A relatively rapid test is the **catalase test**. This involves placing a drop or two of H_2O_2 either directly on the specimen in the Petri dish, or streaking an organism onto a glass slide then applying the H_2O_2 onto the streaked organism.

The reaction, driven by catalase, proceeds as

$$H_2O_2 \text{ ----------> } 2\ H_2O + O_2$$

Catalase is often present in aerobes and facultative anaerobes.

A fourth type of differential media is **peptone iron agar (PIA)** (using test tubes), which detects H_2S production. Sulfur reduction is an anaerobic process. If H_2S is produced from the organism, it is combined with iron to produce iron sulfide (FeS), which appears as a black precipitate.

Yet another test determines if an organism can use citrate as its sole carbon source. This test is done using slants or tubes of **Simmons citrate agar**. Here, ammonium phosphate is the sole nitrogen source and bromothymol blue is used as the pH indicator. Media will appear green at pH 6.9 or lower and blue at 7.6 or higher.

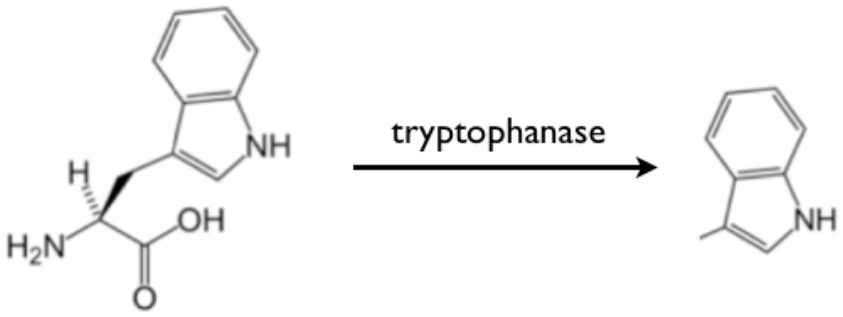
A useful test to determine the ability to reduce nitrate is called the **nitrate reduction test** and uses nitrate broth (it contains potassium nitrate). After incubation, sulfanilic acid is mixed with α-naphthylamine and a small dropper full is added. This might result in a red color, which indicates nitrate reduction. In other words it determines if the following reaction has occurred

$$NO_3^- \text{ ----------> } NO_2^-$$

The enzyme that catalyzes this reaction is called nitrate reductase.

Finally, **SIM media** is used to detect reduction of sulfur, production of indole, and motility. Sulfur reduction is observed if the sodium thiosulfate is reduced and combines with iron, giving a black precipitate. Tryptophan is present in SIM

media and if an organism breaks it down, indole is produced. The reaction, catalyzed by tryptophanase, proceeds as



Pyruvate is liberated as a result of tryptophanase and can be used in respiration or fermentative pathways. Motility is detected as turbidity or the SIM tube turning black in a diffuse manner.

Materials:

- Triple sugar iron agar slant
- PRB glucose, PRB sucrose, & PRB lactose
- Peptone iron agar (in test tubes)
- Simmons citrate agar (slants)
- Nitrate broth
- SIM tubes

Methods:

- Inoculate four TSI slants with *E. coli, P. aeruginosa, P. hauseri,* and *S. cholerasuis.* Start at the back of the slant and slowly work to outer portion of the slant with a streak pattern.
- Inoculate PRB glucose, PRB sucrose, and PRB lactose with *E. coli, P. vulgaris,* and *P. hauseri.*
- Inoculate peptone iron agar with *E. coli, P. vulgaris, and Salmonella cholerasuis*

- Inoculate the Simmons citrate slants with *E. coli* and *Enterobacter aerogenes* or *Klebsiella.* Start at the back of the slant and slowly work to outer portion of the slant with a streak pattern.

- Inoculate nitrate broth with *E. coli* and *P. aeruginosa.* Add a dropperful of sulfanilic acid that has been mixed with α-naphthylamine.

- Inoculate (stab) SIM tubes as with *E. coli, S. aureus,* and *S. cholerasuis.*

- Incubate all media at 37°C.

Results:

Exercise 16: Blood Agar & Hemolysis

Introduction:

Some organisms require special media to grow in order to determine specific properties. For example, different species (and even strains) of bacteria can produce exoenzymes that lyse blood cells and then use the resulting materials for their metabolism and growth. Blood agar is used to determine the different hemolytic properties of *Streptococcus* and *Staphylococcus* species.

While blood agar can also be used to cultivate fastidious organisms, it is more commonly used to detect hemolytic ability. Exoenzymes are produced by many Gram-positive microbes, which release the exoenzyme through pores in their peptidoglycan cell walls. Exoenzymes are a type of exotoxin. Hemolysins in turn are a specific type of exoenzyme. These toxins are released and destroy red blood cells and hemoglobin, which can supply iron to the growing microbe. Hemolysins produced by *Streptococci* such as *S. pyogenes* are called streptolysins. Since iron can be a limiting factor for cells, this and other nutrients are important to obtain.

Blood agar is composed of tryptic soy agar and (often) sheep blood (whole cells). The blood cells are added after the TSA has been autoclaved and cooled so as to not lyse the cells prior to inoculating.

Three major types of hemolysins are

1. Alpha (α) hemolysins

- partial destruction of red blood cell.
- turns green due to oxidation of hemoglobin
- visible as a greenish zone around colonies
- *Streptococcus viridans* shows alpha hemolysis

2. Beta (β) hemolysis

- complete break down of red blood cells
- produces a zone of clearance around colonies
- this includes extremely dangerous organisms such as *Streptococcus pyogenes*, which causes gas gangrene

3. Gamma (γ) hemolysis

- non-hemolysis or lack of hemolysis
- growth without hemolysis
- often seen with *Streptococcus mutans*

Although the above types of hemolysis often are associated with the corresponding organisms, strains may vary.

Materials:

- Overnight cultures of *S. mutans*, *S. viridans, and S. pyogenes*
- Blood agar plates

Methods:

Divide the blood agar plate into three sections

Inoculate each section with one species of bacteria

Incubate for ~20 hours at 37°C

Results:

Exercise 17: *Salmonella*

Introduction:

There are thousands of *Salmonella* serovars, or serological variants. Most are pathogenic. There are two famous members of the *Salmonella* genus, *S. enterica* and *S. typhi*. According to the CDC, there are about 21 million *S. enterica* infections in developing countries every year. There are roughly 400 cases each year in the U.S., with about 3/4 of these from international travel. Isolation of *Salmonella* can be difficult since *Salmonella* must be isolated from stool (fecal) specimens. Many other bacteria are in such samples and usually in greater abundance.

Selective media have been developed to retard the growth of some microbes while enriching for *Salmonella* species. Common media used for isolation of *Salmonella* includes selenite cysteine broth. Lactose is the sugar and cysteine is the sulfur source. *Salmonella Shigella* (SS) agar is also employed in *Salmonella* species isolation. On this media *Salmonella* will show H_2S reduction and is apparent in colonies with black centers. Lactose fermenters appear pink due to the presence of neutral red color indicator. Brilliant green dye, bile salts, and sodium citrate are present to inhibit Gram positives.

Materials:

- Overnight cultures of *Salmonella, Proteus,* and *E. coli*
- SS agar
- Selenite broth

Methods:

- Use a sharpie to divide the SS agar plate into three sections
- Streak each species of bacteria in each section
- Inoculate the selenite broth with each species from the overnight cultures
- Incubate at 37°C for ~24-48 hours

Results:

Exercise 18: Enumeration of Microbes

Introduction:

Determining the number of microbes in a sample is important for several reasons. It is useful to know their numbers in river systems, healthy soil compared to contaminated soil, agricultural fields, areas in oceans, and food samples. In one gram of soil, it is estimated that 1×10^8- 1×10^9 bacteria are present and only ~1% of these can be cultured in a lab. Some estimates suggest that there are about 25,000 species of bacteria in one gram of soil.

For soil samples, a dilution is made and the number of microbes present is estimated by working backwards mathematically. Although there are ways to determine the number of all microbial cells in a sample (direct count methods), this lab will focus on how to determine the number of living (viable) cells are in a sample.

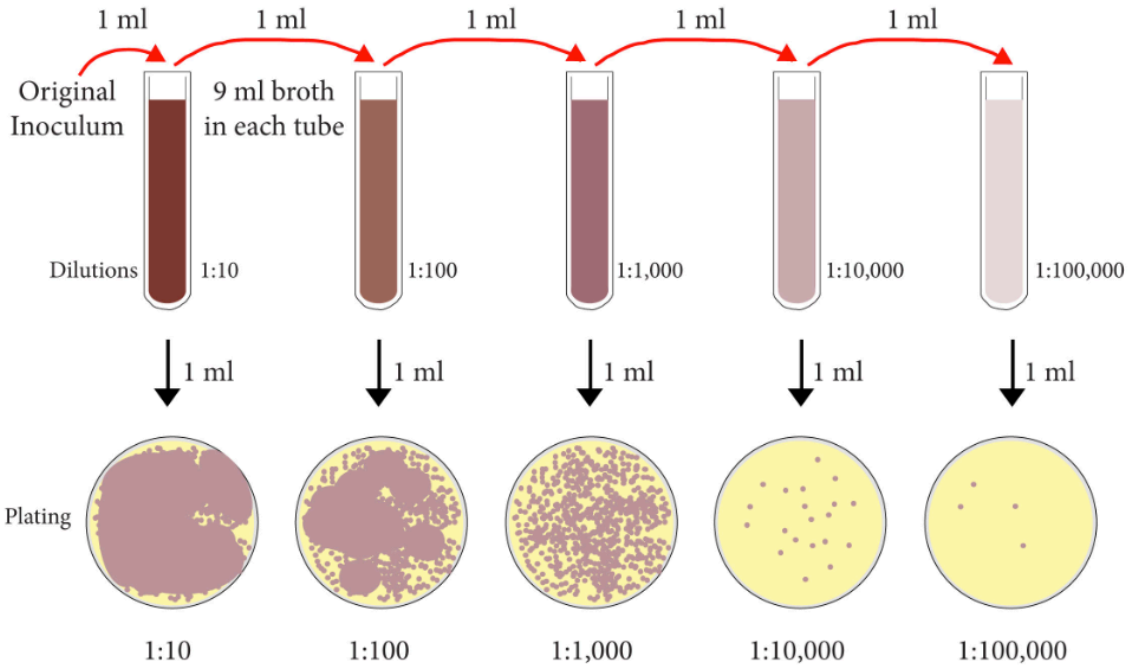
The viable count (or plate count) method counts only cells that can grow when cultured. Two types of viable count methods are

1. pour plate method

2. spread plate method

Both of these methods require dilutions. It is based on the fact that individual cells will grow into colonies. Using this method, there is no way to confirm one cell gave rise to one colony since there is a chance that two cells could start close on the Petri dish, then merge and appear as one colony. Instead of bacterial cells, bacterial colonies are counted and the term colony forming unit (CFU) is used.

Samples with 30-300 colonies often give accurate counting results-outside of this range CFUs become increasingly difficult to count (if too many) or the numbers are too low to be reliable (if too few). The dilution technique begins with measuring a soil sample and adding water. Although the example below shows one gram of soil, more or less can be used as a starting point, but this will, of course, change the method to determine CFUs. For simplicity, we will use one gram of soil dissolved in 9 ml of water. To determine CFUs/ml multiply the number of colonies on the plate after incubation by the reciprocal of the dilution sample. Also, be sure to account for the amount of suspension plated.



(Modified after Willey et al., 2011)

In this example, the dilution uses water. However, dilutions can also use molten agar and is subsequently called the pour plate method. This lab will calculate the CFUs in a soil sample.

Materials:

- Soil sample
- Four test tubes with 9 ml of H_2O
- Plastic transfer pipette
- P200 pipette (to transfer 100 µl)
- Four Petri dishes with general purpose media
- Sterile, L-shaped glass spread rod (L-rod or "hockey stick")

Methods:

- Prepare test tubes for a serial dilution so that each test tube has 9 ml of water.

- Carefully weigh 1 gram of soil sample and place into a glass test tube.
- Cover with parafilm and shake vigorously for 1 minute. Be sure all soil is dissolved.
- Use 1 ml of the dissolved soil sample and transfer it to 9 ml of water. Continue the dilution, taking careful note of each dilution.
- Plate 100 µl of each of the last 3 dilutions onto general purpose media and use the hockey stick to spread the sample evenly.

Results:

Exercise 19: Food Microbiology

Introduction:

There are several factors that can affect microbial growth, which is highly important when considering food spoilage. Intrinsic factors and extrinsic factors are usually the two broad categories when discussing food spoilage. Intrinsic factors include anything that relates to the food and can be things such as fungi or molds. These organisms often weaken the outer skins of fruits, end up penetrating into the soft tissues, and breaking down the tissues.

Some molds such as *Claviceps purpurea* (an ascomycete) can produce ergot, a hallucinogenic alkaloid often associated with rye bread. Some bacteria such as *Erwinia carotovora* secrete pectinase, an enzyme that will also soften fruit tissue, leading to spoilage.

Sometimes amine compounds are produced as byproducts in an anaerobic process of protein breakdown called putrefaction. This results in a highly foul smell that emanates from the food. Anytime the food is ground or mixed, bacteria and other microbes are distributed throughout the food, speeding up the process of food spoilage.

Sometimes intrinsic factors of food inhibit or slow bacterial growth. These include

- coumarins
- lysozyme
- aldehydic and phenolic compounds
- allicin in garlic
- eugenol in cloves

Control of food spoilage can also include extrinsic factors, which are more easily controlled and therefore are used more frequently than relying on intrinsic factors. These include

- salt
- smoking (typically limited to meats)
- low temperature storage

- introduction of high temperatures just before canning, usually 115°C for 25-100 minutes (The range of time is dependent upon the nature of the food (green beans are more basic and hence, more difficult.)

- pasteurization

- manipulation of water availability

- radiation and packaging

Foods with readily available sugars can also be targets of food spoilage. Milk has both sugars and protein available and is used by microbial organisms if given the opportunity. Some organisms that are commonly found associated with milk are *Lactobacillus* species, *Lactococcus lactis*, yeast and molds that degrade acids, and protein-digesting bacteria. However, sometimes it is desirable to intentionally inoculate milk to bring about a desired affect.

Lactic acid bacteria and include *Lactococcus, Lactobacillus,* and *Leuconostoc*. Starter cultures are needed, which are carefully selected organisms that initiate the fermentation process. LABs can be either mesophilic (grow best at 20-30°C) or thermophilic (grow best at 45°C). Probiotic is the term used for live microbes that can give a health benefit to the consumer when ingested in high numbers.

Some examples of fermented foods include

- kefir- often uses goat milk and a mix of lactic acid bacteria, which appear as small clumps or grains, hence starter cultures are usually referred to as kefir grains. it is not the grain that is consumed by the liquid portion that results after fermentation

- yogurt- fermented milk often using *Lactobacillus acidophilus*

- bread- relies on *Saccharomyces cerevisiae* that have amylases that break down starch and release CO_2 making leavened (raised) bread

- cheese- ranges from soft to hard depending on the age and organisms used

- silage- pieces of grass, ground or chopped corn, alfalfa, and other animal feeds in a process called mixed acid fermentation

- beer/wine- uses *Saccharomyces cerevisiae* to convert simple sugars to ethanol and CO_2. (Percent ethanol is measured using a hydrometer, which estimates changes in density.)

- kombucha- another fermented drink that contains bacteria (*Acetobacter*) and fungi (various yeasts)

In this exercise we will focus on making fermented food and drink. Do not eat or drink the product to avoid risk of ingesting unwanted microbes. Work as a table.

Materials:

- whole milk
- skim milk
- cheese cloth
- weak acid such as lemon juice
- *Lactobacillus acidophilus* starter culture
- kefir starter culture
- *Saccharomyces cerevisiae* (brewer's yeast)
- juice
- five 200 ml beakers

Methods:

Kefir

- In one beaker add 100 ml of skim milk and another beaker add 100 ml of whole milk.
- Add a small amount of starter culture.
- Incubate at room temperature for two days.

Yogurt

- In one beaker add 100 ml of skim milk and another beaker add 100 ml of whole milk.
- Add a small amount of starter culture.

- Incubate at 33°C for two days.

Kombucha

- Make a hot tea concentrate and allow to cool.
- In a beaker add 10 grams of sugar and 200 ml of water, add tea concentrate.
- Allow to ferment at least 1 week and view the organism with a microscope.

Cheese

- Heat whole milk until it is warm to the touch.
- Carefully add a few drops of lemon (or lime) juice.
- Remove the curds from the whey using the cheese cloth.

Wine

- Take an initial density reading with the hydrometer.
- Add yeast to the juice and let sit at room temperature in the fermentation vessel.
- Take an end reading 2 days and 1 week later to determine the ethanol concentration.

Results:

Exercise 20: Fungi

Introduction:

Fungi are one of the most understudied groups of organisms yet ironically contribute immensely to diverse aspects of life. Fungi, most often separated based on their mode of reproduction, include basidiomycota (club fungi), ascomycota (sac fungi), zygomycota (several molds-multicellular, filamentous fungi), and oomycota (water molds). There have been several revisions of these phyla since the rising of molecular phylogenetics and to cover them all here is beyond the scope of this lab.

Basidiomycota include the familiar mushrooms, some of which are deadly if consumed, even in small amounts. These include *Amanita* (with several exceptions), *Garlerina*, and *Omphalotus*. However, several mushrooms are edible and are considered a delicacy. Edible basidiomycetes include boletes, *Coprinus*, and *Gymnopilus*.

Ascomycetes include yeasts, a great majority of the mycobiont of lichens, and some edible species such as truffles. Zygomycetes include the common bread mold, *Rhizopus stolonifera*. Water molds are extremely common, especially in stagnant waters. This exercise seeks to demonstrate the diversity of fungal forms, some of their reproductive methods, and cellular processes that are important for all life on earth.

Common media used to culture fungi include Sobauraud dextrose agar (SDA), potato dextrose agar (PDA), and yeast peptone dextrose (YPD) agar. Slide cultures are often used when observing fungi, since moving a fungal specimen to a glass slide often results in its destruction (or too much disruption). We will use SDA to isolate and culture *Aspergillus niger, Penicillium notatum, and Rhizopus stolonifera*. To more easily study molds, a special procedure is used. This involves obtaining a glass slide and flanking it with paraffin wax. A cover slip is placed on top of the wax and warm agar is pipetted between the cover slip and the glass slide. The fungus of choice is then gently applied to the cooled media.

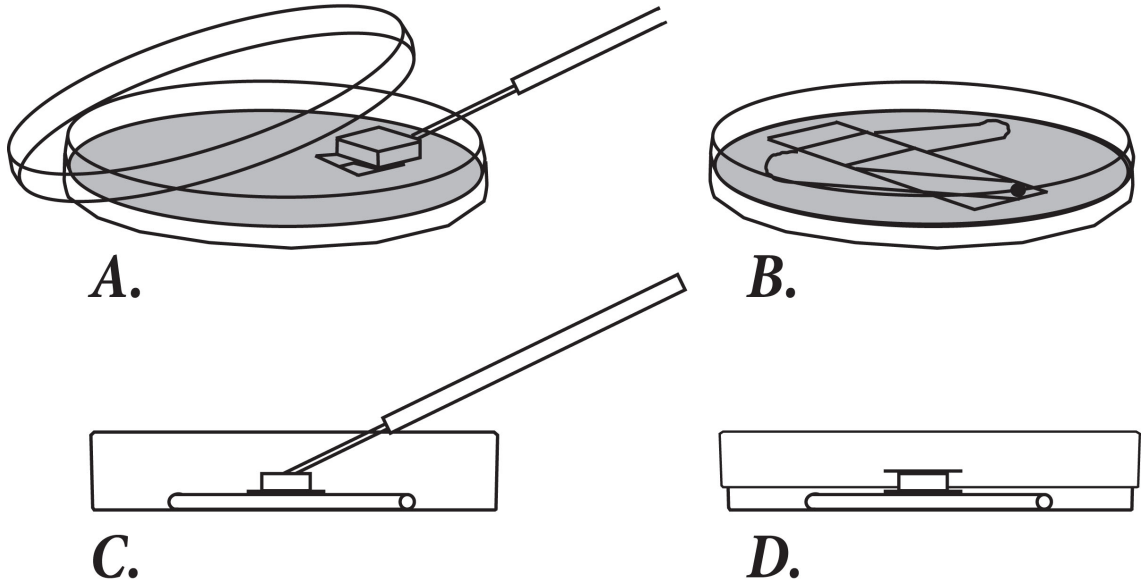


Figure 8. Preparing a fungal sample for microscopic viewing (Modified after Cappuccino and Sherman, 2004).

The slide culture is placed in a humid Petri dish. This is constructed by elevating the slide above a moist paper towel. We will also look for spores and mycelia on the other fungal representatives available in the culture box. What is the yellow organism growing in the culture box?

Materials:

Cultures of basidiomycete (*Coprinus*), ascomycete (Candida and lichen), and zygomycete (*Rhizopus*), and oomycete (water mold), and *Candida albicans*

Yeast peptone dextrose (YPD) agar

Compound light microscope, slides, and inoculating loop

Methods:

Inoculate the different fungal organisms onto yeast peptone dextrose media (YPD). Streak for isolation.

Inoculate the glass slide apparatus and place into a Petri dish.

Incubate the Petri dishes at 30°C for two days.

Examine the different morphologies of the fungi available.

Draw and label each fungal organism and determine apparent size of magnification on your drawing and the size you are observing through the microscope.

Results:

Exercise 21: Epidemiology

Introduction:

The scientific study of the incidence, distribution, and spread of disease is called epidemiology. Epidemic literally means "spread of disease" but epidemiology also attempts to understand how to control disease as well. Epidemics occur when a disease is prevalent among a localized region. This contrasts to much larger spreads of disease such as pandemics, where the disease is among an entire country or is global. If a disease is endemic, it means that it is only found among a certain area or specific population.

Microbes that cause illness can spread in various ways such as through bodily fluids (e.g. HIV) or can be disbursed through the air, as in the case of the common flu.

A famous epidemiology case was that of Mary Mallon, who is perhaps better known as "typhoid Mary". Mary was a cook but also a carrier of *Salmonella typhi*, the cause of typhoid fever. Although she herself was asymptomatic, she infected dozens (perhaps more) of other people. Mary was quarantined twice before she eventually died. The spread of bacteria can often be curtailed with hand washing. Mary Mallon was well-known to not wash her hands (she was in doubt that she carried the disease).

This exercise will demonstrate how quickly a disease can spread if one individual is a carrier. Among the collection of test tubes is one "diseased" test tube. We will see how quickly the disease is spread over a series of transfers between individuals. The experiment is performed twice to demonstrate the variability in spread.

Materials:

- One test tube per individual
- Plastic Pasteur pipette
- Phenolphthalein pH indicator

Methods:

- Choose a sample from the test tube rack.
- Exchange half your sample with half of another person's sample.

- Continue exchanging samples until you have exchanged samples with 4 individuals.

- Add 1-2 drops of phenolphthalein.

Results:

Exercise 22: Determination of an Unknown

Introduction:

Throughout the semester we have used various types of media and experiments to demonstrate the different bacterial species that are able to grow under certain conditions with (or without) particular components in growth media. While some organisms need or require a metabolite, other organisms do not and grow fairly well on several types of media.

It should be clear that the addition of reagents in media can inhibit the growth of some microbes while selecting for the growth of other microbes. We have used differential media to help differentiate among species of bacteria as well, when we look for color changes, precipitate, or turbidity.

This exercise will combine the semester's work into a series of tests that will be carried out over 2-3 weeks of investigative work. Each student will have an unknown microbe, which can be one of the following

Escherichia coli
Klebsiella pneumoniae
Proteus hauseri
Proteus mirabilis
Pseudomonas aeruginosa
Staphylococcus aureus
Salmonella choleraesuis
Staphylococcus epidermidis
Streptococcus viridians
Clostridium sporogenes
Rhodospirillum rubrum
Bacillus subtilis
Mycobacterium phlei
Enterobacter aerogenes
Enterococcus faecalis
Klebsiella pneumoniae
Streptococcus mutans

Only one microbe growing in TSB will be given to one student. A series of tests will need to be performed on the cultures to determine the species.

What first few steps are you going to take before deciding on your tests?

Each student must turn in a lab report with an introduction, materials and methods, and results section. Write this as you would a scientific manuscript with at least two citations. Use regular 12 point type font (black ink) and be sure that the report is double-spaced. Check the schedule to determine the due date.

Materials:

- Media and staining materials will be provided.

Methods:

- Students must determine what steps are needed in order to determine the unknown.

Results:

Appendices

Appendix A: Units of Measure

Basic units

l = liter	g = gram (10^0)
ml = milliliter	mg = milligram (10-^3) = 1/1,000 g ("migs")
μl = microliter	μg = microgram (10^{-6}) = 1/1,000,000 g ("mikes")

Unit qualifiers

m = milli = 10^{-3} = 1/1,000
μ = micro = 10^{-6} = 1/1,000,000
n = nano = 10^{-9} = 1/1,000,000,000
p = pico = 10^{-12} = 1/1,000,000,000,000

Appendix B: Dilutions

Good dilution skills are required for success in science (thanks Dr. Schountz). Dilutions are important because they allow quantitative measurements. Making a dilution requires a stock solution (ss)- a solution containing a high concentration of a solute (anything that dissolves in water)
diluent- solution in which the stock solution is diluted (e.g. water, saline, phosphate buffered saline)

Step 1. Find your dilution factor (df)
The dilution factor is simply a ratio of stock solution to diluent. "What you have by what you need."

(stock conc.)/(needed conc.) = df
1:df = dilution to be made

Example: ss = 50 mg/ml
need 50 µg/ml
(50,000 µg)/(50 µg) = 1,000 = 1:1,000 dilution

Step 2: Determine ss & diluent needed

1. (needed volume)/df = vol of ss
2. (needed volume) - (vol ss) = vol of diluent
3. (vol of ss) + (vol of diluent) = needed vol at needed concentration

Example: need 100 ml

1. (100)/1,000 = 0.10 ml = 100 µl of ss
2. (100) - (0.10) = 99.9 ml of diluent
3. (0.10 ml ss) + (99.9 ml diluent) = 100 ml
   (0.10) + (99.9) = 100 ml at 50 µg/ml

Appendix C: Culture Media (Modified from sigmaaldrich.com).

There are hundreds of different growth media types used in microbiology. Here is a sample of some you might encounter. Note the

Azide Blood Agar Base- used to select and isolate Streptococci and Staphylococci from fecal & sewage samples; the addition of blood helps determine hemolytic reactions

Typical Components	g/l
Tryptose	10.0
`Lab-Lemco' powder (meat extract)	3.0
Sodium chloride	5.0
Sodium azide (NaN_3)	0.2
Agar	12.0
pH 7.2	

Bile Esculin Agar - helps isolate and determine presence of Enterococci

Typical Components	g/l
Peptone	8.0
Bile salts	20.0
Ferric citrate	0.5
Aesculin	1.0
Agar	15.0
pH 7.1	

Brain heart infusion agar (BHI)- an enriched medium used for growing fastidious organisms

Typical Components	g/l
Brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose peptone	10.0
Sodium chloride	5.0
Glucose	2.0
Disodium phosphate	2.5
Agar	10.0
pH 7.4	

Brilliant Green Agar- selects for *Salmonella* species except *S. typhi*

Typical Components	g/l
Proteose peptone	10.0
Yeast extract	3.0
Lactose	10.0
Sucrose	10.0
Sodium chloride	5.0
Phenol red	0.08
Brilliant green	0.0125
Agar	12.0
pH 6.9	

Eosin Methylene Blue- used to isolate and differentiate organisms in the Enterobacteriaceae

Typical Components	g/l
Peptone	10.0
Lactose	10.0
Dipotassium hydrogen phosphate	2.0
Eosin Y	0.4
Methylene blue	0.065
Agar	15.0
pH 6.8	

Fluid Thioglycolate Medium- used to determine the different aerotolerances of different microbes

Typical Components	g/l
`Lab-Lemco' powder	1.0
Yeast extract	2.0
Peptone	5.0
Glucose	5.0
Sodium chloride	5.0
Sodium thioglycollate	1.1
Methylene blue	0.002
Agar	1.0
pH 7.2	

Hektoen Enteric Agar- used to differentiate and select *Shigella* and/or *Salmonella* species from fecal samples

Typical Components	g/l
Proteose peptone	12.0
Yeast extract	3.0
Lactose	12.0
Sucrose	12.0
Salicin	2.0
Bile salts No.3	9.0
Sodium chloride	5.0
Sodium thiosulphate	5.0
Ammonium ferric citrate	1.5
Acid fuchsin	0.1
Bromothymol blue	0.065
Agar	14.0
pH 7.5	

Lysine Iron Agar- used to help determine presence of *Salmonella* members

Typical Components	g/l
Bacteriological peptone	5.0
Yeast extract	3.0
Glucose	1.0
L-lysine	10.0
Ferric ammonium citrate	0.5
Sodium thiosulphate	0.04
Bromocresol purple	0.02
Agar	14.5
pH 6.7	

MacConkey Agar- used to isolate and differentiate coliforms from water samples or dairy products

Typical Components	g/l
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0
pH 7.4	

Mannitol Salt Agar- selects for Staphylococci

Typical Components	g/l
`Lab-Lemco' powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0
pH 7.5	

Potato Dextrose Agar (PDA)- primarily used for cultivation of fungal organisms; Sabourad Dextrose agar is also commonly used for fungi

Components	g/l
Potato extract	4.0
Glucose	20.0
Agar	15.0
pH 5.6	

Salmonella Shigella Agar- selective for Salmonella and Shigella

Typical Components	g/l
`Lab-Lemco' powder	5.0
Peptone	5.0
Lactose	10.0
Bile salts	5.5
Sodium citrate	10.0
Sodium thiosulphate	8.5
Ferric citrate	1.0
Brilliant green	0.00033
Neutral red	0.025
Agar	12.0
pH 7.3	

Sulfur Indole Motility (SIM) Medium- used to determine sulfur reduction, indole production, and motility

Typical Components	g/l
Tryptone	20.0
Peptone	6.1
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Agar	3.5
pH 7.3	

Simons Citrate Agar- helps differentiate members of the Enterobacteriaceae based on their ability to use citrate as the sole carbon source

Typical Components	g/l
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate, tribasic	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0
pH 7.0	

Tryptic Soy Agar-

Typical Components	g/l
Peptone from casein	15.0
Peptone from soymeal	5.0
Sodium chloride	5.0
Agar	15.0
pH 7.0	

Triple Sugar Iron Agar- helps differentiate members of the Enterobacteriaceae based on color changes from the various sugar fermentations and H_2S production

Typical Components	g/l
`Lab-Lemco' powder	3.0
Yeast extract	3.0
Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0
pH 7.4	

Appendix D: Best Practices for the Microbiology Lab

Several of the microorganisms used in the microbiology classroom are closely related to human pathogens. Although the strains we will use are considered non-pathogenic, you should still regard them as dangerous. To do so you must practice safe lab techniques. Failure to abide by rules will result in automatic removal from the laboratory and possible removal from the course.

- Do not eat or drink any cultures. Be aware of where your hands are relative to your mouth and nasal passages at all times.
- Food or drinks are not allowed in the lab at any time. This includes bottled water and gum.
- Upon entering the lab, clean the bench top by wetting the paper towel (not the bench) with disinfectant (instructor demonstration). When you are finished in your area, clean your area again.
- After you are finished with your lab work, wash your hands to remove any surface bacteria.
- Always treat unknown bacteria with caution and handle them appropriately.
- Petri plates should remain closed and liquids should be transferred carefully.
- Clean up spills appropriately by first placing a paper towel on top of the spill. Minimize aerosolizing bacteria.
- All used Petri dishes are to be discarded in the orange biohazard bag at the front of the room. Used broth cultures should also be brought to the front of the room and placed on the community bench.
- Any used toothpick or swab must also go into the biohazard bag.
- When sterilizing your loop pay attention. Loops will melt if kept in the bacticinerator longer than necessary. Always sterilize your loop when you are finished using it and between inoculations.
- Use gloves when instructed. There will be a few instances when gloves must be worn.

- Observe where the eye wash and nearest sink is relative to your location. Quickly wash any spill on the skin with water and/or flush eyes. Assist others if necessary.

- You may wear a lab coat if desired however, they will not be mandatory. Some of the stains we will use can damage clothes. Lab coats can be ordered online or through the chemistry department. Stains can also stain cells-even yours. Do not get stains on your skin or if you do, wash immediately.

- No sandals or shorts are allowed in lab.

- No running in lab. Do not rush your experiments.

- Always ask questions if you are unsure about a technique or lab result.

This page intentionally left blank.