MB352 GENERAL MICROBIOLOGY LABORATORY 2021



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MB352 General Microbiology Laboratory

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Licensing

*A detailed breakdown of this resource's licensing can be found in **Back Matter/Detailed Licensing**.*

CHAPTER OVERVIEW

1: Laboratory Safety

1.1: Safety Procedures for the Microbiology Laboratory

1.2: Biosafety levels and PPE

1.3: Review Questions

Review Questions II

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1.1: Safety Procedures for the Microbiology Laboratory

Learning Outcomes

- Explain & practice safe microbiological procedures, protective measures, and emergency procedures.
- Identify safe laboratory practices when working in a microbiology lab

What is Laboratory Safety?

Safety in a microbiology laboratory is important in the prevention of infection that might be caused by the microorganisms being studied. In addition, many of the reagents, equipment, and procedures used are potentially hazardous. Attention to proper procedures and prudent laboratory practices are required for your safety and protection.

This laboratory does not require the use of any highly virulent human pathogens. However, some of the organisms used are potentially pathogenic. This means that, although they may not cause disease in a normal healthy human, they might if the body's antimicrobial defense mechanisms are impaired. Impairment can arise in a number of different ways: wounds and cuts on the skin surface or lowered overall resistance to infection due to: another disease, surgery, stress, or immune system disability (including autoimmune diseases or the use of immuno-suppressive drugs). In addition, infection can occur, albeit very rarely, by relatively nonpathogenic organisms in healthy individuals.

In addition to organisms, there are some chemicals used in this laboratory that are potentially harmful. Finally, many procedures involve equipment, glassware, open flames, heat sterilizers, and sharp objects, which can cause injury if used improperly.

Although none of the organisms, procedures, or materials used in this laboratory is very dangerous, proper safety techniques and precautions should be understood and become part of your reflexive laboratory technique. The following laboratory rules and regulations should be adhered to at all times, **NO EXCEPTIONS**.

In addition, specific laboratory rules must be followed for containment of microbial cultures in the laboratory, for the safety of all. For this laboratory, these practices are listed below.

General Laboratory Safety Practices and Procedures

1. **If you are taking immune-suppressants, are pregnant, or have a known medical condition that would prevent full participation in the laboratory, please contact the course instructor before the first day of lab.**
2. Read and understand each laboratory exercise **before** you come to class.
3. Do not eat, drink, smoke, or chew pens in the laboratory.
4. You must wear close-toed shoes while in the laboratory and long pants.
5. No hats of any kind will be allowed in lab, unless allowed by University policy and cleared with the instructor.
6. Long hair should be pulled back to keep it away from bacterial cultures, bacticinerator or open flames.
7. Follow precautionary statements given in each exercise.
8. Personal electronic devices will be turned off and stored while in this laboratory. *The unauthorized use of any electronic device (phone, tablet, computer) in lab will result in a loss of course points.
9. Know where specific safety equipment is located in the laboratory, such as the fire extinguisher, safety shower, and the eyewash station.
10. Recognize the international symbol for biohazards, and know where and how to dispose of all waste materials, particularly biohazard waste. Note that all biohazard waste must be sterilized by autoclave before it can be included in the waste stream.
11. Keep everything other than the cultures and tools you need OFF the lab bench. Only necessary work material should be at or on the laboratory bench. Coats, backpacks, and other personal belongings will not be allowed on the laboratory bench top. Store them in a place designated by your instructor. This is to prevent cluttering of the workspace and to avoid exposing them to permanent stains, caustic chemicals, and microorganisms used in the exercises.



12. Leave all laboratory facilities and equipment in good order at the end of each class. Before leaving the laboratory, check to make sure the bacticinerator heat sterilizer is turned off.
13. Never, under any circumstances, remove equipment, media, or microbial cultures from the laboratory.

14. No pets are allowed in the laboratory.

Figure 1: Biohazard Symbol

Microbiology Specific Laboratory Safety Practices

During the course of the semester in the laboratory you will be taught the methods used in the proper handling of microorganisms. Although you will not be working with any that are human pathogens, exercise caution in handling all material coming in contact with live microbial cultures. All cultures should be handled with respect and proper aseptic technique *as if they were potential pathogens.* This is called **"universal precaution"**. Specific instructions that should be followed:

1. Remember that all bacteria are potential pathogens that may cause harm under unexpected or unusual circumstances. If you as a student have a compromised immune system or a recent extended illness, you should share those personal circumstances with your lab instructor.
2. Wear gloves when working with cultures, and when your work is completed, dispose of the gloves in the biohazard garbage. Lab coats, safety glasses or goggles are also required. These will be stored in the laboratory each week in a ziplock bag.
3. Disinfect your work area both BEFORE and AFTER working with bacterial cultures.
4. Cultures of live microorganisms and any material coming in contact with live cultures must be properly sterilized after use in the laboratory. Your instructor will inform you of specific procedures. Follow the general rules outlined below.

    a. Glassware such as test tubes, bottles, and flasks may be reused and washed after sterilization. These are normally placed on a cart at the front of the laboratory after you have finished an experiment or exercise. BE SURE TO REMOVE LABELS before placing any glassware on the cart. Your instructor will sterilize and then wash these items.

    b. Some materials, such as plastic petri dishes, plastic pipettes, microscope slides, and swabs, are considered disposable. These are used once and if they become contaminated by contact with live microorganisms are sterilized and discarded. All of these disposable contaminated materials should be placed in the designated waste container containing a BIOHAZARD autoclave bag.

5. Never place contaminated pipette tips (or pipettes), inoculating loop, or any other contaminated material on the bench top. Sterilize loops before and after each use. Place contaminated pipette tips in the orange biohazard buckets on your bench. Place all other contaminated materials in their designated waste containers. Do not place or put anything containing live microorganisms in the sink.
6. Aerosols should be avoided by the use of proper technique for sterilizing the inoculating loops and by performing any mixing of cultures and reagents in such a way as to avoid splashing.
7. Cultures or reagents should always be transferred with an automatic pipettor that will be provided. In no case should one employ mouth pipetting.
8. Always keep cultures capped and in proper storage racks when not being used during an exercise.
9. In the event of an accidental spill involving a bacterial culture, completely saturate the spill area with disinfectant, then cover with paper towels and allow the spill to sit for 10 minutes. Then carefully remove the saturated paper towels, dispose of them in the biohazard waste, and clean the area again with disinfectant. Notify your instructor about the spill. If the chemical is marked "danger" or "caustic" you should notify the instructor who will handle this type of spill.
10. Immediately report all accidents such as spills, cuts, burns, or other injuries to the instructor
11. Make sure that lab benches are completely cleared (everything either thrown away or returned to storage area) before you leave the lab.
12. Clothing worn in the microbiology laboratory should be washed before being subsequently worn in a facility such as a hospital, clinic or nursing home, or in an area of public food preparation.
13. In the event of a fire alarm, follow the directions of your instructor, and meet at the place designated by your instructor.

Watch these Videos on basic laboratory procedures:

*We will review specific safety procedures for our lab during our first in-person lab session



Watch Video 1: start video at 9:55 - end video at 10:26, covers different types of bench surfaces and cleaning methods



Watch Video 2: How to put on and take off gloves

NOTE: The following recommended practices and procedures for working safely on microbiology projects in a teaching laboratory environment are based on "*Guidelines for Biosafety in Teaching Laboratories*," from the American Society for Microbiology (ASM). The full documents may be viewed at this URL: https://asm.org/getattachment/3c1eb3...Guidelines.pdf

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- Microbiology: a Laboratory Experience. **Authored by**: Holly Ahern. **Provided by**: SUNY Adirondack . **Located at**: https://textbooks.opensuny.org/microbiology-a-laboratory-experience/. **Project**: Open SUNY Textbooks. **License**: *CC BY-NC-SA: Attribution-NonCommercial-ShareAlike*

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1.2: Biosafety levels and PPE

Learning Outcomes

- Recognize distinctions between Biosafety Levels (BSL) identify PPE used for working in the lab under BSL classifications
- Demonstrate and assess the proper use of Personal Protective Equipment (PPE) and be prepared to safely conduct research

BioSafety Levels

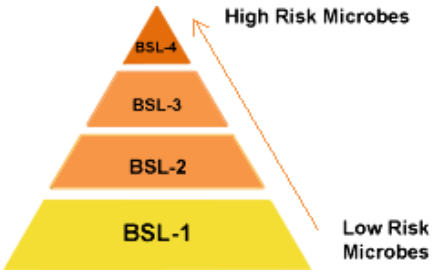


Figure 1: Biosafety levels and risk. Image from CDC. https://www.cdc.gov/training/quicklearns/biosafety/

Handling microbes requires specialized laboratory facilities and techniques. **Biosafety** is the application of safety precautions that reduce a scientists' risk of exposure to a potentially infectious microbe and limit contamination of the work environment. Bacteria pose varying degrees of risk both in a controlled laboratory environment and in their natural settings. Therefore, the level of containment necessary for working safely with bacterial cultures also varies according to a system that classifies microbes into one of four **biosafety levels (BSL)**, which provides minimum standards for safe handling of microbes at each level. BSLs are defined and containment practices are detailed by the Centers for Disease Control and Prevention (CDC) for laboratories in the United States. The full document, "*BiosafetyinMicrobiologicalandBiomedicalLaboratories,*" can be viewed in its entirety at http://www.cdc.gov/biosafety/publications/bmbl5/index.htm.

We designate most of our labs under 4 special hazard categories called biosafety levels (BSLs). Each level has specific controls for containment of microbes and biological agents. Each biosafety level builds on the controls of the level before it. All of these levels follow "*standard microbiological practices*" : which are those practices common to all labs, which include not eating, drinking, or applying cosmetics, washing hands after working in the lab, routinely decontaminating work area.

BSL1: If you work in a lab that is designated a BSL-1, the microbes there are not known to consistently cause disease in healthy adults and present minimal potential hazard to laboratorians and the environment. An example of a microbe that is typically worked with at a BSL-1 is a nonpathogenic strain of *E. coli*. Work can be done on an open lab bench, requires a sink. Requires personal protective equipment (PPE) such as lab coats, gloves, eye protection.

BSL 2: BSL-2 builds upon BSL-1. If you work in a lab that is designated a BSL-2, the microbes there pose moderate hazards to laboratorians and the environment. The microbes are typically indigenous and associated with diseases of varying severity. An example of a microbe that is typically worked with at a BSL-2 laboratory is *Staphylococcus aureus*. It includes various bacteria and viruses that cause only mild disease to humans, or are difficult to contract via aerosols in a lab setting, such as *Clostridium difficile*, most Chlamydiae, hepatitis A, B, and C, influenza A viruses, *Salmonella*. BSL-2 differs from BSL-1 in that: laboratory personnel have specific training in handling pathogenic agents and are directed by scientists with advanced training; access to the laboratory is limited when work is being conducted; extreme precautions are taken with contaminated sharp items; and certain procedures in which infectious aerosols or splashes may be created are conducted in biological safety cabinets or other physical containment equipment. **The Microbiology Teaching labs at NC State are designated as BSL-2 laboratory space.**

BSL-3: is required for work involving indigenous or exotic agents, and they can cause serious or potentially lethal disease that are transmitted through the air (via aerosols). Respiratory transmission is the inhalation route of exposure. Laboratory personnel must receive specific training in handling pathogenic and potentially lethal agents, and must be supervised by scientists competent in handling infectious agents and associated procedures. Lab personnel are under medical surveillance and might receive immunizations for microbes they work with. All procedures involving the manipulation of infectious materials must be conducted within Biosafety Cabinets (BSCs) (shown at the top image), or other physical containment devices, or by personnel wearing appropriate personal protective equipment (eg respirators). A BSL-3 laboratory has special engineering and design features that prevent the release of microorganisms to the environment. Facilities have hands free sink, exhaust air cannot be recirculated, entrance is through two sets of self closing and clocking doors. Microbes that are worked on in BSL3 facilities are *Mycobacterium tuberculosis* which cause tuberculosis, etc.

BSL-4 labs builds on the containment requirements of BSL-3 and is the highest level of biological safety. BSL-4 labs is required for work with dangerous and exotic agents that pose a high individual risk of life-threatening disease, aerosol transmission or unknown risk of transmission. The microbes in BSL4 labs cause infections that are frequently fatal and generally there are no vaccines or treatments for these infections. There are only a small number of such labs in the U.S (<10) and the world. Laboratory staff must have specific and thorough training in handling extremely hazardous infectious agents. Access to the laboratory is controlled by the laboratory supervisor. All handling of agents must be performed in a gas tight Class III Biosafety Cabinet or by personnel wearing a positive pressure protective suit. BSL-4 Laboratories have special engineering and design features to prevent microorganisms from being released into the environment. The lab is in a separate building or isolated restricted zone of the building, and has a dedicated supply and exhaust air, as well as vacuum lines and decontamination systems. Personnel mush change clothing before entering and shower upon exiting. Most of the pathogens worked on are viruses: Crimean-Congo hemorrhagic fever caused by Ebola, Junin, Lassa, Machupo, Marburg viruses , and tick-borne encephalitis virus complex (including Absettarov, Hanzalova, Hypr, Kumlinge, Kyasanur Forest disease, Omsk hemorrhagic fever, and Russian Spring-Summer encephalitis).

Personal Protective Equipment (PPE)

Personal Protective Equipment, or PPE, is the clothing and equipment that forms the last line of defense between you and harmful materials in the laboratory environment. It’s essential that you know what you should be wearing, when you should be wearing it, and how it should be stored, cleaned, maintained and disposed of. (1)

Basic PPE provided in the Microbiology laboratory includes: Disposable gloves, lab coat, safety glasses. We will have disposable face masks.

Disposable gloves serve as a barrier between your hands and any chemical, biological, or physical hazards that can enter your body through your skin. These gloves cannot protect you from all barriers, and most disposable gloves we use in a microbiology lab are made of some synthetic material, such as nitrile or latex. Any prolonged exposure to a chemical agent can permeate these types of gloves, so it is critical that you safely remove your gloves if you are exposed to a chemical spill and it comes in contact with your gloves. Gloves should be changed when they are contaminated by biological or chemical hazards. There are many other types of gloves, such as ones for handling very hot items out of the autoclave, or for very cold items out of a -80degC freezer or for handling liquid nitrogen. The type of glove you use depends on the work you are doing in the lab.

Lab coats protect the user's skin and personal clothing from accidental contact with biological or chemical hazards. They also prevent the spread of contamination outside of the lab (provided they are not worn outside the lab). Lab coats serve as a removable barrier in the event of an a spill or splash of hazardous substances. They should fit the user well (not too large or it will get caught on things and not too small as it may hinder movement), be long-sleeved, a secure cuff, knee-length or longer, fire resistant material, high buttons to provide exposure of the chest or neck area. They come in a variety of materials and provide varying degrees of protection. There are splash resistant coats, static free coats, chemical resistant coats and flame resistant coats. Most lab coats we use in a microbiology teaching lab are made of a synthetic material that is fire resistant. Always select a coat that provides the type of protection that is appropriate for your needs.

Safety glasses/goggles provide a barrier to your eyes and prevent exposure to chemical (eg chemical reagents) physical (eg dust, flying objects), and biological (eg bacterial culture splashes) hazards. Most microbiology labs have standard safety glasses or goggles that wrap around the eyes and avoid splashes, they may have features such as UV light barriers, impact features, and some are designed to be worn over your regular glasses, these are called "Over the glasses" (OTG) safety glasses. There are also labs that have high intensity light sources such as UV light, lasers and the appropriate safety glasses must be used.

Face shields should be worn whenever the entire face needs protection. Such as when there is a potential that an aerosol of chemical or biological hazardous material may be created or whenever chemical or biohazards could splatter, or whenever there is the potential for flying particles or sparks. A face shield should always be worn whenever handling tissue samples or animals where there is the potential for infectious transmission. Safety glasses or goggles should always be worn underneath a face shield for maximal protection.

Masks that are disposable may need to be worn, especially during the COVID-19 pandemic and will limit the transmission of infectious agents. Disposable masks come in a variety of materials and levels of protection. Typical facemasks are loose-fitting, disposable masks that cover the nose and mouth, such as surgical masks and nuisance dust masks. Facemasks are not approved by the National Institute for Occupational Safety and Health (NIOSH) for protection against any regulated hazardous material.

Facemasks help stop droplets from being spread by the person wearing them. They also keep splashes or sprays from reaching the mouth and nose of the person wearing the facemask and are therefore useful when cleaning up spills of infectious materials. They are not designed to protect you against breathing in gases, vapors, or very small particles. Facemasks should be used once and then disposed of. There are different types of masks used for different purposes, specialty disposable face masks such as the N95 or KN95 masks are respirators which are approved by NIOSH for use against certain selected airborne particulates when used as part of a respiratory protection program.

Watch video 1

Video 1: proper usage of PPE. Your instructor will explain how to locate, use, and store your PPE in the lab.



Watch Video 1: WHO: Good Microbiological Practices & Procedures. Personal Protective Equipment (PPE) (19:41)
URL: https://youtu.be/Cuw8fqhwDZA

References:

1. WHO. World Health Organization Biosafety Series. https://www.who.int/ihr/publications...deo-series/en/

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1.3: Review Questions

1. Discuss 2 safety precautions in a BSL-2 lab.

2. Why do we have to tie long hair back in a microbiology lab? give me 2 reasons

3. True or False? Food and drinks are allowed in the lab, as long as they are kept out of sight.

4. Why must microbiology tools and implements be sterilized?

5. Describe 2 pieces of PPE and why they are important in a microbiology lab.

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Review Questions II

"I would literally fall asleep if I was having a conversation or doing anything that involved my brain," she says.

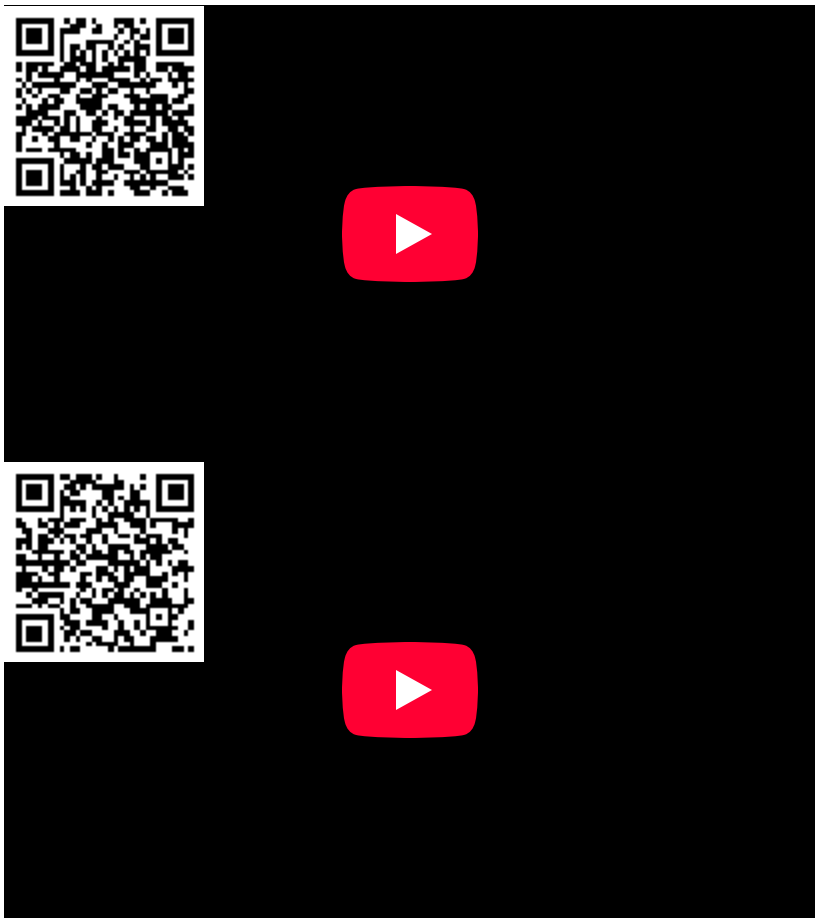
"I would literally fall asleep if I was having a conversation or doing anything that involved my brain," she says.

"I would literally fall asleep if I was having a conversation or doing anything that involved my brain," she says.

lab safety is important

UrL: https://ehs.stonybrook.edu/programs/...b-safety-guide

watch this video



watch video 1: General lab safety by the Amoeba sisters. URL: https://youtu.be/MEIXRLcC6RA

Lab safety Part III

lab safety is important

Lab safety Part I

Exercise 1

Add exercises text here.

Answer

Add texts here. Do not delete this text first.

Review this

Learning Objectives

- Explain the following laws within the Ideal Gas Law

dwreewrew

Learning Objectives

- Explain the following laws within the Ideal Gas Law

Note

The ideal gas law is easy to remember and apply in solving problems, as long as you get the **proper values a**

Example 1

Add text here.

Solution

Add text here.

a;ioejem;wemf

kafj;eoijeoirj

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Review questions III

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CHAPTER OVERVIEW

2: Cultivation of Microbes

2.1: Introduction Growth Media

2.2: Introduction to Bacterial Growth and Aseptic Techniques

2.3: Examples of Bacterial Growth Characteristics in Broths, Slants and Plates

2.4: Lab Procedures- Prepare solid media, Aseptic Technique, T-streaking

2.5: Results

2.6: Review Questions

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2.1: Introduction Growth Media

Learning Outcomes

- Recognize various types of growth media: solid, broth.
- Understand the uses of selective and differential growth media
- Determine the properties of some common bacterial types when grown on selective and differential growth media

Growth Media

To study bacteria and other microorganisms, it is necessary to grow them in controlled conditions in the laboratory. Growth media contain a variety of nutrients necessary to sustain the growth of microorganisms. There are two commonly used physical forms of growth media: liquid media and solid growth media. A liquid medium is called a **broth** (image 2).

Solid growth media usually contains **agar** (image 1), which is a mixture of polysaccharides derived from red algae. It is used as a solidification agent because it (1) is not broken down by bacteria, (2) contains no nutrients that can be used by bacteria and (3) melts at high temperatures, and yet is solid at temperatures used for most bacterial growth. Solid growth media is used in the following forms: agar plates, agar slants, and agar deeps. To make agar deeps or agar slants, melted agar is poured into a test tube and then allowed to solidify vertically (agar deep), or at a slant (agar slant). Agar plates are made by pouring melted agar into a petri dish.



Image 1: Solid Agar slant

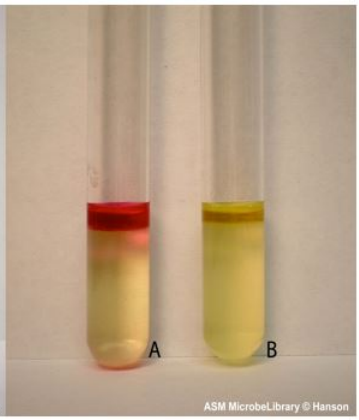


Image 2: Broth media in test tube. Images by Anne Hanson, University of Maine, Orono.

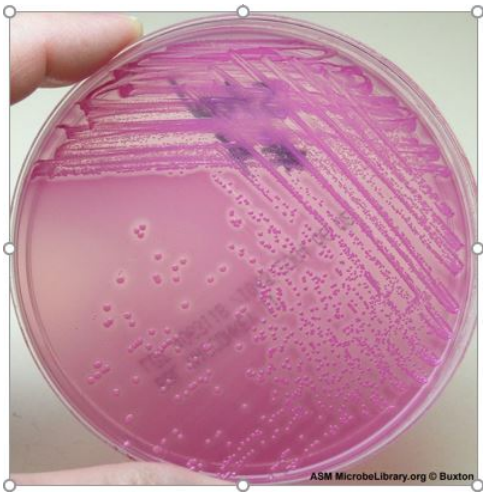


Image 3: MacConkey solid agar plate with pure culture of *Salmonealla* species bacteria. Image by Rebecca Buxton, University of Utah, Salt Lake City, UT.

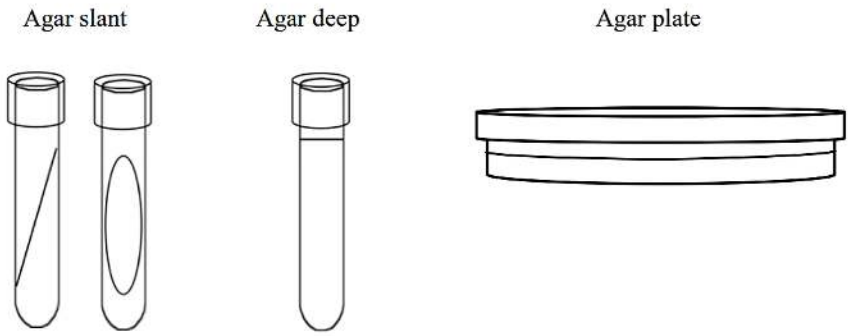


Figure 1:
Solid growth media forms

Broths can be used to determine growth patterns in a liquid medium, and for certain types of inoculations and metabolic tests. They are also the method of choice for growing large quantities of bacteria. Agar slants are commonly used to generate stocks of bacteria. Agar plates can be used to separate mixtures of bacteria and to observe colony characteristics of different species of bacteria . Deeps are used for several different types of differential metabolic tests (e.g., the caseinase hydrolysis test)

Types of Growth Media

Growth media can be categorized based on their chemical constituents, or the purpose for which they are used.

- **Complex (also called Rich) growth media** contain ingredients whose exact chemical composition is unknown (e.g. blood, yeast extract, etc.) Eg TSA, LB agar.
- **Synthetic** (also called **chemically defined**) **growth media** are formulated to an exactly defined chemical composition.

- **Example:** A **general purpose growth medium:** e.g. tryptic soy agar (TSA) or Luria broth (LB) is used to grow a wide variety of non-fastidious bacteria. This type of medium is often **a complex growth medium.**



Image 4: General purpose, Complex/Rich media: LB agar plate streaked with *Bacillus cereus* and incubated at room temperature for 24 hours. Image by Kevin Hedetniemi and Min-Ken Liao, Furman University, Greenville, SC.

Specialized media types

- A **selective growth medium** contains chemicals that allow some types of bacteria to grow, while inhibiting the growth of other types. An example of a purely selective growth medium is PEA, phenylethyl alcohol agar, which allows Gram positive bacteria to grow while inhibiting the growth of Gram negative bacteria.



Image 5: *Staphylococcus aureus*, a Gram positive organism, grows on this PEA plate while *Serratia marcescens*, a Gram negative organism, does not. Image by WelcometoMicrobugz. URL: https://www.austincc.edu/microbugz/p...cohol_agar.php

- A **differential growth medium** is formulated such that different types of bacteria will grow with different characteristics (e.g. colony color). An example of a differential growth medium is blood agar, which differentiates among bacteria based on their ability to break down red blood cells and hemoglobin. Blood agar is also a complex growth medium because it contains blood.



Image 6: Blood agar plate. Image by Rebecca Buxton, University of Utah, Salt Lake City, UT.

- A growth medium can be both **selective and differential.** For example, **EMB (eosin methylene blue agar)** inhibits the growth of Gram-positive bacteria. Gram negative bacteria that grow on this medium are differentiated based on their ability to ferment the sugars lactose and sucrose. Strong fermenters of lactose or sucrose will produce large amounts of acid and will appear dark purple to black. *Escherichia coli*, a strong fermenter will frequently appear as colonies with dark black with a green metallic sheen (Image 7). Weaker fermenters will produce pink colonies. Clear or colorless colonies indicate that bacterium does not ferment either sugar and is not a fecal coliform. (Note: the Gram staining procedure divides bacteria into 2 main groups: Gram-positive bacteria and Gram-negative bacteria, based on their cell wall structure.).

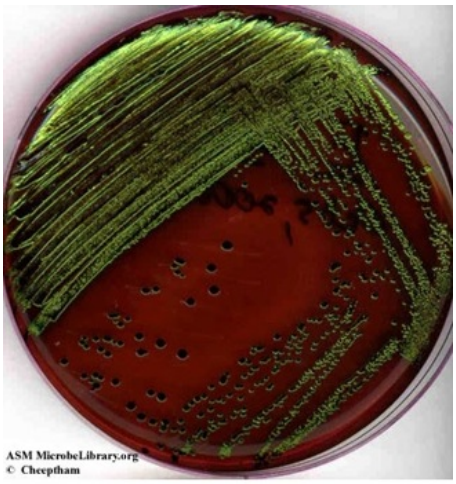


Image 7: Eosin-methylene blue (EMB) agar plate inoculated with *Escherichia coli* (a Gram-negative coliform bacterium) showing good growth of dark blue-black colonies with metallic green sheen indicating vigorous fermentation of lactose and acid production which precipitates the green metallic pigment. (Naowarat Cheeptham, Thompson Rivers University, Kamloops, BC, Canada)

Making Media

I. Making culture media requires patience and attention to detail. Watch Video 1: Making Microbiological Media



Watch Video 1: Making Microbiological Media by Bio-Rad. URL:https://youtu.be/BH4ESgWU_Eo

II. Pouring Agar plates. Watch Video 2: Solid Media Preparation, video was filmed at NC State Microbiology labs.



Watch Video 1: Solid media preparation, video was filmed at NC State Microbiology labs. URL:https://youtu.be/P7M_MCXbZjc

Recap: Media Types

Tryptic soy agar (TSA): General purpose rich/complex growth medium.

Mannitol-salt agar (MSA): Differential and selective growth medium. This medium contains 7.5% NaCl, the carbohydrate mannitol and the pH indicator phenol red (yellow at pH 8.4). It is selective for staphylococci due to the high concentration of NaCl, and differentiates based on the ability to ferment mannitol. Staphylococci that ferment mannitol produce acidic byproducts that cause the phenol red to turn yellow. This produces a yellow halo in the medium around the bacterial growth.

Table 1: MSA Agar

Selectivity	Interpretation	Identification
Growth	Growth Organism not inhibited by NaCl	E.g., *Staphylococcus*, *Micrococcus*
No Growth	Organism inhibited by NaCl	Not *Staphylococcus*
Differentiation		
Yellow Halo	Organism ferments mannitol	Probable *S. aureus*
No Yellow Halo	Organism does not ferment mannitol	*Staphylococcus* species (other than *S. aureus*); *Micrococcus* (yellow colonies)

Eosin-methylene blue agar (EMB): Differential and selective growth medium. This medium contains peptone, lactose, sucrose and the dyes eosin Y and methylene blue. Gram positive organisms are inhibited by the dyes, so this medium is selective for Gram negative bacteria. The medium differentiates based on the ability to ferment lactose (and/or sucrose.) Organisms that cannot ferment either of the sugars produce colorless colonies. Organisms that ferment the sugars with some acid production produce pink or purple colonies; organisms that ferment the sugars and produce large amounts of acid form colonies with a green metallic sheen. This medium is commonly used to detect the presence of fecal coliforms (like *E. coli*)—bacteria that grow in the intestines of warm-blooded animals. Fecal coliforms produce large amounts of acid when fermenting lactose and/or sucrose; non-fecal coliforms will produce less acid and appear as pink or purple colonies.

Table 2: EMB Agar

Result	Identification	Interpretation
No or poor growth	Organism inhibited by dyes	Organism is Gram-positive
Good growth	Organism not inhibited by dyes	Organism is Gram-negative
Colorless growth	Organism does not ferment sucrose or lactose	Non-coliform
Growth is pink and mucoid	Organism ferments lactose and/or sucrose with some acid production	Coliform bacteria
Growth is dark (purple to black with or without green metallic sheen)	Organism ferments lactose and/or sucrose, with large amounts of acid production	Possible fecal coliform (E. coli)

References

1) Dr. Gary Kaiser (COMMUNITY COLLEGE OF BALTIMORE COUNTY, CATONSVILLE CAMPUS)

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2.2: Introduction to Bacterial Growth and Aseptic Techniques

Learning Outcomes

- Describe general characteristics of bacterial growth on agar plates
- Explain how to inoculate growth media using proper aseptic procedures
- Describe the process for inoculating sterile media
- Describe the procedure (T-streak) for isolation of single bacterial colonies

Characteristics of Bacterial Growth

Even on general purpose growth media, bacteria can exhibit characteristic growth patterns. On agar plates, bacteria grow in collections of cells called **colonies**. Each colony arises from a single bacterium or a few bacteria. Although individual cells are too small to be viewed, masses of cells can be observed. Colonies can have different forms, margins, elevations, and colors. Observing colony characteristics is one piece of information that microbiologists can use to identify unknown bacteria. Shown below are isolated colonies of *S. aureus* on a blood agar plate. **Colonies that are visible to the human naked eye contains tens of thousands or even millions of individual bacteria!!**

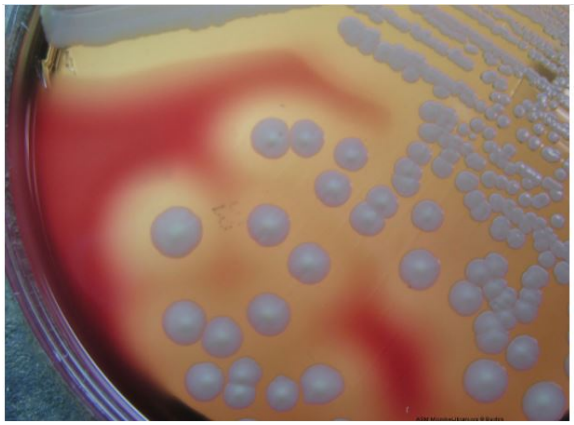


Image 1: Notice individually isolated colonies: Large, creamy white, circular, beta-hemolytic colonies typical o*f Staphylococcus aureus* cultured on Blood agar. Image by Rebecca Buxton, University of Utah, Salt Lake City, UT.

Colony morphology can be an aid in the identification of microorganisms.

Although colony morphology cannot be employed as the sole identifying criterion, it is a useful trait in the classification of many common types of microorganisms. Six parameters are normally used to describe microbial colonies growing on an agar surface:

a. *Size:* pinpoint, small, medium, or large; range: < l mm - 3cm

b. *Color:* absolutely white, various degrees of pigmentation

c. *Texture:* the texture of the colony as determined by touching the colony with a needle; smooth (buttery), dry (granular), or mucoid (slimy) and the appearance as judged by the manner in which the colony refracts light; clear, glistening, dense, opaque, or translucent.

d. *Form:* the shape of the colony; circular, irregular, filamentous, or rhizoid

e. *Elevation:* the degree to which colony growth is raised; flat, raised, convex or umbonate

f. *Margin:* the shape of the edge or margin of the colony

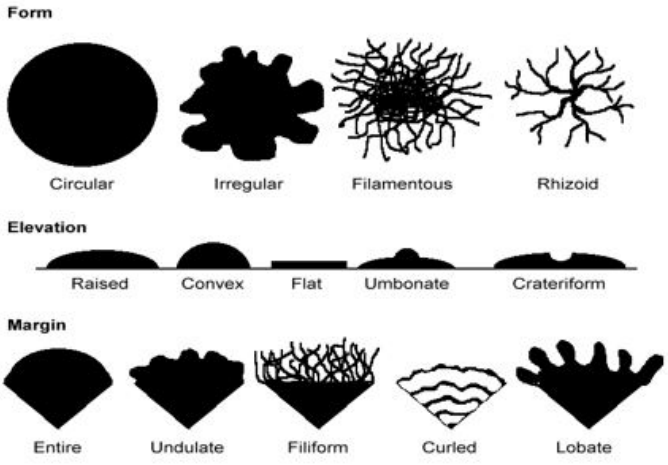


Figure 1: Different colony morphologies/characteristics

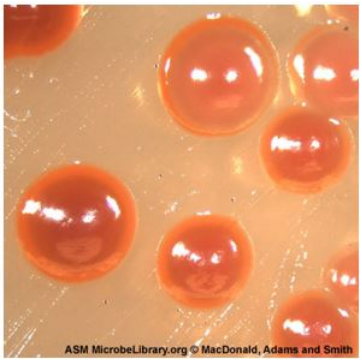


Image 2: Example of circular formed colonies--*Serratia marcescens colonies* cultivated on trypticase soy agar. Image by Bryan MacDonald, Christopher Adams, and Kyle Smith, Brigham Young University, Provo, UT.

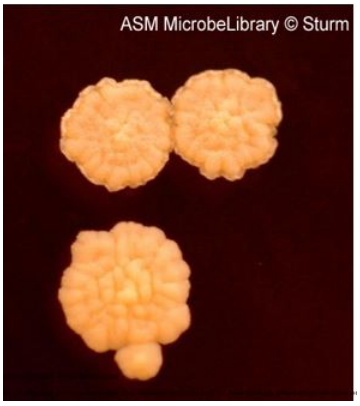


Image 3: Undulate form colonies-- Streak plate isolation of *Mycobacterium smegmatis* on trypticase soy agar (TSA) incubated for 96 hours at 37oC. Note the rough texture of colonies characteristic of this organism. Image by Tasha L. Sturm, Cabrillo College, Aptos, CA.

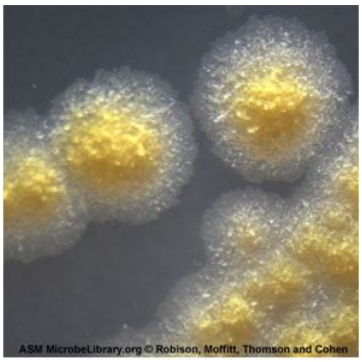


Image 4: Irregular form colonies-- *Mycobacterium marinum* cultivated on Mycobacterium 7H11 agar with oleic acid-albumin-dextrose-catalase enrichment. Image by Richard A. Robison, Gable Moffitt, Neal Thomson, and Marissa Cohen, Brigham Young University, Provo, UT.

Aseptic Technique and Inoculation

In nature, microorganisms usually exist as mixed populations of different species of bacteria, fungi, and even viruses. If we are to study, characterize, and identify microorganisms, we must have the organisms in the form of a pure culture, that is of only one species of microorganism. A **pure culture** is one in which all organisms are descendants of the same organism. In working with microorganisms we must also have a sterile nutrient-containing-medium in which to grow the organisms. Anything in or on which we grow a microorganism is termed a **medium**. A **sterile** medium is one which is free of all life forms. It is usually sterilized by heating it to a temperature at which all contaminating microorganisms are destroyed. Finally, in working with microorganisms, we must have a method of transferring growing organisms (called the **inoculum**) from a pure culture to a sterile medium without introducing any unwanted outside contaminants. This method of preventing unwanted microorganisms from gaining access is termed **aseptic technique**. (1)

Inoculation is the purposeful introduction of bacteria into a **sterile** growth medium. A material is sterile when it has no living organisms present; **contamination** is the presence of unwanted microorganisms. **Aseptic techniques** are practices that prevent the contamination of growth media.

When working in a microbiology laboratory, you must always remember that bacteria are present on all surfaces in the lab, as well as on your own hands and clothing. Aseptic techniques are designed to prevent the transfer of bacteria from the surrounding environment into a culture medium. These techniques require care and concentration. Pay attention to what you are doing at all times!

Aseptic techniques include the following practices:

1. Minimize the time that cultures and growth media are open to the environment.

2. Disinfect the work area before and after use.

3. Do not touch or breathe into the sterile culture media or the stock cultures.

4. Loops, needles, pipets, etc. should be sterilized before they are used.

5. When working with tubes, the tube caps should not be placed on the table top; they should be held in your hand while inoculating.

6. When removing the caps from test tubes, heat/flame the lip of the test tube after the cap is removed. This heats the air inside the tube, so the air moves out of the tube, preventing contaminants from entering the tube.

7. Information about the use of the bacticinerator can be found in the Procedures page and view videos 2 and 3 below.

Watch this video 1: Aseptic Technique Tips



Watch Video 1: Aseptic Techniques tips. Video by Dr. Gary Kaiser (CCBC). (5:56) URL: https://youtu.be/_tMM0F0Pr60

General Procedure for inoculating media

1. Sterilize an inoculating loop or needle in the flame of a Bunsen burner. The portion of the loop or needle that will contact the stock culture or the growth medium must turn bright orange for effective sterilization. For the most rapid sterilization, place the loop at the top of the inner blue cone of flame—this is where the temperature of the Bunsen burner is the hottest. Or if it is a bacticinerator, make sure the loop is in the body of the bacticinerator and heat for 10 sec. Remove the loop from the flame after it is properly heated- keeping the loops in the flame for too long will eventually cause them to crack.

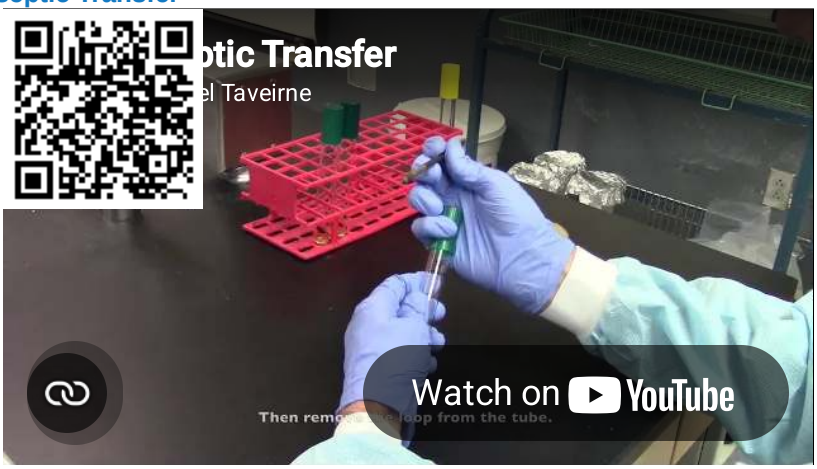
2. If you are picking a colony from a plate, cool the inoculating loop on agar that does not contain any bacterial colonies.

3. Pick a small amount of bacteria (you do not need much). If you are inoculating a tube of broth or an agar slant, remove the cap of the tube (do not set the cap down on the table) and flame the lip of the tube. Throughout the procedure, hold the tube at an angle to reduce the probability of particles entering the opening. Insert the loop into the tube and transfer bacteria to the growth medium. Be careful that only the sterilized part of the loop touches the tube or enters the growth medium.

4. Flame the lip of the test tube before replacing the cap.

5. Sterilize the inoculating loop again.

Watch this video 2: Aseptic Transfer



Watch Video 2: Aseptic transfers: a variety of transfers from solid to liquid. This video was filmed in the Microbiology teaching labs at NC State. (7:39) URL: https://youtu.be/sFbJmJeTIFc

Streaking for single colonies: T-streak

In the real world outside the laboratory, bacteria grow in communities made of many bacterial species. If you need to identify the types of bacteria present in environmental or medical samples, you must have a way to separate out the different types and produce pure cultures. A **pure culture** contains a single bacterial species, whereas a **mixed culture** may contain many different types of bacteria. The **T-streak method** describes the method that you will use to separate different types of bacteria in a mixture.

To maintain a pure culture or to obtain a pure culture of a microorganism, a quadrant/T/isolation/streak plate is performed. The T-streak is a form of dilutions on a solid surface (Franklund, 2018). In this technique, the plate is divided into sections. Bacteria are deposited in the first section at full strength from the original source. Then the inoculating loop is sterilized. From this point on, no *additional* cells are added to the agar surface. The sterile loop is used to spread out cells that have already been placedon the plate. After spreading cells from the first area to the second, the loop is sterilized again. This eliminates extra cells from the loop. The sterile loop is used to spread some cells from the second area into the third area diluting them further. (In a quadrant streak, cells are spread into a forth area as described.) After all the regions have been inoculated, the hope is that in the last section cells are far enough apart so that they grow up into isolated colonies. This technique allows one to observe isolated colonies and characterize them and determine if your observations are consistent with our expectations for the organism you are working with. If you are working with a pure culture, you would expect that all the colonies would look the same, similar size, color, shape etc. One or more different looking colonies indicates your culture was contaminated or you created contamination by poor aseptic technique.

Recall that each individual colony that you see on an agar plate represents billions of bacteria that originated from a single cell, and therefore should be clones of each other. If you want a pure culture then, there should only be 1 species or strain of that bacterium on that plate. We typically do a T-streak in the lab where you draw a large “T” on the back of the agar plate to divide it into 3 sectors. Watch video 3 below for a demonstration.



Image 5: Agar plate with T-streak of *E. coli* . This is a pure culture and notice the isolated colonies in the last sector of the plate, they appear as single, individual, round colonies. Image by Rebecca Buxton, University of Utah, Salt Lake City, UT.

Watch video 3: Isolating bacterial colonies using a T-streak method

Watch video 3: Isolating bacterial colonies using a T-streak. Excellent summary video of many things we talked about in this section: the difference between a cell vs a colony, how to separate bacterial species using a T-streak isolation method, common mistakes when doing a T-streak, and the results of a T-streak. They use disposable loops here and sterilize the loop with 70% ethanol between each section when doing the T-streak. You can also discard the loop (in biohazard) when streaking between sections, or use a metal loop (usually made of nickel-chrome wire) that you sterilize using a bunsen burner or a bacticinerator.
Video by ID laboratory videos (9:17) URL: https://youtu.be/c1onYow0O58

Watch Video 4: Doing a T-streak at NC State



Watch video 4: How to perform a T-streak for isolated colonies. This video was filmed in the Microbiology laboratories at NC State. It will familiarize you with the use of a bacticinerator for sterilizing a metal inoculating loop. (7:39)
URL:https://youtu.be/NsQv7QOmdXo

Notes about Labeling and Incubating Plates

1. Always label your plates/tubes BEFORE you do your inoculations. You can use Sharpies on the plates, but wax markers ONLY on tubes. When labeling tubes, label the tube itself—don't label the cap!

2. Make sure you label the **bottom** of the plates (the part of the plate that holds the agar).

3. Place plates inverted (upside down) for incubation. This prevents condensation from falling on the surface of the agar and disrupting the streaking pattern.

Contributions and Attributions

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2.3: Examples of Bacterial Growth Characteristics in Broths, Slants and Plates

Even on general purpose growth media, bacteria can exhibit characteristic patterns of growth. Some examples are shown below. While these growth patterns are an important piece of information when identifying a bacterial species, they are not sufficient for a positive identification. Staining procedures and metabolic tests must be used for a definitive identification.

Growth Characteristics in Broths

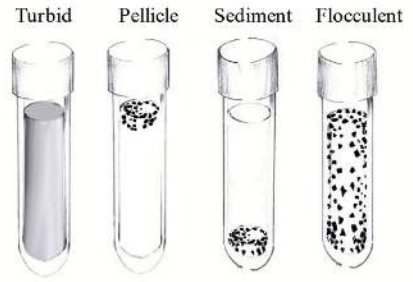


Figure 2.6.1: *Bacterial growth in broths*

Growth Characteristics on Slants

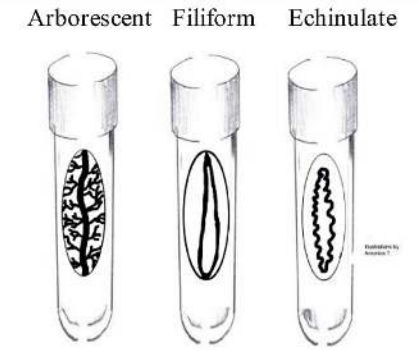


Figure 2.6.2: *Bacterial growth on slants*

Growth Characteristics on Plates

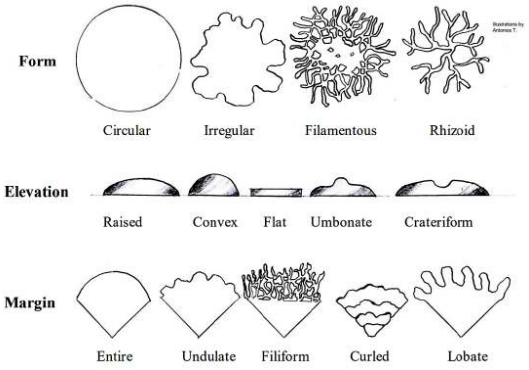


Figure 2.6.3: *Bacterial growth on plates*

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2.4: Lab Procedures- Prepare solid media, Aseptic Technique, T-streaking

Learning outcome

- To acquaint you with the two types of culture media, **Nutrient broth** and **Nutrient agar**.
- To familiarize you with aseptic technique.
- To learn how to isolate a pure culture.
- To observe the ubiquity and diversity of microorganisms

Aseptic Technique

The average human is made up of approximately 10^{13} eukaryotic animal cells and 10^{14}associated eukaryotic and prokaryotic microbial cells. This means that 90% of "your" cells are not human.

The purpose of this exercise is to familiarize you with aseptic techniques. Aseptic technique can be thought of as the manipulation of sterile instruments or culture media in such a way as to maintain sterility.

In nature, microorganisms almost always exist as mixed populations of many widely differing types. However, before most properties and characteristics of a particular organism can be determined, the organism must first be isolated in **pure culture.** A pure culture is one that contains a single strain of microorganism. For example, a pure culture of the bacterium *Escherichia coli* contains only *Escherichia coli* cells of a particular strain - no other living microorganisms are present. The term strain refers to a population of cells all descended from a single cell. The isolation and maintenance of pure cultures is one of the most important procedures in microbiology, and one with which all students must be familiar.

Like all other organisms, microorganisms require nutrients and a favorable environment to grow and multiply. Microbes are generally grown in a **culture medium** that contains essential nutrients and provides a suitable environment (e.g. proper pH). Microbes are essentially grown in a liquid medium**, broth**, or on a solid medium, **agar.**

Since most laboratory studies are made with pure cultures, it is necessary to **sterilize** culture media, that is, completely eliminate all living organisms. This medium must be maintained in a **sterile** condition, free from living organisms, until inoculated with a pure culture.

To grow a microbial culture in a sterilized medium, a number of the cells, the inoculum, are transferred, inoculated, into or onto the medium with special precautions to maintain the purity of the culture. The procedures used in the microbiology laboratory to prevent contamination of pure cultures are commonly referred to as **aseptic technique**.

The general rules in following aseptic technique are: 1) put nothing into sterile material that is not itself sterile, except the specific organism you are studying and, 2) do not expose sterile materials to sources of contamination, for example, laboratory air.

Following inoculation, a microbial culture is grown **(incubated)** in a specific environment favorable for growth. Growth of microbes is usually defined in terms of **population growth**, an increase in numbers of cells in the population, as opposed to **cellular growth,** an increase in the size of an individual cell. The resultant growth is visible as cloudiness, turbidity, in a liquid medium or as an isolated mass**, colony**, on a solid medium.

Materials:

Cultures

Escherichia coli (broth culture)

Serratia marcescens

mixed broth culture of *Escherichia coli* and *Serratia marcescens*

Media

one bottle sterile Nutrient agar

three tubes sterile Nutrient broth

three Nutrient agar plates, pre-dried for 2 days at room temperature

Supplies

four sterile plastic petri dishes

sterile cotton swabs

The purpose of this exercise is to familiarize you with aseptic techniques. All the procedures have been designed to minimize contamination. Observe the demonstration by your laboratory instructor and follow the procedures outlined in the manual exactly. Place the bacticinerator in front of you and put all tubes and other equipment in a suitable location that will allow you to reach them without any difficulty and without burning yourself.

At first these procedures for manipulating the loop, tubes, and caps will be difficult, but with practice these manipulations will become more rapid and less cumbersome.

Operation of the Bacticinerator:

The bacticinerator is a gasless, flameless, heat sterilizer for use with inoculating loops, needles, glass tube/pipette mouths and various metal & borosilicate glass instruments. Sterilization is accomplished through infrared heat. **OPERATION:** Turn on the toggle switch to the **"High" position**; the red indicator light will come on when the switch is in the correct position. **Sterilizer must be heated for 15mins prior to use.** Holding the inoculating loop by the handle, gently insert inoculating loop to be sterilized into cylindrical sterilization area (see image below). Always ensure that the item is held with an insulated material. Do not directly touch the sterilized item. Do not "park" the loop in there unintended, it will **MELT.** Avoid scraping sides of element to ensure the longevity of the loop and heater element. **Hold loop for 5 seconds.** It is not necessary for loop to be glowing. The outer, metal shield of the heater element can reach temperatures as high as 400°F. **Do not touch outer metal shield** or introduce any flammable or volatile materials in the area surrounding the sterilizer.

Turn off bacticinerator after you have finished using it for the lab exercises for the day.



Lab Procedures:

A. Preparation of Solid Media

Solid medium is usually made by adding a solidifying agent to a broth medium. The most common solidifying agent is agar, a substance obtained from marine algae and available in dried purified form. Although different agars vary considerably in their physical properties, the usual melting point is 97-100°C. Thus, solid agar can be added to liquid culture media and melted during heat sterilization. After sterilization, this liquid media can be poured into test tubes, bottles, or petri dishes. On cooling, this medium containing agar solidifies at about 42°C. Once solidified, agar media may be incubated over the entire range of temperatures a bacteriologist is likely to use (up to 70°C, perhaps) without melting.

An **agar slant** results when the hot molten agar media is allowed to harden in a test tube held in a tilted position. Pouring a molten agar medium into plastic or glass Petri dishes makes **agar plates**.

1. Place the four sterile petri plates right side up in front of you.

The bottom of a petri plate is smaller and deeper than the top, often called the lid. Therefore, right side up is when the bottom is in contact with the bench and the larger lid covers the bottom.

2. Turn on your bacticinerator.

3. You must work quickly now to prevent the agar from solidifying before you are finished pouring the plates. Remove a bottle of melted Nutrient agar medium from the 55°C water bath. Carefully wipe the adhering water from the bottle so that, when you tip the bottle, this contaminated water will not run into the plate.

4. Loosen the cap of the bottle but do not remove it. When the cap is loosened all the way, remove it by encircling the cap with your right or left little finger and the outside edge of your palm. See Figure 1-1 a. Do not set the cap down on the bench-top.



Figure 1-1a. Aseptically removing a bottle cap, freeing the rest of the hand to manipulate a plate lid, pipette, etc.

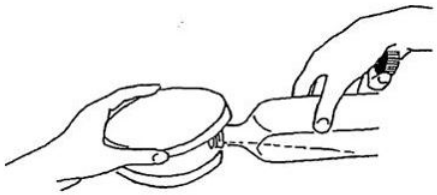


Figure 1-1b. Agar poured into a petri dish. The lid shields the lower plate and its contents from airborne contamination.

5. While still holding the cap, grasp the bottle securely with the thumb and forefinger of your right hand. See Figure 1. Lightly pass the lip of the bottle in front of the bacticinerator to burn off any adhering dust and to also to set up a negative air current. The lip of the bottle would be sterile from the autoclaving procedure.

6. Lift the lid of a petri plate with your left or right hand just enough to allow you to pour some of the agar medium into the bottom of the petri plate. See Figure 1-1b.

7. Pour enough medium into the petri plate bottom to just cover the bottom surface. (You should be able to easily pour four plates and possibly five with 100 mL of medium.)

8. Replace the lid to cover the bottom of the petri plate.

9. Carefully and slowly slide the poured plates to an undisturbed (back) portion of your bench-top to cool and solidify. Do not disturb these plates for at least 20 minutes.

10. These plates will be used in part D of this exercise.

B. Aseptic Transfers

To the microbiologist, transfer of cultures from tube to tube and from agar plates to tubes is a common and simple procedure but requires careful attention to certain details. Transfer of microorganisms is often done using a wire loop. Proper use of this inoculating loop will help insure maintenance of a pure culture.

1. Label the 3 tubes of sterile Nutrient broth. Label one "sterile control", one "broth transfer", and one "colony transfer".

2. The broth cultures of *Escherichia coli* and plate culture of *Serratia marcescens* are at the end of your bench.

3. Holding your loop like a pencil insert the loop into the cylindrical area of the bacticinerator. Heat only the wire in the sterilizer, **NOT** the wire holder. If done properly it should only take 5 seconds for the entire length of the wire to be sterile. Once this has occurred, remove the loop. The wire will now be **sterile and very hot.** Allow the loop to cool for several seconds in the air before using it. This avoids killing the cells to be transferred and splattering that can lead to the production of contaminating aerosols.

3a. Now pick up the tube containing the pure culture *of Escherichia coli* with your other hand, while still holding the **sterile** loop. With the hand holding the loop, remove the cap from the culture tube by encircling it with your little finger and the outer edge of your palm. See **Figure l**. **DO NOT** put the cap down on your bench. Lightly pass the lip of the tube in front of the bacticinerator.

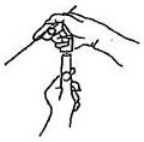


Figure 1. Removal of a tube cap while holding a loop.

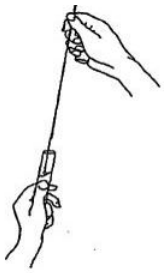


Figure 2. Obtaining inoculum from a broth tube while holding the cap and maintaining cap sterility

4. Now insert the loop into the *E. coli* broth culture and then remove it, carrying out a loopful of culture from the tube. See **Figure 2**. Replace the tube cap and return the tube to the storage rack.

5. Pick up and remove the cap from the tube of sterile Nutrient broth labeled "broth transfer" in the same manner as outlined in step 3a above.

6. Tilt the tube at about a 45°angle and insert the loop containing the culture into the open tube. Immerse the loop in the broth, swirl the loop gently, then remove it. Pass the tube in front of the bacticinerator and replace the cap and return the tube to the storage rack.

7. Sterilize the loop before putting it down by repeating step 3. This is necessary to avoid contaminating of the bench with the culture.

8. Now, with your other hand, lift the lid of the petri plate containing a pure culture of the *Serratia marcescens.* Obtain an inoculum by **JUST TOUCHING ONE** of the isolated single colonies on the agar surface. **Do not dig into the agar. Do not scrape the loop across the surface of the plate.** Replace the lid of the plate immediately.

9. Pick up and remove the cap from the tube of sterile Nutrient broth labeled "colony transfer" in the same manner as outlined in step 3a above.

10. Repeat steps 6 and 7.

11. To test your ability to properly sterilize your loop, pick a loopful of the culture from the *E. coli* broth culture tube following the procedures outlined in steps 3, 3a, 4. Properly sterilize the loop containing the bacterial culture, as in step 7.

12. Pick up and remove the cap from the tube of sterile Nutrient broth labeled "sterile control" in the same manner as outlined in step 3a above and insert the loop into the broth as described in step 6.

13. Repeat steps 11 and 12 a few times with the same "sterile control" tube.

C. Streak Plates

The streak plate technique is a rapid and simple method of isolating bacteria by mechanically spreading them over the agar surface of a petri plate. The purpose is to obtain well-isolated colonies, **each arising from a single bacterium,** so that a pure culture of each desired species in a mixture can be established. Proper streaking of plates is a relatively simple yet indispensable skill required of any microbiologist.

The basic method for the preparation of a streak plate involves the spreading of a single loopful of inoculum over the surface of the agar medium. There are many different techniques used for streaking plates. Choosing a technique is a matter of individual preference and judgment. Any method can be used as long as the objective, production of well-isolated colonies, is attained and there is no evidence of contamination. The procedure described below is commonly called a "T streak", after the marking made on the bottom of the plate.

1. Draw one line, approximately one third of the way down, across the entire **BOTTOM** of the plate. Then draw a second line from the middle of the first through the center to the other side of the plate. This should make a large "T" on your plate, dividing it into three sectors. See Figure 1-4.

Figure 1-4

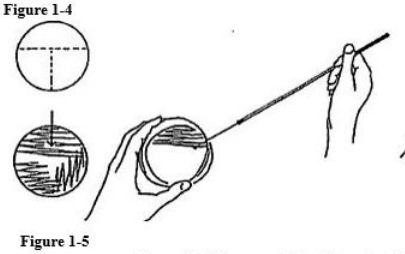


Figure 1-5

Figure 1-6a. Downward streaking of a plate with the upper lid ajar. The lid shields the agar from airborne contamination

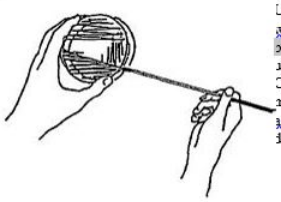


Figure 1-6b. Upward streaking while holding the bottom dish upside-down. Contamination is minimized as the agar surface faces downward.

2. Label the **bottom** of each of the 3 prepared Nutrient agar plate with your name or initials, your lab section, and the bacterial species to be used. In addition, label one "from broth", one "from agar", and one "mixed culture".

3. Use the aseptic techniques you learned in part B. **Sterilize the loop and allow it to cool**.

Obtain an inoculum of bacterial cells from:

- the broth tube containing a pure culture of *Escherichia coli*
- the agar plate of *Serratia marcescens*
- the broth tube containing a mixed culture supplied by your instructor.

When obtaining samples from colonies growing on agar plates, it is **ONLY** necessary and is advised that the loop **JUST TOUCHES THE SURFACE** of **ONE** colony.

When obtaining samples from broth cultures, be sure to **MIX** the contents to ensure even distribution of microorganisms.

4. Inoculate the surface of the agar plates using the pattern shown in figure 1-5 and the technique described below.

a) deposit the inoculum by touching the loop to the surface of the plate near the edge, in the top sector of the plate. Draw the loop **lightly** across the surface of the agar for 2 cm, from the edge towards the center.

The handling of the plate can be accomplished in number of ways, all of which attempt to minimize possible contamination. Your lab instructor will demonstrate one or several possible techniques. For all of the following do not keep the agar surface of the plate unnecessarily uncovered, only expose the surface during the streaking process.

- The plate remains right side up on the bench, and the lid is lifted but kept over the plate while streaking. See figure l-6a.
- The plate is inverted and while the lid remains on the bench, the bottom is lifted and the agar surface is streaked from underneath. See figure l-6b.

Then sterilize the loop and allow it to cool in the air for a few seconds. Touch the loop to an unused edge (sterile area) of the agar surface to cool it completely before continuing.

b) Beginning at the edge of the plate near the initial inoculum deposit, make several (10-20) parallel streaks passing through the original inoculum deposit. Cover one-third of the plate, starting at the edge and moving towards the center of the plate. Do this by swinging the loop holder back and forth on the surface of the agar with very gentle and even pressure, using *only a wrist motion.* The loop should almost retrace its path with each swing as it moves down the agar surface from the edge towards the center of the plate. Try to keep the loop, wire, and loop holder in a plane parallel to the surface of the agar throughout the streak. Try not to lift the loop off of the agar. Try to keep the loop in contact with the surface from the beginning of the streak to the end. *Do not dig the loop into the agar medium*,

*** Sterilize the loop and allow it to cool as described above.**

c) Rotate the plate 90°.

Pull the loop through one edge of the previous streaks 2 or 3 times to re-inoculate the loop. Now streak the second third of the plate with several (10-20) more parallel streaks again starting at an edge and moving towards the center of the plate. After the initial re-inoculation, *avoid touching the already streaked first third of the plate.*

***Sterilize the loop and allow it to cool as described above.**

d) Rotate the plate 90°.

Pull the loop through one edge of the streaks in the second third of the plate to re-inoculate the loop. Now streak the last third of the plate as above, being careful to *avoid the already streaked first and second thirds of the plate.*

***Sterilize the loop after the last streaking**.

D. Ubiquity of Microorganisms

Microorganisms are everywhere. The laboratory is populated with many different types of microbes, in the air, on the benches, on the floor, and on your clothing and body. This omnipresence of microorganisms is one reason why you have learned good aseptic techniques in parts A, B, and C of this exercise. This part of the exercise is designed to give you some idea of the kind and numbers of microbes that are present in the laboratory. There is also some freedom to allow you to satisfy your curiosity about the microorganisms all around you. Finally, you will be required to isolate one of the bacterial species you obtain from this exercise for use in the next two exercises.

1. Check to be sure the agar has solidified in your plates from part A by carefully tilting the plates and looking for movement. If the agar is solid, you may work with the plates. Label one plate on the bottom as "sterile control". ***Do not open this plate at any time.*** This is used to check if you have good aseptic technique when you poured your plates.

2. Use one plate to test for the natural contamination of articles in your environment. Label this your “test plate” and with the source “finger, cell phone, etc.” Suggested experiments include: lightly *rubbing your fingers (no gloves on) over the agar surface of the plate, call this the “test plate, finger”*, or opening a plate to the air for 15-20 minutes, or coughing into a plate, or gently wiping the agar surface with an object from your pocket or backpack. It is also possible to sample a surface, such as the bench-top,

floor, sink, clothing, or skin. If you wish to do so, wipe the surface with sterile cotton swab then streak the *entire* agar surface of the plate with the swab. Use a light touch. The swabs are packaged individually and are sterile until you contaminate them with something of your choosing. Make sure the cotton tip of the swab does not come in contact with anything other than the surface you are testing. A moist swab works best for collecting samples from a dry surface. You can moisten a swab on the surface of the nutrient agar plate (at a corner), then sample the area of interest.

3. Leave the remaining two plates unlabeled and unopened on the bench, these are extra plates for the TA to collect and use for the next class.

4. Remember to label the bottom of your sample plate. Include your name or initials, your lab section, date, media type (NA for nutrient agar) and what was sampled (source).

E. Incubation

In order for microorganisms to grow they need a suitable environment. One of the most critical environmental parameters affecting growth of microorganisms is temperature. This will be discussed in lecture and is part of a later exercise. For most organisms used in this laboratory,

30°C is an acceptable temperature for growth. Constant growth temperatures are maintained by incubating microbial cultures in thermostatically controlled rooms or small ovens called incubators.

1. Incubate all tubes from part B in the 30°C incubator. Make sure *labeling of your tubes is clear and complete.* Place tubes in racks provided by your instructor.

2. Incubate all plates from parts C and D in the 30°C incubator. Make sure *labeling of your plates is clear and complete.*

Because of the high concentration of water in the agar medium, some water condensation forms in petri plates during incubation. If plates were incubated right side up, moisture would likely drip from the cover to the surface of the agar and spread out. This would disrupt individual colony formation and result in a confluent mass of growth. To avoid this***, petri plates are routinely inverted (bottom-side on top) during incubation.***

F. Observation

Detailed observations are the key to performing, understanding, and designing scientific experiments. A keen eye and an attention to subtle differences will help you successfully complete the laboratory report sheets. After incubation, plates and tubes should be observed for evidence of microbial growth. Record your observations on the outlined report sheets provided. These are only a guide, feel free to modify or add to them, as you deem necessary. Remember these report sheets constitute your lab notebook and are the basis for lab grades and evaluations. They should be kept up to date and brought to lab at all times.

DO NOT DISCARD YOUR SAMPLES UNTIL YOU ARE SURE OF THE RESULTS AND ARE CERTAIN THE SAMPLES ARE NOT NEEDED FOR THE NEXT EXERCISE.

Watch Video 1: Aseptic techniques Inoculating broth, slant, and stab tubes



Watch Video 1: Aseptic techniques Inoculating broth, slant, and stab tubes. URL: https://youtu.be/nMoM8Ku5-8A

Watch Video 2: How to perform a T-streak for isolated colonies.



Watch Video 2:How to perform a T-streak for isolated colonies. This video was filmed in the Microbiology laboratories at NC State. URL:https://youtu.be/NsQv7QOmdXo

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2.5: Results

Record the results of your experiments in the tables below.

A. Pouring plates:

Observe that your plates were poured properly--the bottom of the petri dish should be about half full and the media should be sterile, even, and contain few or no bubbles.

B. Aseptic transfers

Look for turbidity in the broth indicating growth of microorganisms. Record the turbidity of the Nutrient broth in the three tubes as + or -. Recall what your sterile Nutrient broth looked like before it was inoculated.

Nutrient broth tube	**Turbidity** *+ or -*
"sterile control"	
"broth transfer"	
"colony transfer"	

Also note any other distinguishing characteristics (amount and location of turbidity, color, etc.) of the bacterial growth in the broth and note and record any differences between species of bacteria. An effective method for recording your results is to draw a picture of the tube and the appearance of the broth due to any growth.

Escherichia coli



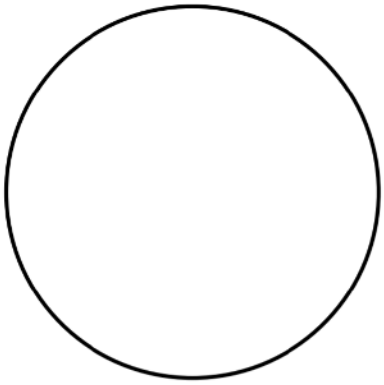
Serratia marcescens



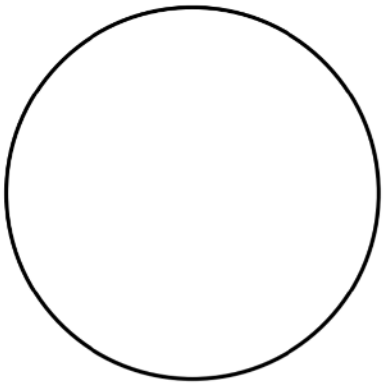
C. Streak Plates

1. Check to see whether you obtained well-isolated single colonies on the agar streak plates. Describe and compare your results for each inoculum.

From Broth: Describe growth characteristics and make a sketch in the circle below.



From Agar: Describe growth characteristics and make a sketch in the circle below.



Based on your results, answer the following questions:

2. Were you able to separate and identify the two different bacterial species in your "mixed culture"?

3. Colony morphology can be an aid in the identification of microorganisms.

Although colony morphology cannot be employed as the sole identifying criterion, it is a useful trait in the classification of many common types of microorganisms. Five parameters are normally used to describe microbial colonies growing on an agar surface.

a. *Size:* pinpoint, small, medium, or large; range: < l mm - 3cm

b. *Color:* absolutely white, various degrees of pigmentation

c. *Texture:* the texture of the colony as determined by touching the colony with a needle; smooth (buttery), dry (granular), or mucoid (slimy) and the appearance as judged by the manner in which the colony refracts light; clear, glistening, dense, opaque, or translucent.

d. *Form:* the shape of the colony; circular, irregular, filamentous, or rhizoid (see Figure 1 -7)

e. *Elevation:* the degree to which colony growth is raised; flat, raised, convex or umbonate (see Figure l-7)

f. *Margin:* the shape of the edge or margin of the colony (see Figure below)

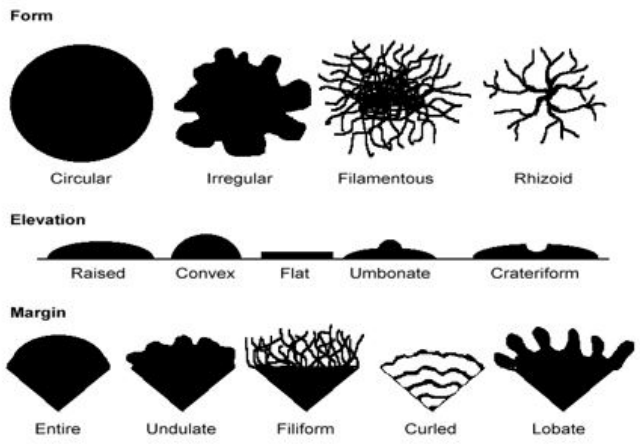


Image 1: Different colony morphologies/characteristics

Compare your bacterial colonies to those of your bench mates. Note and record any differences in the way various bacterial species grow on agar surfaces.

Escherichia coli:

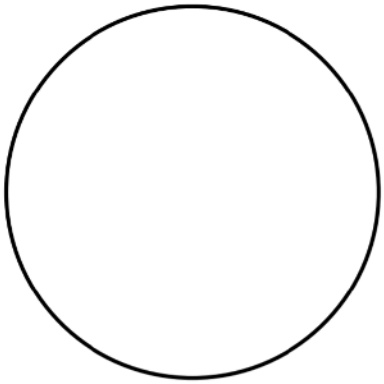
Serratia marcescens:

D. Ubiquity of Microorganisms

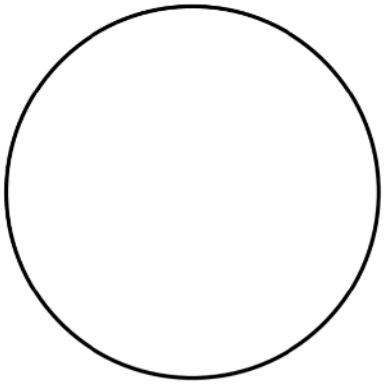
Do not throw these test plates away until you have read Exercise 2, part D ("test plate isolate").

Check to see if your "sterile control" plates remained sterile and were not contaminated. Observe, record, and **DESCRIBE** the numbers and varieties of different microbial colonies that appear on your test plate. Indicate the sample source (finger plate or other) or exposure method for the test plate.

"sterile control":



"test plate" and source:



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2.6: Review Questions

PRACTICE QUESTIONS FOR REVIEW

1. What general type of growth medium would you use to:

(a) grow one type of bacteria but inhibit the growth of another type?

(b) discriminate between different types of bacteria?

2. Why is it necessary to sterilize the loop between streaks when streaking for single colonies?

3. Define and/or explain the use of the following:

(a) synthetic medium

(b) agar

(c) broth

4. A bacterial species is inoculated on EMB agar.

(a) The bacteria do not grow. Why?

(b) If the bacteria ferment lactose, what would you expect to see?

(c) The bacteria produce clear colonies. Why?

5. What medium would you use (TSA, EMB, MSA) if you wanted to determine if a *Staphylococcus* isolate could ferment mannitol? Describe what you would see on this medium.

6. If you were testing water for the presence of fecal coliforms, what sort of medium would you use: TSA, EMB agar or MSA agar? If fecal coliforms were present, what would their growth characteristics be on this medium?

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CHAPTER OVERVIEW

3: Microscopy

3.1: Introduction to the Microscope

3.2: Comparison of Sizes and Shapes of Microorganisms

3.3: Lab Procedures- Operating a Microscope

3.4: Results

3.5: Review Questions

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3.1: Introduction to the Microscope

Learning Outcomes

- Review the principles of light microscopy and identify the major parts of the microscope.
- Learn how to use the microscope to view slides of several different cell types, including the use of the oil immersion lens to view bacterial cells.

Early Microscopy

The first microscope was developed in 1590 by Dutch lens grinders Hans and Zacharias Jansen. In 1667, Robert Hooke described the microscopic appearance of cork and used the term cell to describe the compartments he observed. Anton van Leeuwenhoek was the first person to observe living cells under the microscope in 1675—he described many types of cells, including bacteria. Since then more sophisticated and powerful scopes have been developed that allow for higher magnification and clearer images.

Microscopy is used by scientists and health care professionals for many purposes, including diagnosis of infectious diseases, identification of **microorganisms** (microscopic organisms) in environmental samples (including food and water), and determination of the effect of pathogenic (disease-causing) microbes on human cells. This exercise will familiarize you with the microscopes we will be using to look at various types of microorganisms throughout the semester.

The Light Microscope

What does it mean to be microscopic? Objects are said to be microscopic when they are too small to be seen with the unaided eye—they need to be magnified (enlarged) for the human eye to be able to see them. This includes human cells and many other types of cells that you will be studying in this class. The microscope you will be using uses visible light and two sets of lenses to produce a magnified image. The total magnification will depend on which objective lens you are using—the highest magnification possible on these microscopes is typically 1000X—meaning that objects appear 1000X larger than they actually are.

Resolution vs. Magnification

Magnification refers to the process of making an object appear larger than it is; whereas **resolution** is the ability to see objects clearly enough to tell two distinct objects apart. Although it is possible to magnify above 1000X, a higher magnification would result in a blurry image. (Think about magnifying a digital photograph beyond the point where you can see the image clearly). This is due to the limitations of visible light (details that are smaller than the wavelength of light used cannot be resolved).

The limit of resolution of the human eye is about 0.1 mm, or 100 microns (see Table 1 for metric review). Objects that are smaller than this cannot be seen clearly without magnification. Since most cells are much smaller than 100 microns, we need to use microscopes to see them.

The **limit of resolution of a standard brightfield light microscope**, also called the **resolving power,** is ~0.2 µm, or 200 nm. Biologists typically use microscopes to view all types of cells, including plant cells, animal cells, protozoa, algae, fungi, and bacteria. The nucleus and chloroplasts of eukaryotic cells can also be seen—however smaller organelles and viruses are beyond the limit of resolution of the light microscope (see Figure 1).

Resolution is the ability of the lenses to distinguish between two adjacent objects as distinct and separate.

A compound light microscope has a maximum resolution of 0.2 µm, this means it can distinguish between two points ≥ 0.2 µm, any objects closer than 0.2um will be seen as 1 object. Shorter wavelengths of light provide greater resolution. This is why we often have a blue filter over our light source in the microscope, it helps to increase resolution since its wavelength is the shortest in the visible light spectrum. Without resolution, no matter how much the image is magnified, the amount of observable detail is fixed, and regardless of how much you increase the size of the image, no more detail can be seen. At this point, you will have reached the limit of resolution or the resolving power of the lens. This property of the lens is fixed by the design and construction of the lens. To change the resolution, a different lens is often the only answer.

Table 1: Metric units commonly used in Microbiology
The basic unit of measurement of length in the metric system is the meter.
There are 1000 millimeters (mm) in one meter. 1 mm = 10^{-3} meter.
There are 1000 micrometers (microns, or µm) in one millimeter. 1 µm = 10^{-6} meter.
There are 1000 nanometers in one micrometer. 1 nm = 10^{-9} meter.

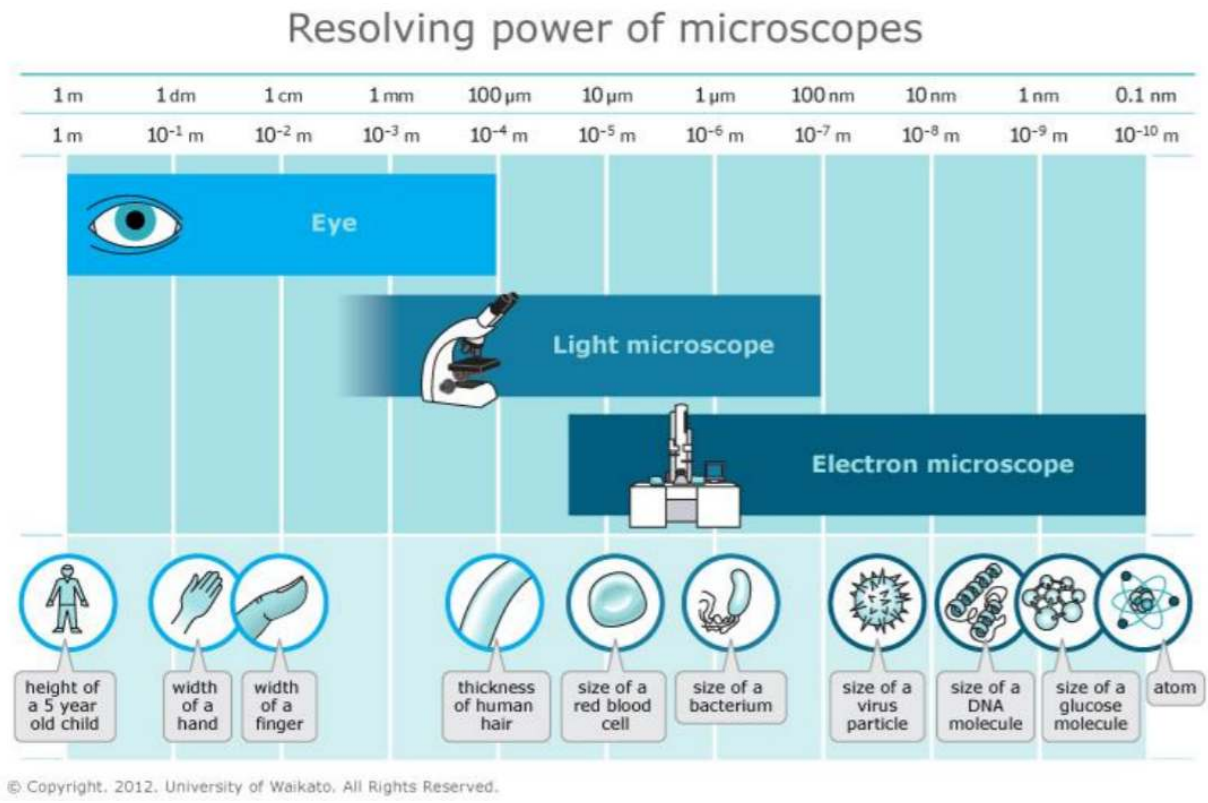


Figure 1: Resolving Power of Microscopes

The microscope is one of the microbiologist's greatest tools. It allows for the visualization of small particles, including microbes, which *individually* are too small to be seen with the human eye. With the help of proper illumination, a microscope can magnify a specimen and optically resolve fine detail. This introduction to microscopy will include an explanation of features and adjustments of a compound **brightfield light microscope,** which magnifies images using a two lens system.

Before reading the following discussion of the theory of the microscope, please familiarize yourself with the names of the microscope parts shown in Figure 2 and their function.

1. **Eyepiece/Ocular lens:** Lens in which the final magnification occurs. Often is at 10X magnification, but can be different.

2. **Revolving nose piece**: Holds multiple objective lenses in place. The base of the nose piece can rotate, allowing each of the lens to be rotated into alignment with the ocular lens.

3. **Objective lenses**: Initial magnification of your specimen occurs here. Most brightfield light microscopes have 3 objective lenses seated into the resolving nose piece base.

4. **Coarse focusing knob**: larger of the two knobs, the coarse adjustment knob moves the *stage* up or down to bring the specimen into focus. It is very sensitive, even small partial rotation of this knob can bring about a big change in the vertical movement of the stage. ONLY use coarse focusing at the beginning with the 4X, 10X low powered objectives in place. If you use it with the higher powered objectives, it can damage the objective if you crash the lens through your glass specimen slide.

5. **Fine focusing knob:** smaller of the two knobs, the fine adjustment knob brings the specimen into sharp focus under low power and is used for all focusing when using high power lenses such as the 100x oil immersion lens.

6/9. **Stage & Mechanical stage**: The horizontal surface where you place the slide specimen is called the stage. The slide is held in place by spring loaded clips and moved around the stage by turning the geared knobs on the **mechanical stage**. The mechanical stage has two perpendicular scales that can be used to record the position of an object on a slide, useful to quickly relocate an object.

7. **Illuminator**: contains the light source, a lamp made either of an incandescent tungsten-halogen bulb or an LED. There is normally a switch to turn on/off or a **rheostat** located on the side that you can use to adjust the brightness of the light.

8. **Diaphragm and Condenser**: the diaphragm controls the amount of light passing from the illuminator through the bottom of the slide, there is a small lever used to achieve the optimal lighting. The condenser is a lens system that focuses the light coming up from the illuminator onto objects on the slide.

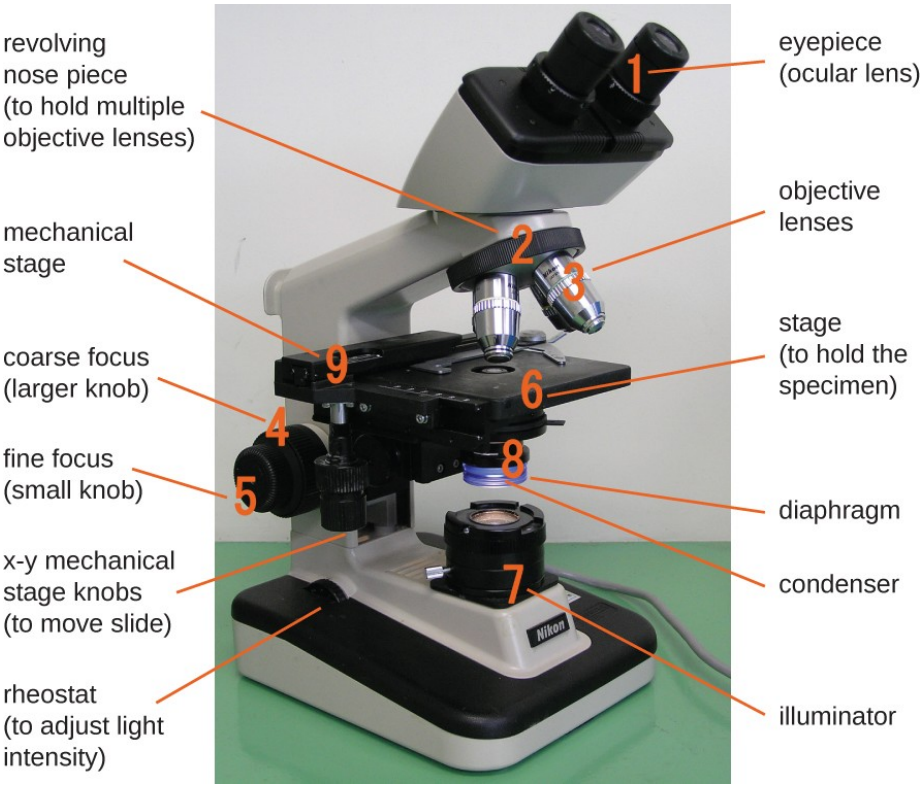


Figure 2: Brightfield light microscope used in a Microbiology lab (Lumen)

The Optical System. The optical system of a compound microscope consists of two lens systems: one found in the objective(s) lens(es) (Fig. 2, part 3); the other in the ocular (eyepiece) (Fig. 2 part 1). The objective lens system is found attached to a rotating nosepiece (Fig. 2, part 2). A microscope usually has three or four objectives that differ in their magnification and resolving power. **Magnification** is the apparent increase in size of an object. ***Resolving power*** is the term used to indicate the ability to distinguish two objects as separate. The most familiar example of resolving power is that of car headlights at night: at a long distance away, the

headlights appear as one light; as the car approaches, the light becomes oblong, then barbell-shaped, and finally it becomes *resolved* into two separate lights. Both resolution and magnification are necessary in microscopy in order to give an apparently larger, finely detailed object to view.

Look at the engravings on the objective lenses and note both the magnification (for example: 10X, 40X, 100X) and the resolution given as N.A. = numerical aperture, from which the limit of resolution can be calculated:

$$\text{limit of resolution} = \frac{\text{wavelength}}{2 \times \text{numerical aperture}}$$

At a wavelength of 550 nm (0.55µm), the 100X objective lens with a N.A. of 1.25 has a resolving power of 0.22 µm. Visible light has of wavelength from about 400-750 nanometers (nm). Since the limit of resolution decreases at the shorter wavelengths, microscopes are usually fitted with a blue filter. The resolving power of the lens separates the details of the specimen, and the magnification increases the apparent size of these details so that they are visible to the human eye. Without both resolution and magnification, you would either see nothing (good resolution, no magnification) or a big blur (poor resolution, good magnification).

The objective lens system produces an image of the specimen, which is then further magnified by the ocular lens (eyepiece). The magnification of this lens is engraved on the ocular. The total magnification of the microscope is determined by the combination of the magnification of the objective lens and ocular lens that is in use, that is:

Total magnification = objective lens X ocular lens (eyepiece)

For example, with a 10X objective lens and a 10X ocular, the total magnification of the microscope is 100X. If the objective lens is changed to a 20X objective, then the total magnification is now 200X, whereas if a 10X objective is used with a 12.5X ocular lens, the total magnification is now 125X. The use of objective and ocular lenses with different magnifications allows greater flexibility when using the compound microscope. Due to the size of most bacteria (ranges widely from ~1um to over 100um), generally we require the use of the 100x *oil immersion lens* with a 10x ocular lense to view bacteria in a standard brightfield light microscope.

The Illumination System. The objective and ocular lens systems can only perform well under optimal illumination conditions. To achieve these conditions, the light from the light source (bulb) must be centered on the specimen. (In most inexpensive microscopes, the manufacturer adjusts this centering. In more versatile microscopes, the centering becomes more critical and is a function performed by the operator.) The parallel light rays from the light source are focused on the specimen by the condenser lens system (see Fig. 2) The condenser can move up and down to affect this focus. Finally, the amount of light entering the condenser lens system is adjusted using the condenser diaphragm. It is critical that the amount of light be appropriate for the size of the objective lens receiving the light. This is important to give sufficient light, while minimizing glare from stray light, which could otherwise reduce image detail. The higher the magnification and resolving power of the lens, the more light is needed to view the specimen.

Objective lenses used for observing very small objects such as bacteria are almost always **oil immersion lenses**. With an oil immersion lens, a drop of oil is placed between the specimen and the objective lens so that the image light passes through the oil. Without the oil, light passing through the glass microscope slide and specimen would be **refracted** (bent) when it entered the air between the slide and the objective lens. This refracted light might still be able to contribute to the image of the specimen if the objective lens is large. However, at the higher magnification, the objective lens is small, so is unable to capture this light. The loss of this light leads to loss of image detail. Therefore, at higher magnifications, the area between the slide and the lens is modified to have the same (or nearly the same) refracting qualities (refractive index) as the glass and specimen by the addition of immersion oil. Watch this NC BioNetwork video (https://youtu.be/-0EvnroWpVc) on oil immersion. For more information, read this article (https://www.microscopeworld.com/t-us...rsion_oil.aspx).

To use an oil immersion lens, place a drop of oil on top of the dried specimen on the slide and carefully focus the microscope so that the objective lens is immersed in the oil. Any lens, which requires oil, is marked "oil" or "oil immersion." Conversely, any lens not marked "oil" should *NOT be* used with oil and is generally not sealed against oil seeping into and ruining the objective.

Watch this Video on how to use a Microscope, filmed at NC State Microbiology labs:



Video 1: Introduction to the Microscope (6:26)

Key Terms

microorganism, magnification, resolution, working distance, parfocal, parcentric, prokaryotic, eukaryotic, bacillus, coccus, spirillum, spirochete, morphology, bacterial arrangements, depth of field, field of view, taxonomic classification

References:

- Contributed by Joan Petersen & Susan McLaughli: Associate Professors (Biological Sciences and Geology) at Queensborough Community College
- Lumen Learning: Figure 3: Brightfield light microscope https://courses.lumenlearning.com/mi...of-microscopy/

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3.2: Comparison of Sizes and Shapes of Microorganisms

Learning Outcomes

- Recognize the shapes and arrangements of some common types of bacteria.

A. BACTERIAL SHAPES, ARRANGEMENTS, AND FORMS

Bacteria are unicellular prokaryotic microorganisms that **divide by binary fission**, a process by which one bacterium splits into two.

- Scanning electron micrograph of *Salmonella typhimurium* undergoing binary fission.

YouTube movie of binary fission in bacteria.

YouTube movie of fluorescing imaging of binary fission in bacteria.

Review of Prokaryotic and Eukaryotic Cells from Unit 1.

There are three common **shapes of bacteria**:

- **coccus (singular) or cocci (plural)**
- **bacillus or rod (singular) or bacilli, rods (plural)**
- **spiral (example of a spirochete, a type of spiral is shown in image 1)**

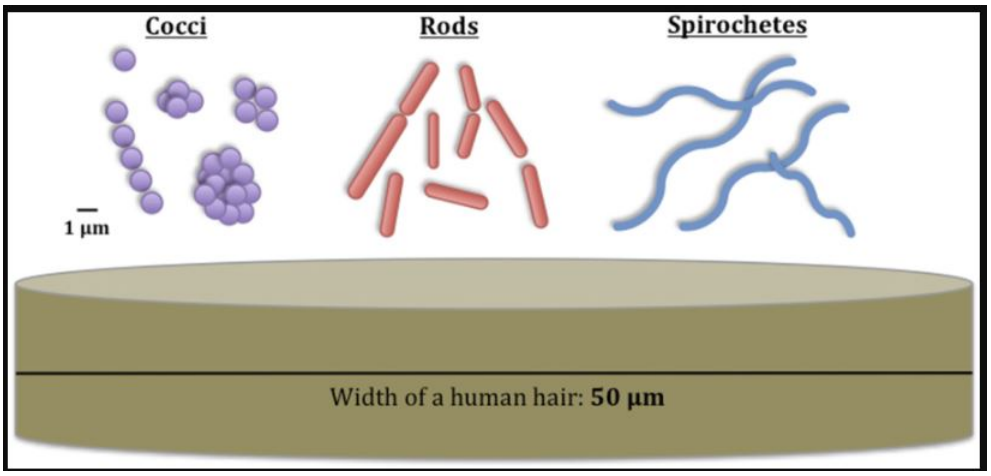


Image 1: These are the different shapes of bacteria and their sizes compared with the width of a human hair.
The unit "µm" is a measurement of length, the "micrometer," or commonly known as the micron. It equals 1×10^{-6} meters that is, one millionth of a meter. Image courtesy of Kestin Schulz, Mariya W. Smit, Lydie Herfort and Holly M. Simon URL: https://commons.wikimedia.org/wiki/F...omparisons.jpg

The cocci come in 5 different arrangements; the bacilli in 3 different arrangements; and the spirals in 3 different forms.

1. Coccus

A coccus-shaped bacterium is usually spherical, although some appear oval, elongated, or flattened on one side. Most cocci are approximately **0.5 - 1.0 micrometer (µm)** in diameter and may be seen, based on their planes of division and tendency to remain attached after replication, in one of the following **arrangements** (see Fig. 1A):

a. Division in **one plane** produces either a **diplococcus** (see Fig. 1A and Fig. 1B) or **streptococcus** (see Fig. 1A and Fig. 1C) arrangement.

1. **diplococcus**: a pair of cocci
- Photomicrograph of a diplococcus
- Scanning electron micrograph of a *Streptococcus pneumoniae*, a diplococcus
- Scanning electron micrograph of a *Neisseria*, a diplococcus; courtesy of Dennis Kunkel's Microscopy

2. **streptococcus**: a chain of cocci
- Photomicrograph of a streptococcus
- Scanning electron micrograph of *Streptococcus pyogenes*; courtesy of Dennis Kunkel's Microscopy
- Transmission electron micrograph of *Streptococcus*
-Scanning Electron Micrograph of *Enterococcus*

b. Division in **two planes** produces a **tetrad** arrangement (see Fig. 1A and Fig. 1D).

- **tetrad**: a square of 4 cocci
  - Photomicrograph of a tetrad
  - Scanning electron micrograph of *Micrococcus luteus*

c. Division in **three planes** produces a **sarcina** arrangement (see Fig. 1A).

- **sarcina**: a cube of 8 cocci
  - Photomicrograph of a sarcina

It is difficult with a conventional light microscope to tell a tetrad arrangement (square of four cocci) from a sarcina arrangement (cube of eight) so in our lab, anytime you see ba square of four cocci, say it is either a tetrad or a sarcina arrangement.

d. Division in **random planes** produces a **staphylococcus** arrangement (see Fig. 1A and Fig. 1E).

staphylococcus: cocci in irregular, often grape-like clusters
- Photomicrograph of a staphylococcus, negative image
- Scanning electron micrograph of *Staphylococcus aureus*; courtesy of Dennis Kunkel's Microscopy
- Scanning electron micrograph of methicillin-resistant *Staphylococcus aureus* (MRSA); courtesy of CDC

As you observe these different cocci, keep in mind that **the procedures used in slide preparation may cause some arrangements to break apart or clump together** (see Figs. 1D and 1E). The correct form, however, should predominate. Also remember that each coccus in an arrangement represents a complete, individual, one-celled organism.

2. Bacillus (rod)

A bacillus or rod is a hotdog-shaped bacterium having one of the following **arrangements** (see Fig 2A):

a. **bacillus**: a single bacillus (see Fig 2A and Fig 2B)
- Photomicrograph of a bacillus
- Scanning electron micrograph of a bacillus
- Scanning electron micrograph of *Escherichia coli* O157H7, a bacillus; courtesy of CDC

b. **streptobacillus**: bacilli in chains (see Fig 2A and Fig 2C)
- Photomicrograph of a streptobacillus

c. **coccobacillus**: oval and similar to a coccus (see Fig 2A, 2D, and 2E)

A single bacillus is typically **0.5-1.0 µm wide** and from **1- 4 µm long**. **Small bacilli or bacilli that are dividing or have just divided by binary fission may at first glance be confused for diplococci or cocci** (see Fig. 2A) so they must be **observed carefully**. You will, however, be able to see bacilli that have not divided and are definitely rod-shaped as well as bacilli in the process of dividing.

3. Spiral

Spiral-shaped bacteria occur in one of three **forms** (see Fig. 3A):

a. **vibrio**: an incomplete spiral or comma-shaped (see Fig. 3A and Fig. 3B)
- Photomicrograph of a vibrio
- Scanning electron micrograph of *Vibrio cholerae*; courtesy of Dennis Kunkel's Microscopy

b. **spirillum**: a thick, rigid spiral (see Fig. 3A and Fig. 3C)
- Photomicrograph of a spirillum

c. **spirochete**: a thin, flexible spiral (see Fig. 3A and Fig. 3D)
- Photomicrograph of a spirochete
- Scanning electron micrograph of the spirochete *Leptospira*; courtesy of CDC
- scanning electron micrograph of the spirochete *Treponema pallidum*; courtesy of CDC

The spirals you will observe range from **5-40 µm long** but some are over 100 µm in length. The spirochetes are the thinnest of the bacteria, often having a width of only 0.25-0.5 µm.

To view a nice interactive illustration comparing size of cells and microbes, see the Cell Size and Scale Resource at the University of Utah.

B. YEASTS

Yeasts, such as the common baker's yeast *Saccharomyces cerevisiae* (see Fig. 4), are **unicellular fungi**. They usually appear spherical and have a diameter of **3 - 5 µm**. Yeasts commonly reproduce asexually by a process called budding. Unlike bacteria, which are prokaryotic, yeasts are eukaryotic.

- Scanning electron micrograph of *Saccharomyces*; courtesy of Dennis Kunkel's Microscopy

Review of Prokaryotic and Eukaryotic Cells from Unit 1.

To view a nice interactive illustration comparing size of cells and microbes, see the Cell Size and Scale Resource at the University of Utah.

C. MEASUREMENT OF MICROORGANISMS

The approximate size of a microorganism can be determined using an ocular micrometer (see Fig. 5) , an eyepiece that contains a scale that will appear superimposed upon the focused specimen.

To view a nice interactive illustration comparing size of cells and microbes, see the Cell size & scale resource at the University of Utah's Learn.genetics site, URL: https://learn.genetics.utah.edu/content/cells/scale/

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3.3: Lab Procedures- Operating a Microscope

Brightfield Light Microscope General Operating Procedure:

The following step-by-step operating procedure should be carefully observed when first using the Nikon Alphaphot-2. Go through these steps now and again when carrying out the procedures for parts B, C, and D. This instrument is designed for fast-simplified use and correct operating technique should soon become automatic.

1. Plug in and turn on the in-base illuminator.

2. Raise the nosepiece using the coarse adjustment knob. This provides greater access to the stage when the slide is in position.

3. Rotate the nosepiece so that the 10X objective is in operating position. You should feel a definite position stop for the objective. Turn the nosepiece clockwise and counter-clockwise until you are familiar with this position stop.

4. Open the iris diaphragm approximately half way.

5. Place the slide in the stage slide holder securely. Be sure the slide has the *specimen side up*. Roughly center the specimen over the light coming from the condenser.

6. Move the microscope condenser by means of the condenser rack and pinion knob until the top of the condenser is approximately the thickness of a piece of paper beneath the slide.

7. Lower the objective using the coarse control knob until it reaches a stop. *Do not* force the knob. The stop should be obvious if you are moving the controls gently. Again, become familiar with *the feel* of the position stop.

8. View through the eyepieces and without disturbing the coarse adjustment setting, slowly rotate the fine adjustment knob in the appropriate direction until the specimen detail is in sharpest possible focus.

9. Adjust the interpupillary distance between the two eyepieces by sliding the eyepiece plates in or out.

10. If your eyes are very different the left eyepiece tube is adjustable to compensate for refraction differences of the eyes. The correct procedure is to bring the specimen into sharpest possible focus with a fine adjustment knob using the right eyepiece only, while closing your left eye. To focus for the left eye, view the specimen with the left eye only and turn the knurled collar until the specimen is in sharp focus. *Do not* adjust the fine adjustment knob during this procedure.

11. Once the specimen detail is in sharp focus using the 10X objective, it is then possible to rotate the nosepiece to other objectives without changing the position of the coarse adjustment knob. Very little refocusing with the fine adjustment is required since the Nikon Alphaphot-2 microscope objectives are *parfocal*. The term parfocal means that if an object is in focus with one objective lens, it will be in focus with all of the lenses.

Make certain that the portion of the specimen you wish to view is exactly centered in the field of the low-power objective. This is necessary because the microscopic field of view is smaller under high magnification than under low magnification.

Remember that the iris diaphragm setting must be changed whenever a different objective is used. As magnification increases the condenser iris diaphragm is opened as required. With these microscopes you are effectively increasing the light reaching the higher power objective. Adjustment of the condenser iris diaphragm results in proper contrast for viewing specimens under varying magnifications.

12. Try the highest magnification, the l00X-oil immersion objective. To focus with the oil immersion lens proceed in the following manner:

a) Revolve the nosepiece so that there is no objective directly over the specimen. This allows enough space so that a drop of immersion oil may be placed on the specimen.

b) Place *one drop* of immersion oil in the center of the circle of light formed on the specimen slide.

c) Turn the nosepiece until the 100X objective snaps into place. The objective should be in the oil but must not touch the slide.

d) Rotate the fine adjustment knob to obtain a sharp focus of the specimen. Remember to make the adjustments noted in step 10.

Problems:

Certain mechanical difficulties, real or apparent, may be encountered while operating your microscope.

A common problem is the failure of the fine adjustment to turn in the direction required for sharp focusing. This indicates that it has been screwed to the limits of its threads, either upward or downward, as the case may be. Screw it back to about one-half the thread distance, use the coarse adjustment to raise or lower the objective sufficiently to bring the specimen into view, and then refocus with the fine adjustment.

If the coarse adjustment fails to lower the objective sufficiently to bring the specimen into view, the fine adjustment has been screwed up too far and should be screwed down to about half its thread distance.

Any other problems? Burned-out light bulb? Focus knobs difficult to turn, grinding as they move, or moving by themselves? Specimen going in and out of focus as the stage is moved? Did you write the word "up" on your microscope slide? Is it still facing up? *Do not attempt to fix these yourself. Get your instructor.*

Proper Care of the Microscope:

The compound microscope used in microbiology is a precision instrument. Its mechanical parts, such as the calibrated mechanical stage and the adjustment knobs, are easily damaged and all lenses, particularly the oil immersion objective, are delicate and expensive. Thus, the instrument must be handled with care. The following rules, cautions, and maintenance should be observed:

1. Use both hands when carrying the microscope; one firmly grasping the arm of the microscope, the other beneath the base. Avoid sudden jars.

2. To keep the microscope and lens systems clean:

- Never touch the lenses. If the lenses become dirty, wipe them gently with lens paper.
- Never leave a slide on the microscope when it is not in use.
- Always use lens paper to remove oil from the oil-immersion objective after its use. *Do not* wipe the lower power objectives with the same piece of lens paper used to clean the oil-immersion objective. If by accident oil should get on either of the lower power objectives, wipe it off immediately with clean lens paper.
- Keep the stage of the microscope clean and dry.

3. To avoid breaking the microscope:

- Never force the adjustments. All adjustments should work freely and easily.
- Never allow an objective lens to jam into or even to touch the slide or cover slip.
- Never focus downward with the coarse adjustment while you are looking through the microscope. Always incline your head to the side with eyes parallel to the slide, so that downward movement can be arrested before the objective touches the slide.
- Never exchange the objectives or eyepieces of different microscopes and never under any circumstance remove the front lenses from the objectives.
- Never attempt to carry two microscopes at one time.

4. Storage of the microscope:

- ***Make sure the immersion oil has been removed from the lens.***
- Put the low power (10X) objective into position and turn the stage all the way down.
- Be sure the slide holder does not extend beyond the *left* edge of the microscope.
- Wrap the electrical cord around the cord hanger.

- Your TA will place the microscope in the cabinet.

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3.4: Results

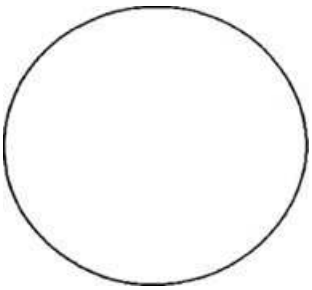
Results

C. Methylene Blue-A Simple Stain

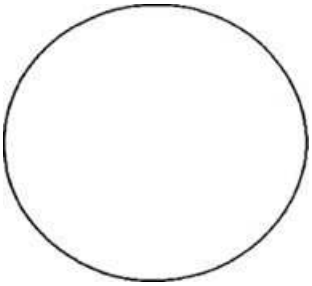
Sketch cellular shape (rods or cocci) and arrangement (single cells, pairs, clusters, chains) of the organisms observed with the oil-immersion lens (total magnification 1000X). Compare size and shape of the three microorganisms. You will need to be familiar with these for the next exercise and for identifying your unknowns.

The only way to become proficient with a microscope is to use it often and be observant while using it.

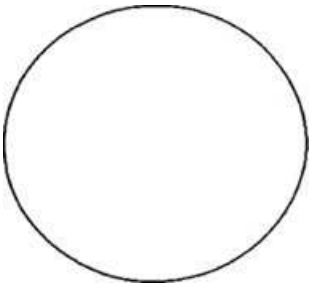
Saccharomyces cerevisiae- Eukaryotic Yeast



Bacillus megaterium



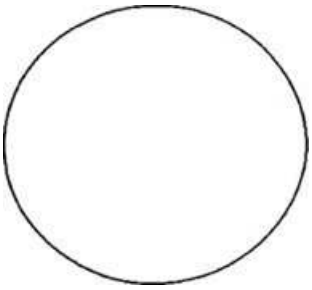
Micrococcus luteus



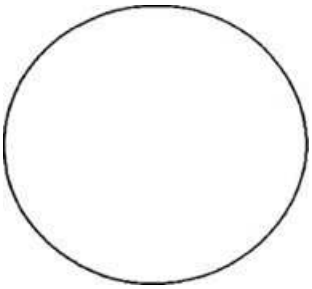
D. Test plate isolate

Sketch cellular shape and arrangement of the isolates from your "test plate" (total magnification, 1000X). In the next laboratory we will do a differential stain of this bacterial culture and show you how to use Bergey's *Manual of Determinative Bacteriology.*

"test plate isolate"



E. Demonstration slide



Sketch the cellular shape of *Spirillum volutans.*

Questions:

1.What are the structures seen on the end of these cells?

2. What is their function?

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3.5: Review Questions

1. Be able to describe the different parts of a microscope and their function

2. Explain this statement: "The limit of resolution for a brightfield light microscope is 0.2um."

3. What is the purpose of using oil in the oil immersion lens?

4. The objective lens is 40X and the ocular lens is 20X, what is the total magnification?

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CHAPTER OVERVIEW

4: Staining Techniques

4.1: Introduction to Staining

4.2: Specialized Bacterial Staining Techniques

4.3: Lab Procedures- Bacterial Smear, Simple and Gram Staining

4.4: Results

4.5: Review Questions

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4.1: Introduction to Staining

Learning Objectives

- Describe the differences between simple staining and differential staining techniques.
- Discuss how to prepare a bacterial smear from cultured organisms.
- Distinguish between Gram-positive and Gram-negative bacteria.
- Describe the process of the Gram stain procedure.
- Use microscopy to examine Gram stained cells.
- Describe select special staining procedures and view examples of these under oil immersion.

Why do we have to stain bacteria?

Most types of cells do not have much natural pigment and are therefore difficult to see under the light microscope unless they are stained. Several types of stains are used to make bacterial cells more visible. In addition, specific staining techniques can be used to determine the cells' biochemical or structural properties, such as cell wall type and presence or absence of endospores. This type of information can help scientists identify and classify microorganisms, and can be used by health care providers to diagnose the cause of a bacterial infection.

The Simple Stain

One type of staining procedure that can be used is the **simple stain**, in which only one stain is used, and all types of bacteria appear as the color of that stain when viewed under the microscope. Some stains commonly used for simple staining include crystal violet, safranin, and methylene blue. Simple stains can be used to determine a bacterial species' morphology and arrangement, but they do not give any additional information. Living bacteria are almost colorless, and do not present sufficient contrast with the water in which they are suspended to be clearly visible. The purpose of staining is to increase the contrast between the organisms and the background so that they are more readily seen in the light microscope. In a simple stain, a bacterial smear is stained with a solution of a single dye that stains all cells the same color without differentiation of cell types or structures. The single dye used here in our lab is methylene blue, a basic stain. Basic stains, having a positive charge, bind strongly to negatively charged cell components such as bacterial nucleic acids and cell walls.



Image 1: Microscopic view of Bacillus (rod) shaped bacteria simple stained with crystal violet. Isolated and imaged by Muntasir Alam, University of Dhaka, Department of Microbiology in 2007. https://commons.wikimedia.org/wiki/F...micrograph.jpg

Watch Video 1: how to apply a simple stain



Watch Video 1: How to apply a simple stain to a bacterial culture by NC BioNetwork (4:05)
URL: https://youtu.be/8ODeT9DLHKI

The Gram Stain

Scientists will often choose to perform a **differential stain**, as this allows them to gather additional information about the bacteria they are working with. Differential stains use more than one stain, and cells will have a different appearance based on their chemical or structural properties. Some examples of differential stains are the Gram stain, acid-fast stain, and endospore stain. You will learn how to prepare bacterial cells for staining, and learn about the gram staining technique.

This very commonly used staining procedure was first developed by the Danish bacteriologist Hans Christian Gram in 1882 (published in 1884) while working with tissue samples from the lungs of patients who had died from pneumonia. Since then, the Gram stain procedure has been widely used by microbiologists everywhere to obtain important information about the bacterial species they are working with. Knowing the Gram reaction of a clinical isolate can help the health care professional make a diagnosis and choose the appropriate antibiotic for treatment.

Gram stain results reflect differences in cell wall composition. **Gram positive** cells have thick layers of a peptidoglycan (a carbohydrate) in their cell walls; **Gram negative** bacteria have very little. Gram positive bacteria also have teichoic acids, whereas Gram negatives do not. Gram negative cells have an outer membrane that resembles the phospholipid bilayer of the cell membrane. The outer membrane contains lipopolysaccharides (LPS), which are released as endotoxins when Gram negative cells die. This can be of concern to a person with an infection caused by a gram negative organism.

File:Gram stain 01.jpg

Image 2: Microscopic image of a Gram stain of mixed Gram-positive cocci (*Staphylococcus aureus* ATCC 25923, purple) and Gram-negative bacilli (*Escherichia coli* ATCC 11775, red). Magnification:1,000. Image by Y Tambe. https://commons.wikimedia.org/wiki/F...m_stain_01.jpg

Figure 1 below shows the major differences between the Gram positive and Gram negative cell walls. The differences in the cell wall composition are reflected in the way the cells react with the stains used in the Gram stain procedure.

Gram stains are best performed on fresh cultures—older cells may have damaged cell walls and not give the proper Gram reaction. Certain species are known as **Gram-variable**, and so both Gram positive and Gram negative reactions may be visible on your slide.

Poor staining technique could lead to inaccurate results. One of the most important steps in Gram staining is the decolorizing step (use of alcohol/acetone). If the decolorizer is not left on long enough, then it will not be able to differentiate between Gram positive and Gram negative bacteria. This step uses decolorizer, made of an alcohol/acetone mixture. Its function in Gram

negative bacteria is to remove the outer cell membrane and thin layer of peptidoglycan. The cell membrane is mostly made of lipids and are sensitive to alcohols. By dissolving these layers, the crystal violet-iodine complex is also removed, and thus Gram negatives are now able to take up the secondary stain, safranin, which is used in the last step of the Gram stain, staining them pinkish-red and differentiating between them and the Gram positives, who with their thick peptidoglycan layer has retained the primary stain, crystal violet, and appears purple/blue. On the flip side, if you use too much decolorizer, it can decolorize your sample on the slide, leading to loss of crystal violet (the primary stain)-iodine complex. The decolorizing step is sensitive because of the cell wall structure. Even Gram positive bacteria with their thick cell walls could become excessively decolorized, resulting in the loss of the peptidoglycan layer and the crystal violet-iodine complex. When the use of the secondary stain, safranin, is applied in the last step, the Gram positive bacteria will pick up this stain and look reddish-pink instead of purple/blue. Watch video 2 for an example of this.

Another common mistake is in the preparation of the bacterial smear, which is in the first step of any staining procedure. This involves applying a thin film of bacteria on your microscope slide and then **heat fixing** it with either your bunsen burner, bacticinerator, or slide warmer. The main purpose of this step is to adhere the bacterial cells to the microscope slide (it also denatures the proteins and kills them too). If you forget to do this step, then the cells will be 'washed' off in all the subsequent steps of your staining process. You will literally have no cells on your slide to stain!

Although the vast majority of bacteria are either Gram positive or Gram negative, it is important to remember that not all bacteria can be stained with this procedure (for example, Mycoplasmas, which have no cell wall, stain poorly with the Gram stain).

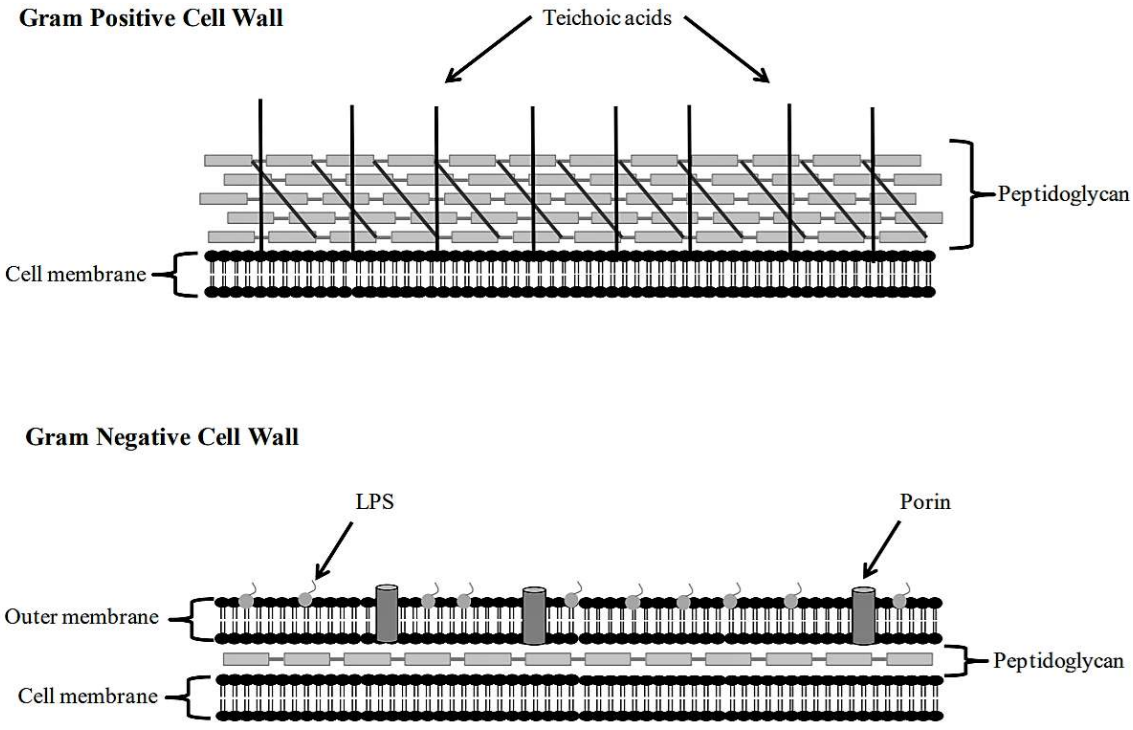


Figure 1: *Gram positive and Gram negative cell walls*

Watch Video 2: Gram Stain Animation and discussion on what is happening at each step.



Watch Video 2: Gram stain animation with description of each step and interpretation of what is happening in each step by Dr.G Bhanu Prakash Animated Medical Videos. (3:37) URL https://youtu.be/AZS2wb7pMo4

Watch Video 3: Gram staining procedure



Watch Video 2: Gram Staining procedure, filmed at NC State Microbiology Labs. (5:58). URL: https://youtu.be/H-fxk1be1hQ

Special Stains

There are a variety of staining procedures used to identify specific external or internal structures that are not found in all bacterial species, such as a capsule stain and a flagella stain. For images and more examples of specialized stains, see below and in the next section, 4.2 specialized bacterial staining techniques.

Capsule Stain

Some bacteria secrete a polysaccharide-rich structure external to the cell wall called a glycocalyx. If the glycocalyx is thin and loosely attached, it is called a **slime layer**; if it is thick and tightly bound to the cell, it is called a **capsule**. The glycocalyx can protect the cell from desiccation and can allow the cell to stick to surfaces like tissues in the body. They may also provide cells with protection against detection and phagocytosis by immune cells and contribute to the formation of a biofilm: in this way a glycocalyx can act as a virulence factor; (contributes to the ability of an organism to cause disease).

Capsules can be detected using a **negative staining** procedure in which the background (the slide) and the bacteria are stained, but the capsule is not stained. The capsule appears as a clear unstained zone around the bacterial cell. Since capsules are destroyed by heat, the capsule staining procedure is done without heat-fixing the bacteria.

Silver Stain

Flagella (long whip-like structures used for bacterial motility) and some bacteria (e.g. **spirochetes**) are too thin to be observed with regular staining procedures. In these cases, a **silver stain** is used. Silver nitrate is applied to the bacteria along with a special mordant; the silver nitrate precipitates around the flagella or the thin bacteria, thus thickening them so they can be observed under the light microscope.

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4.2: Specialized Bacterial Staining Techniques

Learning outcomes

- Describe the purpose of each of the stains listed: simple, Gram, Acid-Fast, Endospore, Capsule, Flagella, Spirochete.
- Recognize the cellular characteristics revealed by each of the stains listed

Simple **Stains:**

Commonly used dyes for simple stains: Crystal Violet, Methylene Blue, Safranin

- Uses one dye
- Used to provide color to otherwise transparent bacterial cells
- Can be used to determine cell size, morphology and arrangement
- All bacteria are the same color when stained with the single dye that is used



Image 1: Simple stain with crystal violet showing rod shaped bacteria. Note that all bacteria are the same color of the dye, crystal violet (purple), regardless of its cell wall composition. Image by Muntasir du, 2007 (https://commons.wikimedia.org/wiki/F...micrograph.jpg)

Gram Stain

Primary stain – crystal violet

Mordant – iodine; decolorizer- 95% Ethanol

Counterstain – Safranin

- Common differential stain
- Gram reaction (positive or negative) reflects cell wall properties
- Also used to determine cell size, morphology and arrangement



Image 2. Gram positive, rod, *Bacillus subtilis.* (Ann C. Smith, University of Maryland, College Park, MD)

File:Gram stain 01.jpg

Image 3: Gram stain showing Gram negative rods (pink) and Gram positive cocci (purple): https://commons.wikimedia.org/wiki/F...m_stain_01.jpg

Acid-Fast Stain

The acid-fast stain is a differential stain used to identify acid-fast organisms such as members of the genus *Mycobacterium* .

Acid-fast organisms are characterized by wax-like, nearly impermeable cell walls; they contain mycolic acid and large amounts of fatty acids, waxes, and complex lipids. Acid-fast organisms are highly resistant to disinfectants and dry conditions. (1)

Because the cell wall is so resistant to most compounds, acid-fast organisms require a special staining technique. The primary stain used in acid-fast staining, carbolfuchsin, is lipid-soluble and contains phenol, which helps the stain penetrate the cell wall. This is further assisted by the addition of heat. The smear is then rinsed with a very strong decolorizer, which strips the stain from all non-acid-fast cells but does not permeate the cell wall of acid-fast organisms. The decolorized non-acid-fast cells then take up the counterstain. (1)

Primary stain – Carbol fuchsin

Decolorizer – acid alcohol

Counterstain – Methylene blue

- A differential stain used to detect bacteria with mycolic acid cell walls (genera *Mycobacterium* and *Nocardia*)
- Developed to detect the bacterial species that causes tuberculosis
- Acid-fast organisms resist decolorization with acid-alcohol



Image 4. A mixed culture of *Mycobacterium smegmatus* (acid-fast, red/pink) and *Staphylococcus epidermidis* (non-acid-fast, light blue/purple). (Alan Schenkel, Peter Justice, and Erica Suchman, Colorado State University, Fort Collins, CO)

Endospore Stain

This stain is used to visualize bacterial endospores produced by members of the genera *Bacillus* and *Clostridium*. The nature of the spore makes it impervious to most ordinary stains and staining methods, but, once stained it strongly resists decolorization and counterstaining. In the **Schaeffer-Fulton** method, a primary stain with malachite green is forced into the endospore by steaming the bacterial emulsion. Malachite green is water soluble and has a low affinity for cellular material, so vegetative (actively dividing) cells may be decolorized with water. Vegetative cells are then counterstained with safranin. Microscopic examination of stained endospores will reveal their relative size and position in the cell, which are distinctive characteristics of each of the spore-forming species.

Primary stain - Malachite green

Counterstain - safranin

- Endospores resist staining with basic stains
- Endospores stain with malachite green; vegetative cells stain with safranin (red)



Image 5. Endospore stain of a *Bacillus cereus* culture using the Shaeffer-Fulton method and viewed at 1,000x total magnification under an oil immersion lens. Vegetative cells of *B. cereus* are in red; endospores are in green. Due to the age of the culture, endospores have been released from the cells. (Derek Weber and S. Finazzo, Broward Community College–Central campus, Davie, FL)

Capsule Stain (Negative staining)

Polysaccharide capsules and slime layers are generally difficult to stain selectively. Instead they are demonstrated by simply outlining them, staining the background and/or the cells without staining the capsule. This *negative stain* can be done several ways. The lab demonstration slide was prepared by staining the background and cells with basic fuchsin. Hence, the capsule appears as a clear zone or halo surrounding the red cell against a red background.

Uses an acidic stain: (Congo red or Nigrosin) and a basic stain: (crystal violet or safranin)

- Negative stains are neither heat-fixed nor rinsed
- The background of the slide is stained by acidic stains (capsule remains unstained)
- The cells within the capsule are stained with Basic stains
- Examples of encapsulated cells: *Bacillus anthracis*, *Streptococcus pneumoniae*, and *Klebsiella pneumonia*



Image 6: Encapsulated *Enterobacter aerogenes* stained with Anthony's capsule stain. (Gary E. Kaiser, The Community College of Baltimore County, Catonsville Campus, Baltimore, MD)

Flagella Stain

This stain coats the thin bacterial flagella with heavy metals or other compounds to make them visible in the light microscope. Once visible, the location and number of the flagella can be used diagnostically. The presence of flagella varies with cultural conditions, so a negative result is not proof of a cell's inability to produce flagella. The demonstration slide was prepared with a silver compound to coat the flagella and a red dye, basic fuchsin, to stain the cells.

Silver nitrate

- Used to see bacterial flagella that are too slender to be seen with other staining techniques
- Silver nitrate makes flagella appear larger than they are
- Can be used to determine arrangement of flagella for identification.
  - Ex: *Proteus vulgaris* has peritrichous flagella



Image 7: *Pseudomonas fluorescens* stained with Presque Isle Cultures Flagella Stain. Arrows in the labeled view point to the flagella. (Jay Mellies and Introductory Biology students, Reed College, Portland, OR)

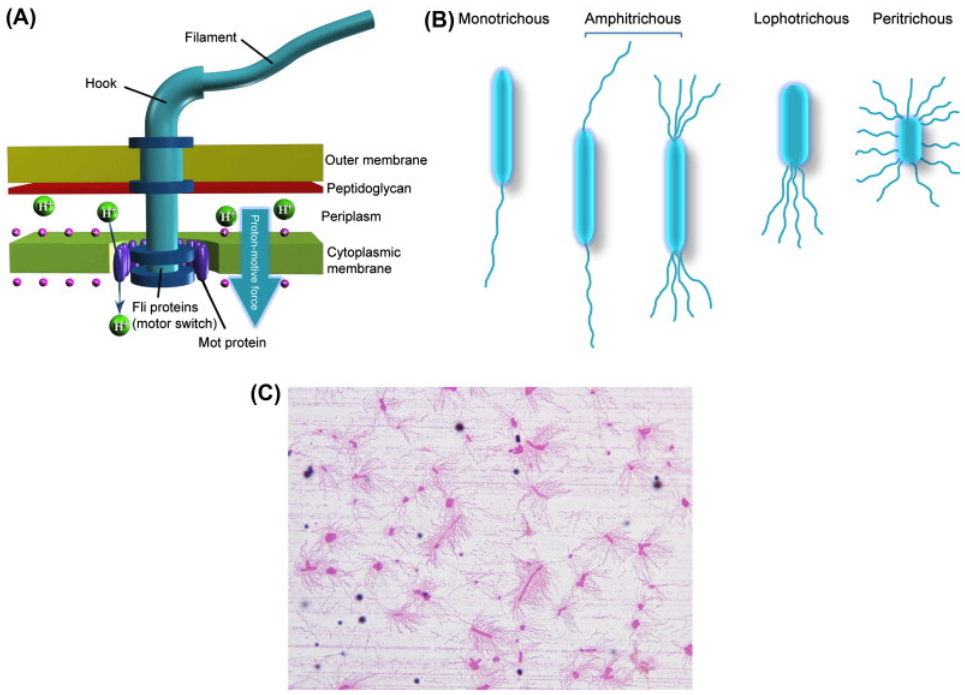


Image 8: (A) Schematic of *E. coli* flagellum. Basal body: Located at the base of the flagellum. The basal body, embedded in the cell wall and cell membrane, is the output device. It acts as an engine to provide energy for locomotion. The nearby switch determines the direction of rotation. Hook: This structure points directly away from the cell and has a sharp bend (about 90°) from which filaments protrude. Filament: This filiform structure protrudes from the bacterial cell. It is a hollow tube made of the protein flagellin. Its acts like a ship's or plane's propeller to move the bacterial cell. (B) Examples of bacterial flagellar arrangement. (C) Periplasmic flagella (flagella staining). The bacterial cell is stained red and the flagella are stained light red around the bacterial cell. From the Atlas of Oral Microbiology, edited by: Xuedong Zhou and Yuqing Li, 2015.

Spirochete stain

Silver nitrate

- Used to visualize slender spirochetes like *Treponema pallidum*

File:Secondary syphilis spirochete eccrine ducts high power.jpg

Image 9: Photomicrograph of skin biopsy showing secondary syphilis. Spirochete organisms are stained bright red by the *Treponema pallidum* immunohistochemical stain. 400x magnification by Jerad M. Gardner, MD. https://commons.wikimedia.org/wiki/F...high_power.jpg

References

1. Welcome to microbugz. Acid Fast Stain https://www.austincc.edu/microbugz/a...mplex%20lipids.

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4.3: Lab Procedures- Bacterial Smear, Simple and Gram Staining

Learning Outcomes

- Prepare microorganisms for microscopic observation.
- Observe the difference in size between bacteria and other unicellular microorganisms.
- Perform a simple stain and a Gram stain.
- Observe stained microorganisms and identify their size, shape, and staining properties.

Part I: Preparation of a Bacterial Smear

This semester you will be performing two staining procedures: A Simple Stain and a Gram stain. Both of these staining procedures begin with the preparation of a bacterial smear.

Materials

- Clean microscope slides
- Staining trays and newspaper
- Water bottle (for rinsing)
- Bacterial cultures: *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus*, *Pseudomonas aeruginosa*,

How to make a Bacterial Smear

1. Label a clean glass slide using a red wax marker. Note that it is important to recognize the side of the glass slide that you put your bacterial sample on.

2. Add a small drop of saline to the slide (you will usually put two bacteria on one microscope slide- Follow your instructor's specific instructions). This can be done by placing a drop of saline onto your inoculation loop and then transferring it to the slide. If you use the saline dropper directly on the slide, do not release a full drop.

3. With an inoculation loop or needle, pick up a small amount of bacteria. Mix it well with the saline and spread the mixture over a wider area of the slide.

    - Be careful not to have the two smears run into each other.

4. Air dry the bacterial specimen on the slide (slide warmers may also be used).

5. When slides are completely air-dry, heat fix the bacterial specimen by passing the slide slowly over the flame twice (your instructor will demonstrate this).

    - Heat fixing kills cells, and adheres them to the slide.
    - Cells will be rinsed off the slides if they are not heat fixed properly.
    - Be careful not to overheat the slides in this procedure

After heat-fixing is complete, you are ready to simple or gram stain your slide.

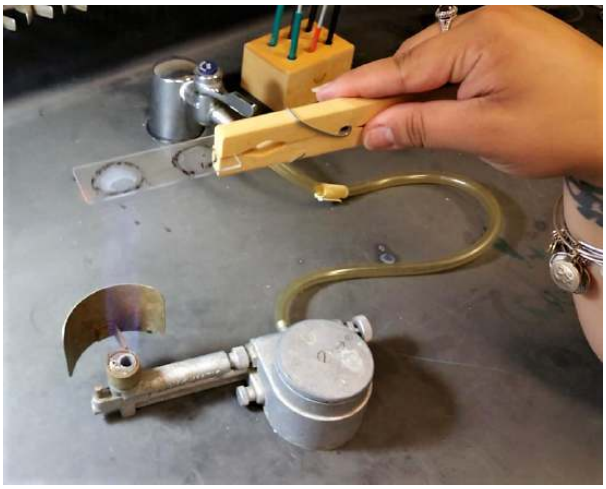


Image 1: Heat Fixing over a bunsen burner

Watch Video 1: on heat fixing using a bacticinerator



Watch Video 1: on heat fixing using a bacticinerator from Dr. G. Kaiser at URL: https://youtu.be/Rh0GrcTzjnU

Part II: Simple Stain

Cultures

Bacillus megaterium

Micrococcus luteus

Saccharomyces cerevisiae - a yeast

Materials

Dropper bottle containing staining solution of methylene blue

Brightfield Light Microscope

Oil immersion

lens paper

bibulous paper

Lab Procedures

A. Review Lab procedures for operating a Brightfield Light microscope

B. Preparation of a Bacterial Smear for Staining

Preparations of bacteria for staining can be made from growth on an agar plate or from a broth culture.

1. To prepare a slide from cells grown on an agar medium, first place a *SMALL* drop, a loopful works well, of water on a clean grease-free slide. Next, with a sterile loop transfer a *SMALL AMOUNT of the* growth to the drop of water and rub the loop around until the material is as evenly distributed as possible to form a just visibly turbid suspension. Spread the drop over a small portion of the slide to make a thin film with lightly visible turbidity.

2. Prepare three separate smears, one each of *Bacillus megaterium. Micrococcus luteus*, and *Saccharomyces cerevisiae.*

3. Next, *heat fix* the slide by placing it on the slide warmer for 5 minutes. Put the sample side facing up and label with your initials on one end of the slide. This process is called *heat fixing* the specimen to the slide. Its purpose is to bind the specimen to the slide so that it does not wash off during staining. Killing the cells with heat fixation also increases their permeability to the dyes used in staining.

Do not under-fix (the smear will wash off) nor over-heat (the cells will be ruptured or distorted) your specimen. The slide should be warm to the touch, *not hot*. If you think you have heated the slide too much, do not touch the slide to your hand to find out. Chances are, your suspicions are correct, and you will burn your hand with the hot glass. Instead, heat-fix the slide for a shorter period of time next time, then test.

4. After cooling the specimen is ready for the simple stain.

C. Methylene Blue – A Simple Stain

Living bacteria are almost colorless, and do not present sufficient contrast with the water in which they are suspended to be clearly visible. The purpose of staining is to increase the contrast between the organisms and the background so that they are more readily seen in the light microscope. In a simple stain, a bacterial smear is stained with a solution of a single dye that stains all cells the same color without differentiation of cell types or structures. The single dye used here is methylene blue, a basic stain. Basic stains, having a positive charge, bind strongly to negatively charged cell components such as bacterial nucleic acids and cell walls.

1. Stain by covering the smear completely with methylene blue. *This should be done over a sink with a slide holder.*

***Avoid getting stain on your clothes, books, and fingers. It is very difficult to remove.**

2. Allow the stain to act 1-2 minutes.

3. Tilt the slide to allow the stain to drain off. Now, rinse the remaining dye off with a *gentle* stream of water from a faucet or wash bottle.

4. Blot the slide dry with bibulous paper. Remove excess water from the slide by touching one corner of the slide to the blotting paper, then place the slide between clean sheets of paper in the blotting pad and *blot* dry. Be sure you *do not rub off* the smear.

5. Examine under the microscope using first the 10X and then the 100X oil-immersion objective.

6. Record your observations on the report sheets.

D. Test plate isolate

1. Check your "test plates" from Lab1: Exercise I, part D (ubiquity of microorganisms) for isolated single colonies to be candidates for your test plate isolate.

2. Attempt a simple stain of your candidate test plate isolate as described in part B of Exercise II. Record your observations.

3. Show an instructor your isolated single test plate isolate and the slide of the simple stain of the specimen you have chosen. This is to ensure you will be working with a bacterial culture instead of a fungal isolate.

4. If you did not obtain any single colony bacterial isolates on your "test plates" use one from another student in the lab, but make sure you choose a different isolate than that student has chosen, or use one from the instructor's plates.

5. After picking a candidate for your test plate isolate it is necessary to make sure it is a pure culture. Use a new Nutrient agar plate to re-streak for single colonies using the technique from Exercise I, part C (the "T" streak). Make certain you try and touch *only one* colony, the one you are interested in using as your unknown. Save your original plate. Incubate until the next laboratory period.

E. Demonstration Slide

You have just prepared slides of *Bacillus* (a rod shape) and *Micrococcus* (a coccus). We have set up a slide to demonstrate a third major cellular shape seen in bacteria. *Spirillum volutans* is a spiral shaped microorganism found in fresh water. View the demonstration slide and record your observations in the Results page.

Part III: Gram Staining

Gram Staining Procedure

- For all steps in the gram staining procedure, add enough of the solution to cover the areas of the slide that have bacteria on them. You do not need to flood the entire slide.
- All staining should be done over a staining tray. Be sure to put newspaper under the tray in case of spillage.
- Gloves should be worn while staining and removed before working with the microscope.

STEPS	EXPLANATION
1. Add a few drops of **crystal violet (primary stain)** to the smear and let it sit for 60 seconds.	
2. Rinse the slide with water.	All cells are **purple** after this step. Stopping here would be a simple stain.
3. Add a few drops of **Gram's iodine (mordant)** to the smear and let it sit for 60 seconds.	Gram's iodine forms a complex with crystal violet
4. Rinse the slide with water.	All cells are **purple** after this step.
5. **Decolorize** with **95% ethanol**: let the alcohol run over surface of slide until no more crystal violet color comes out of the smear (time varies—no more than 5-10 seconds).	Gram positive cells retain crystal violet and remain **purple**. Gram negative cells lose crystal violet and are now **colorless**
6. Rinse with water.	Water rinse stops the decolorization process
7. Add a few drops of **safranin (counterstain)** and let it sit for 60 seconds.	Safranin is a pink/red dye
8. Rinse with water. Blot dry on bibulous paper. Be careful not to wipe off the bacteria.	Gram positive cells remain **purple**; gram negative cells are now **pink/red**
9. Observe your slide under the microscope.	For each organism, determine morphology, arrangement and Gram reaction

Watch Video 2: on Gram Staining



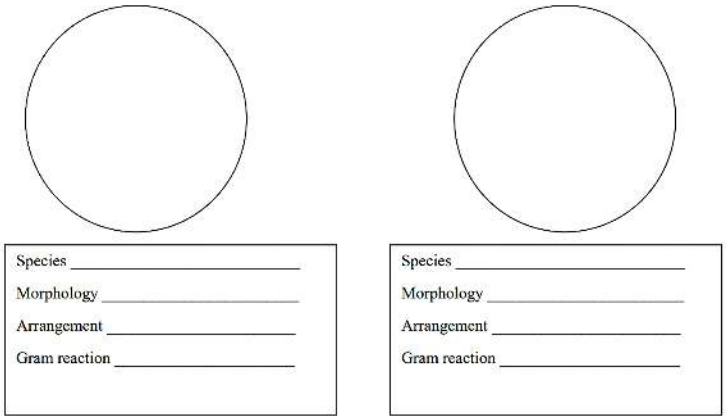
Watch Video 2: on Gram Staining. Video filmed at NC State. URL https://youtu.be/H-fxk1be1hQ

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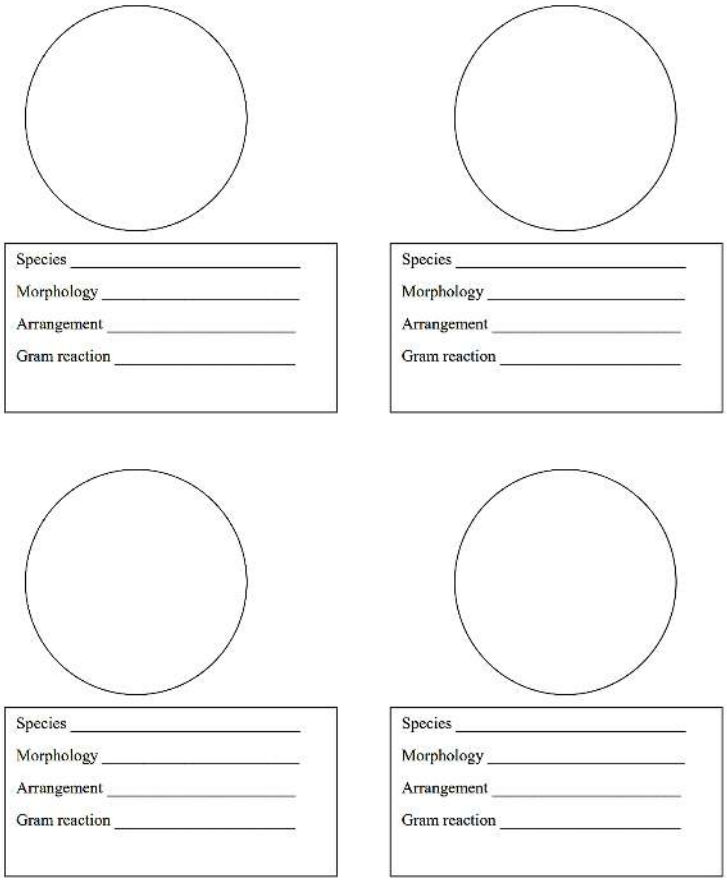
4.4: Results

Simple Stain Results

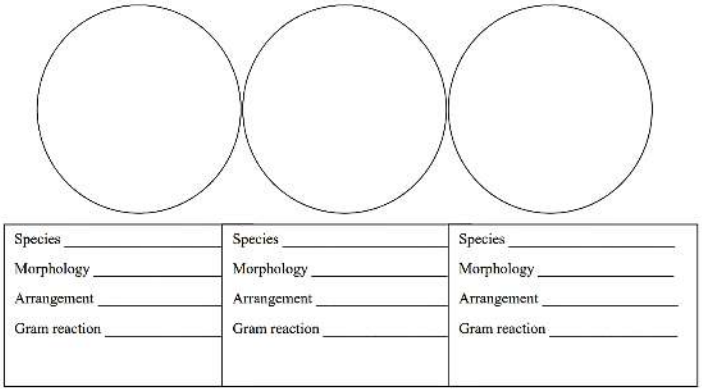


Gram Stain Results

Draw sketches for each type of bacteria that you observe. Identify its morphology, arrangement, and Gram reaction.



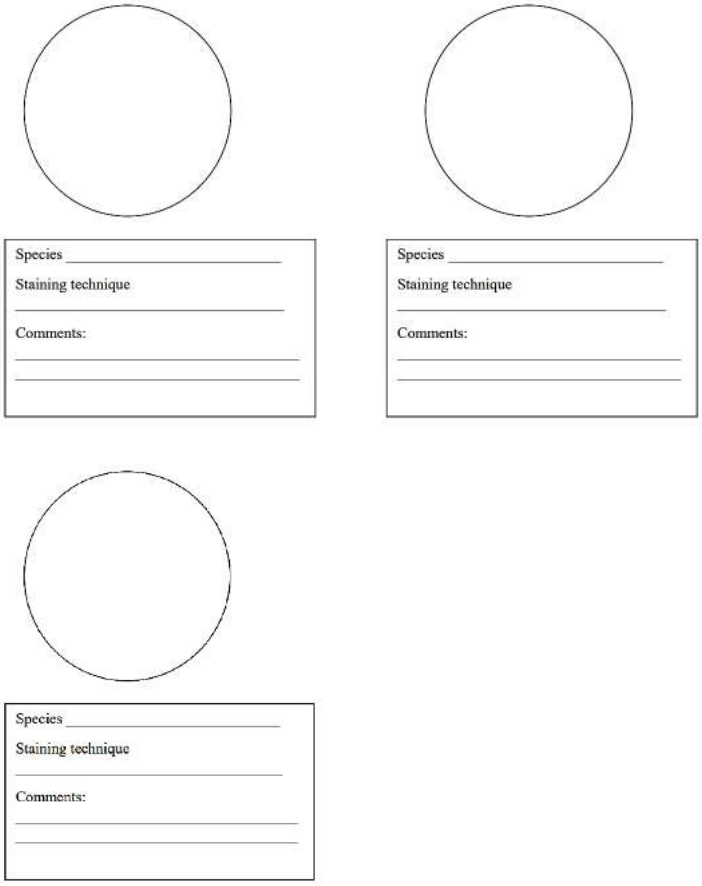
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results.png

Special Stain Results

Observe the special stains set up at the demo microscopes at the back of the lab. Make sketches below.



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4.5: Review Questions

1. Explain the major differences between the gram positive and gram negative cell wall.

2. *Salmonella typhi* is a gram negative organism.

a. What color will it appear when simple stained with crystal violet?

b. What color would it be if it was gram stained correctly?

3. Explain how bacterial cells would look in the gram staining procedure if the following mistakes were made:

a. Decolorizer left on too long

b. Decolorizer not left on long enough

c. Slide not heat-fixed before staining

4. What is the difference between a simple stain and a differential stain?

5. Explain why it is important to use only a small amount of bacteria when preparing a smear.

6. What are the two things that are stained in a capsule stain? What is NOT stained in a capsule stain?

7. Why would a health care provider be interested in knowing the gram reaction of a pathogenic bacterium?

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CHAPTER OVERVIEW

5: Enumeration of Bacteria

5.1: Introduction to Enumeration of Bacteria

5.2: Lab Procedures- How to operate a Pipettor

5.3: Lab Procedures- Viable Plate count

5.4: Results

5.5: Review Questions

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5.1: Introduction to Enumeration of Bacteria

Learning Outcomes

- Introduction to dilution theory
- Estimate the number of microbes in a sample using serial dilution techniques and standard/viable plate counts

Enumeration of Bacteria

Often one needs to determine the number of organisms in a sample of material, for example, in water, foods, or a bacterial culture. For example, bacterial pathogens can be introduced into foods at any stage: during growth/production at the farm, during processing, during handling and packaging, and when the food is prepared in the kitchen (1). In general, small numbers of pathogenic bacteria are not dangerous, but improper storage and/or cooking conditions can allow these bacteria to multiply to dangerous levels (1).

Fecal contamination of water is another one of the ways in which pathogens can be introduced (1). **Coliform bacteria** are Gram-negative non-spore forming bacteria that are capable of fermenting lactose to produce acid and gas. A subset of these bacteria are the **fecal coliforms**, which are found at high levels in human and animal intestines. Fecal coliform bacteria such as *E. coli*, are often used as **indicator species,** as they are not commonly found growing in nature in the absence of fecal contamination (1). The presence of *E. coli* suggests feces are present, indicating that serious pathogens, such as *Salmonella* species and *Campylobacter* species, could also be present (1).

Methods of Enumeration

Many approaches are commonly employed for enumerating bacteria, including measurements of the *direct microscopic count, culture turbidity, dry weight of cells*, etc. In a microbiology lab, we frequently determine the total viable count in a bacterial culture.

The most common method of measuring viable bacterial cell numbers is the ***standard or viable plate count or colony count.*** This is a viable count, NOT a total cell count. It reveals information related only to ***viable or live*** bacteria. Using this method, a small volume (0.1 - 1.0 mL) of liquid containing an unknown number of bacteria is spread over the surface of an agar plate, creating a **"spread plate."** The spread plates are incubated for 24-36 hours. During that time, each individual viable bacterial cell multiplies to form a readily visible colony. The number of colonies is then counted and this number should equal the number of viable bacterial cells in the original volume of sample, which was applied to the plate.

For accurate information, it is critical that each colony comes from only one cell, so chains and clumps of cells must be broken apart. However, many bacterial species grow in pairs, chains, or clusters, or they may have sticky capsules or slime layers, which cause them to clump together. It is sometimes difficult to separate these into single cells, which in turn makes it difficult to obtain an accurate count of the original cell numbers. Therefore, the total number of viable cells obtained from this procedure is usually reported as the number *of* ***colony-forming units (CFUs).***

A bacterial culture and many other samples usually contain too many cells to be counted directly. Thus, in order to obtain plates, which are not hopelessly overgrown with colonies, it is often necessary to ***dilute*** the sample and spread measured amounts of the diluted sample on plates. Dilutions are performed by careful aseptic pipetting of a known volume of sample into a known volume of a sterile buffer or sterile water. This is mixed well and can be used for plating and/or further dilution. If the number of cells in the original sample is unknown, then a wide range of dilutions are usually prepared and plated. The preparation of dilutions and the calculation and use of dilution factors to obtain the number of microorganisms present in a sample are important basic techniques in microbiology.

Method:

Aliquots from a stepwise or ***serial dilution*** of the original sample are spread on plates. Only a few of the plates following incubation will contain a suitable number of colonies to count; those plated from low dilutions may contain too many colonies to count easily while those plated from high dilutions may contain too few colonies or none at all. Ideally plates containing **30-300**

colonies per plate should be counted. At this colony number, the number counted is high enough to have statistical accuracy, yet low enough to avoid mistakes due to overlapping colonies.

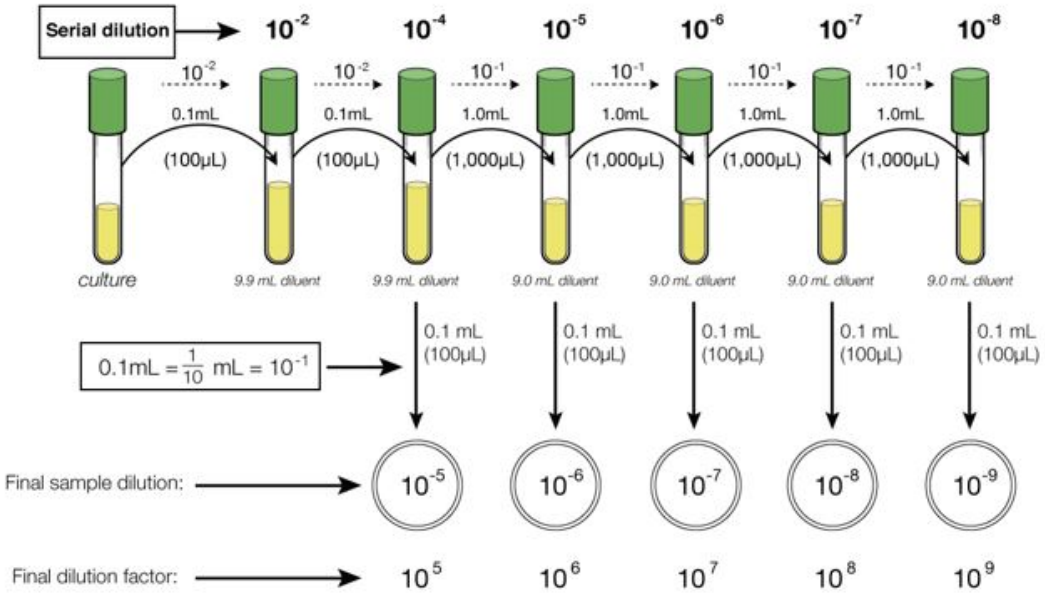


Figure 1: Serial dilution series and plating. A wide series of dilutions (e.g. 10^{-2} to 10^{-8}) is normally performed on the sample culture and spread plates created from the dilutions. A number of spread plates is needed because the exact number of live bacteria in the sample is usually unknown. Greater accuracy can be achieved by plating duplicates or triplicates of each dilution.

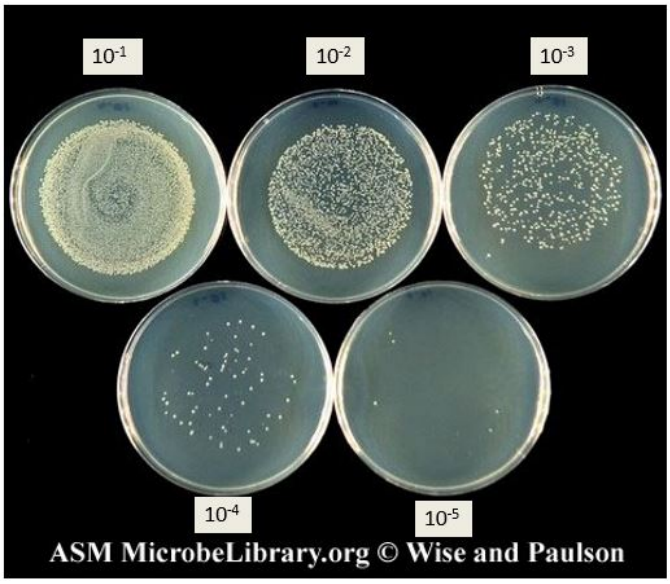


Image 1: Picture of spread plates showing bacterial growth (*Escherichia coli*, 40 hours, 25°C) on five plates prepared from a ten-fold dilution series. Care was taken to avoid spreading to the edges of the plates as it is more difficult to count colonies along the edge of the agar. Note how many colonies are in the plate from the 10^{-1} & 10^{-2} dilution plates. These plates have densely packed colonies, are too numerous to count, and most likely more than 300 CFUs. On the other hand, plates 10^{-3} , 10^{-4} and 10^{-}

[5] have a countable number (between 30-300) of CFUs. Image by Kathryn Wise and Darel Paulson, Minnesota State University, Moorhead, MN.

Calculations

In order to make the calculation of the number of cells/mL in the original sample less formidable, dilutions are designed to be easy to handle mathematically. The most common dilutions are ten-fold and multiples of ten-fold. A 1/10 or 10^{-1} dilution can be achieved by mixing 1 mL of sample with 9 mL of sterile dilution buffer. A subsequent 1/10 dilution of this first ten-fold dilution, made by mixing 1 mL of this first dilution with 9 mL of fresh sterile dilution buffer, would give a total dilution of the original sample of 100-fold (1/10 X 1/10 = 1/100 or 10^{-1} X 10^{-1} = 10^{-2}). Alternatively, a 100-fold dilution can be made directly from the original sample by mixing 1 mL of sample and 99 mL of buffer. These dilutions can be made in successive steps or a series to give a wide range for any given sample.

Once the dilution is made, an aliquot can be spread on an agar plate to create a spread plate. After incubation, the colonies which arise can be counted and the number of cells (more precisely the number of colony-forming units or CFUs) in the original sample can be calculated. For example in the image below, you did a serial dilution of a culture of the red pigmented bacterium, *Serratia marcescens* and made a series of spread plates. In plate 1, this was the 10^{-1} dilution, in plate 2 is the 10^{-2} dilution, and in plate 3 is the 10^{-3} dilution. After incubation, you count 241 colonies were present on the plate 10^{-2} dilution.

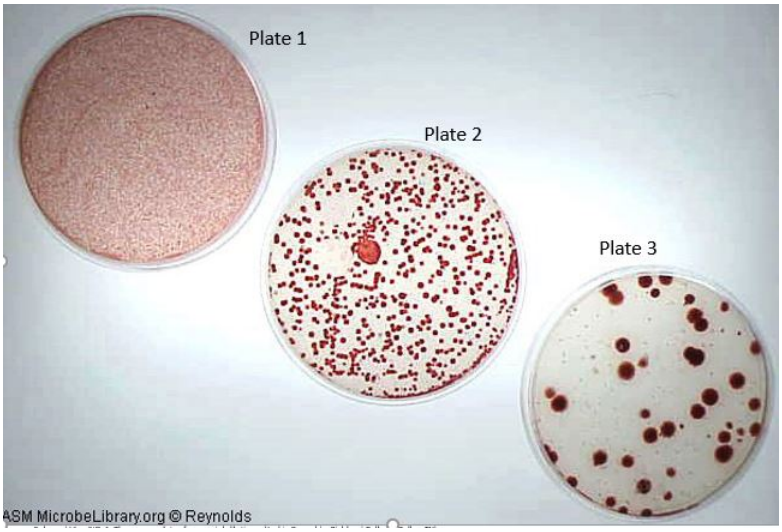


Image 2: Three spread plates from serial dilutions. Image by Jackie Reynolds, Richland College, Dallas, TX.

Therefore, there were 241 X 10^2 CFU/mL in the original sample or expressed 2.41 x 10^4. To arrive at this final number you only need to multiply the final number of colonies on the plate, 241, times the *total dilution factor.* The dilution factor is defined as the *inverse of the dilution.* So in this case, the dilution factor is the inverse of 1/100 or 100. In other words, the dilution factor is how many times the sample was diluted.

It usually works better to spread only 0.1 mL of a sample on a standard-size petri plate. If the above example were changed such that 0.1 mL of a 100-fold dilution of the same sample was plated, there would ideally have been 24 colonies on the plate. This number represents the number of CFU in *only 0.1 mL of the dilution* plated. Therefore, to calculate the *CFU/ml* in the sample it is necessary to multiply the number of colonies on the plate by 10 (there are ten 0.1 mL units in 1.0 mL) and then by the dilution factor (100) to arrive at the final answer: 24 CFU x 10 x 100 = 24000 or 2.4 x 10^4 CFU/mL.

The only way to understand dilution theory well is to practice it, so you should work practice problems until you feel confident in using dilution factors and calculating CFU/mL in original samples. You should also be able to determine the proper dilutions to use

to obtain 30-300 colonies on a plate if the original number of CFU/mL in a sample is known.

Watch Video 1 on how to perform a serial dilution using "pour plates" and the associated calculations:



Watch Video 1: Serial dilutions and pour plate technique. The example here using a "pour plate" technique to spread the dilutions out instead of the "spread plate" discussed above, but the outcome of both techniques of spreading the dilution sample out is the same. (10:48) Video by Microbial Zoo. URL:https://www.youtube.com/watch?v=nViO9Y4Yxfk

Watch video 2 on how to perform a serial dilution and make spread plates.



Watch Video 2: Serial dilutions with a bacterial sample and the 'spread plating' technique at NC State Microbiology labs. (10:30) URL: https://youtu.be/IJcw4fRsYnU

References

1. Contributed by Joan Petersen & Susan McLaughlin Associate Professors (Biological Sciences and Geology) at Queensborough Community College

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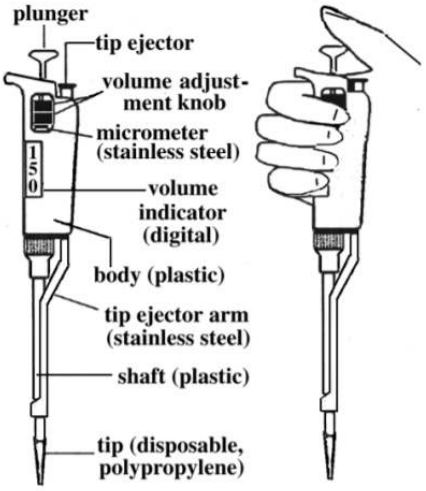
5.2: Lab Procedures- How to operate a Pipettor

Getting to Know Your Pipette

A pipet is a commonly used laboratory piece of equipment used to transfer a measured volume of liquid. A mechanical pipettor, shown in Fig. 1, can come in range of volumes it can transfer. Careful attention to its use, operation, and practice is needed to be proficient.

1. Find the following parts on your pipette:

Fig. 1 Pipettor



- Volume adjustment dial
- Tip ejector button
- Plunger button
- Stainless steel micrometer
- Digital volume indicator
- Stainless steel ejector arm (removable)
- Plastic shaft
- Disposable yellow or blue tip

2. Practice holding your pipette correctly, placing a tip on our pipette, and ejected the tip. Do this at least three times.

3. The total volume a pipette can hold is stated on the top of the plunger. It is listed as "µl" or microliter volumes. We will be working with the following three volume pipettes:

- P-20: 0.02 µl – 20 µl
- P-200: 20 µl – 200 µl
- P-1000: 200 µl – 1000 µl

4. Based on the volume pipette you have the numbers in the digital display have a different meaning.

P20		
min.	int.	max.
0	1	2
2	2	0
0	5	0
2 µl	12.5 µl	20 µl

P200		
min.	int.	max.
0	1	2
5	2	0
0	5	0
50 µl	125 µl	200 µl

P1000		
min.	int.	max.
0	0	1
2	7	0
0	5	0
200 µl	750 µl	1000 µl

5. Rotate the volume adjustment knob until the digital indicator reaches the desired volume, then place a disposable tip on the shaft of the pipette (practice all three volumes min, int, max)

6. Press down on the plunger to the First Stop. (You will be able to push past this point, but there is enough resistance to stop the movement if you try to be aware of it.)

7. Hold the pipette vertically and immerse the disposable tip into the sample. Use the colored water and the microcentrifuge tubes provided to you.

8. Allow the plunger button to return slowly to its original position. Do not allow the button to snap up.

9. To dispense the sample: place the tip against the side wall of the receiving tube and push the plunger down to the first stop. Wait 2-3 seconds, then depress the plunger to the second stop in order to expel any residual sample in the tip.

10. While the plunger is still pushed down, remove the pipette from the tube and allow the plunger to slowly return to its original position.

11. Practice.

Warning

- Never rotate the volume adjustment knob past the upper or lower range of the pipetman.
- Never lay the pipetman down on its side or hold it horizontally when it contains liquid.
- Never immerse the shaft of the pipetman into the fluid.

Watch this video 1: Micropipetting



Watch Video 1: how to use a micropipettor URL: https://youtu.be/NgosWmRjjAo (5:02)

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5.3: Lab Procedures- Viable Plate count

Learning Outcomes

- Learn microbiological skills such as pipetting, performing serial dilutions, prepare spread plates for a viable plate count, and performing a colony count.
- Estimate the number of microbes in a sample using serial dilution techniques, including: a. correctly choosing and using pipettes and pipetting devices b. correctly spreading diluted samples for counting c. estimating appropriate dilutions d. extrapolating plate counts to obtain the correct CFU in the starting sample.

Materials:

Cultures: Stationary phase broth culture *of Serratia marcescens*

Media: Dilution tubes of sterile water

Supplies: Nutrient agar plates, P-1000 Pipetman, sterile tips, Sterile L-shaped blue cell spreaders ("hockey stick" or "spreader")

Procedure:

A. Sample Dilution and Spread Plating

1. Label one plate on the bottom for each of the following dilutions: 10^{-5},10^{-6} , 10^{-7}, 10^{-8},and 10^{-9}. See Image 1.

2. Label the dilution tubes as follows: label the two tubes (Navy blue caps) containing 9.9 mL sterile water as 10^{-2} and 10^{-4}; label the four tubes (Green caps) containing 9.0 mL sterile water as 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}.

3. *Carefully* and *aseptically* remove 0.1 mL (100µL) of the *Serratia marcescens* culture and pipette it into the tube marked 10^{-2}. Mix the tube completely, *being careful not to spill any of the contents.* The vortex mixer will help you mix the contents of the tube.

- You have now prepared a 10^{-2} (1/100) dilution. Why is it 10^{-2}? Because 0.1 mL of undiluted culture was diluted into 9.9 mL of water, giving a total volume of 10.0 mL in the dilution tube. That makes this a 10^{-2} dilution (0.1/0.1 + 9.9 = 0.1/10.0= 1/100= 10^{-2}).

4. *Change the pipette tip*, and take 100µL (0.1 mL) from the 10^{-2} dilution and pipette it into the next 9.9 mL dilution tube marked 10^{-4}. Mix well. You have now prepared a successive 1/100 dilution, resulting in 10^{-4} dilution of the **original** culture (1/100 multiplied by 1/100 = 1/10,000 = 10^{-4}, which is the same as 10^{-2} x 10^{-2}=10^{-4}).

5. *Change your pipette tip again.*

- Why keep changing pipettes? Because any fluid left in the pipette tip from the previous dilution will contain many more cells per mL than any successive dilution and, if used, will grossly confuse the final results by indicating a higher number of cells than were actually present in the original sample.

Now we will start doing a series of 1/10 dilutions. Set your Pipetman to 1,000µL and remove a 1,000µL (1.0 mL) aliquot from the 10^{-4} dilution tube. Transfer it to the 9.0 mL dilution tube marked 10^{-5} and mix well. The ***original culture*** has now been diluted 1/100,000, or 10^{-5} (10^{-4} x 10^{-1} = 10^{-5}),

- You have just prepared a 10^{-1} (1/10) dilution of the **previously diluted culture**. Why is it 10^{-1}? Because 1.0 mL of diluted culture was further diluted into 9.0 mL of water, giving a total volume of 10.0 mL in the dilution tube. (1.0/1.0 + 9.0 = 1.0/10.0 = 1/10 = 10^{-1}). Now you know why these series of dilutions are referred to as ***serial dilutions.***

- Continue your dilution series, as indicated in Image 1, through to the 10^{-8} dilution tube.

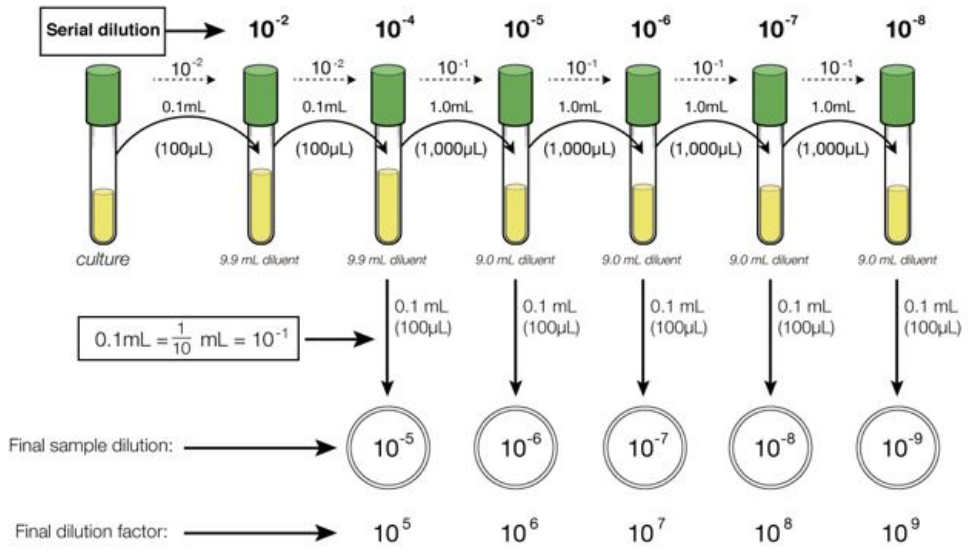


Image 1: Serial dilution and plating

6. *Using a new pipette tip*, transfer 100µL (0.1 mL) of the 10^{-8} dilution onto the center of the agar surface of the plate marked 10^{-9}.

Consider why this is a 10^{-9} plate after you put 100uL (0.1 mL) of inoculum from the 10^{-8} dilution tube on it. Remember that you are trying to determine the number of viable cells **in each 1.0 mL aliquot** of the original *Serratia marcescens* sample, and you only put 10% of 1.0 mL (0.1 mL) on the nutrient agar plate.

Repeat with the rest of the dilutions, transferring 100µL (0.1 mL) of each dilution tube onto the appropriate agar surface, using a new pipette tip between each dilution tube.

7. **Spread plates:** Using good aseptic technique, take one sterile blue L-shaped cell spreader out of the bag and reseal the bag. Starting with the plate marked 10^{-9} , using the spreader, gently push the liquid inoculum applied to the center of the plate, two or three times clockwise around the dish, then several times counterclockwise, turning the plate on the turn table as needed to obtain *complete* coverage.

Continue with the rest of the spread plates ***using the same* cell spreader**. Make sure you are continuing to work *backwards* (from 10^{-9} , then 10^{-8} , then 10^{-7}, then 10^{-6} etc.) from the most dilute suspension to the most concentrated suspension to minimize the amount of carryover from plate to plate. Again, use good aseptic technique, work quickly, do not touch the cell spreader on surfaces other than the agar plate you are using. If you accidentally "contaminate" the spreader by touching a surface (table or other), then dispose of it and get a new spreader. We are trying to minimize the use of the spreaders to reduce waste, but if it gets compromised, then replace it with a new sterile spreader.

Dispose of the cell spreader in the small orange biohazard bin on your bench when you are done with your spread plates.

Remember that the plates should be labeled as a ten-fold higher dilution than the dilution tube of the 0.1 mL sample being plated. For example, 0.1 mL of the 10^{-6} dilution tube should be plated on the agar plate marked 10^{-7}. Again, if you need help visualizing this, see Figure 6-1.

It is generally desirable to make duplicate *or* triplicate plantings of each dilution and to average the resulting counts. However, since your lab sample comes from the same stock culture, the class average should give an accurate enumeration of the original stock culture.

8. After the spread plating, leave plates agar side down for at least 30mins. in order for the inoculum to absorb onto the agar, then invert the plates and incubate at 30°C.

B. Counting colonies on plates

1. Looking at your dilution plates prepared last period, choose the plates that have from 30-300 colonies on them. As this might take some practice in plate counting, you might need to choose all plates with what looks like a reasonable number of colonies to count.

a. Those plates that have no microbial growth can be recorded as **0** or **NG**, No Growth.

b. Those plates on which colonies are not individually distinct (their edges run together) can be recorded as **TNTC**, **T**oo **N**umerous **T**o **C**ount.

c. Those plates on which you cannot distinguish any individual colonies, the entire surface is covered with microbial growth, can be recorded as *confluent*.

2. Count each colony to give a total colony count for each plate chosen. You will avoid counting a colony twice by marking off the colonies on the bottom of the plate as you count them. This requires, of course, that the plate be upside down. Be sure to count any small colonies. Record your results on the report sheet.

C. Calculation of number viable cells/mL in the original sample

- Choose the plate containing between 30 and 300 colonies.
- Multiply the *number of colonies on the plate* by *the final dilution factor.* This gives the total viable cells/mL in the original sample.
- Calculate the colony-forming units, CFU, per mL for the *Serratia marcescens* culture.

D. Watch this video on how to perform a serial dilution and make spread plates.



Watch Video 1: Dilutions and Plating at NC State Microbiology labs. URL: https://youtu.be/IJcw4fRsYnU

5.3: Lab Procedures- Viable Plate count is shared under a not declared license and was authored, remixed, and/or curated by LibreTexts.

5.4: Results

Enumeration and Dilution

1. Read lab procedure section on how to obtain colony counts and CFU/mL in the original *Serratia marcescens* sample.
2. Record data from your *Serratia marcescens* plates here:

final dilution	**colony count**
10^{-5}	
10^{-6}	
10^{-7}	
10^{-8}	
10^{-9}	

CFU/mL of original *Serratia marcescens* sample =_____

3. Record the class data here for the *Serratia marcescens* broth culture:

Individual Student data	**Dilution with 30-300 colonies**	**Number of colonies on plate**	**CFU/mL in original *Serratia marcescens* sample**
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			

Class average:_____

4. Practice question

Final sample dilution	Colony Count*	CFU/mL in original sample
1/1000	135	
10^{-6}	48	
10^{-8}		6.5×10^{9}
	99	$9.9 \times l0^{6}$

*Remember the 30-300 colonies per plate rule.

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5.5: Review Questions

1. Why do we report bacterial counts as CFUs/mL?

2. A viable plate count was performed on a river sample. Using the table below, how many bacteria were present in the original river sample?

Dilution	Amount plated	Number of colonies counted
10^{-4}	1.0mL	310
10^{-5}	1.0mL	28
10^{-6}	1.0mL	7

2. How would you prepare a series of dilutions to get a final dilution of 10^{-10}? outline each step.

3. When determining the number of bacteria from a sample using a viable plant count, why do you often have to do a 'serial' dilution of the sample first? Why don't you just plate out 1ml or 0.1ml of the sample on a spread plate?

4. Why is it called a "viable" plate count? what does that mean?

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CHAPTER OVERVIEW

6: Microbial Physiology

6.1: Introduction to Oxygen Requirements

6.1.1: Determining Oxygen Requirements and Anaerobes

6.2: Temperature, pH, and Osmotic Requirements

6.3: Bacterial Growth Dynamics

6.4: Bacteriophages

6.5: Lab Procedures- Testing Oxygen requirements

6.6: Lab Procedures- Plaque Assay

6.7: Results

6.8: Review Questions

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6.1: Introduction to Oxygen Requirements

Learning Outcomes

- Recognize the effects of Oxygen on bacteria
- Explain the various oxygen requirements of the microbes, observe and interpret the growth of microbes in thioglycollate agar deep media
- Discuss methods of culturing anaerobic bacteria

Environmental Requirements: Oxygen requirements

How does Oxygen affect bacterial growth?

Bacteria can differ dramatically in their ability to utilize oxygen (O_2). Under aerobic conditions, oxygen acts as the final electron acceptor for the electron transport chain located in the plasma membrane of prokaryotes. Bacteria use this process to generate ATP, the energy source for most cellular processes. In the absence of oxygen (O_2), some bacteria can use alternative metabolic pathways including anaerobic respiration and/or fermentation. During anaerobic respiration, other alternative molecules are used as the final electron acceptor for the electron transport chain such as nitrate (NO_3), sulfate (SO_4), and carbonate (CO_3).

Bacteria and many microorganisms are very sensitive to oxygen concentrations. Some will only grow in its presence and are called **obligate aerobes.** **Facultative aerobes** will grow either aerobically or in the absence of oxygen (anaerobic conditions), but they generally do better with oxygen. **Aerotolerant anaerobes** don't require oxygen, but can grow in its presence, while strict **obligate anaerobes** cannot use oxygen and cannot grow or survive in its presence. **Microaerophiles** use oxygen, but at lower concentrations than atmospheric oxygen levels (which is ~20%).

One can determine a bacterium's oxygen requirements by cultivating them in a special medium called **thioglycollate agar tubes.** The bottom of the tube of medium is kept anaerobic by cystine and thioglycollic acid, which chemically react with, and tie up any oxygen that diffuses in. Any un-reacted oxygen in the tube will be indicated by resazurin, a dye that turns pink in the presence of oxygen. It is common for the top centimeter or so to be pink. One can inoculate a thioglycollate tube with your bacterium and observe where the bacterium grows in the tube to determine its oxygen requirements (see Image 1)

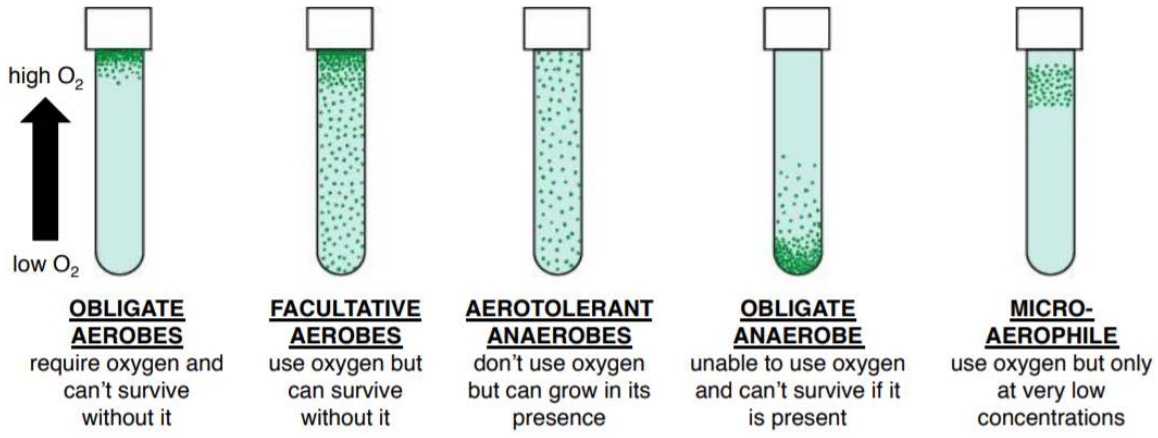


Image 1: Microbial oxygen requirements determined using thioglycollate agar tubes. Green dots represent bacterial colonies within in the agar or on its surface. The surface of the agar tube is directly exposed to atmospheric oxygen, and will be aerobic. The oxygen content of the thioglycollate medium decreases with depth until the medium becomes anaerobic towards the bottom of the tube.

Cultivation of Anaerobes

The cultivation of anaerobes can be done in anaerobic chamber (image 2). This is a special chamber where you can work with and cultivate strict obligate anaerobes without exposing them to oxygen. Anaerobic chambers contain a hydrogen (H_2) gas mixture that is circulated through a heated palladium catalyst to remove oxygen (O_2) by forming water (H_2O). Anaerobic chambers use a gas mixture of H_2 and nitrogen gas (N_2) (5/95%) or N_2/carbon dioxide (CO_2)/H_2 (85/10/5 %) to remove oxygen. An airlock is used to reduce O_2 levels prior to the transfer of samples in and out of the chamber.

Another way of culturing bacteria anaerobically on plates is to use a GasPak anaerobic system. In these systems, hydrogen and carbon dioxide are generated by a GasPak envelope after the addition of water. A palladium catalyst in the chamber of the GasPak system catalyzes the formation of water from hydrogen and oxygen, thereby removing oxygen from the sealed chamber (image 3 and 4). These systems are compact, easy to use, and less expensive than an anaerobic chamber. They come in jars (image 3) or in a box format (image 4).



Image 2: Anaerobic chamber. https://coylab.com/products/anaerobi...robic-chamber/





Image 3: GasPak system jars
https://www.fishersci.com/shop/produ...rs-3/p-4902079

Image 4: GasPak system boxes.

Watch Video 1: Basics of thioglycollate media



Watch Video 1: explanation on how thioglycollate media works and examples. (9:33) URL: https://youtu.be/AJG18sQd8mU

Watch Video 2: how to prepare an anaerobic jar



Watch Video 2: how to set up an anaerobic jar. The process is similar for an anaerobic box. (3:03) URL: https://youtu.be/aFDYx-7ceS8

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6.1.1: Determining Oxygen Requirements and Anaerobes

Learning Objectives

- Identify the 3 major categories of microbes based on oxygen requirements.
- Learn different ways to culture anaerobic bacteria.

HOW TO DETERMINE OXYGEN REQUIREMENTS

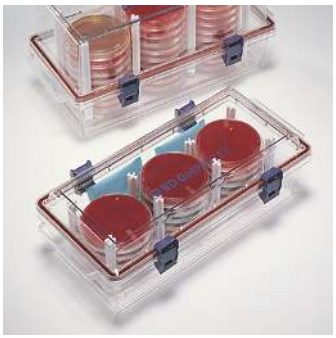
An excellent way to determine the oxygen needs of your bacterium is to grow it in different oxygen environments---atmospheric oxygen of 22%, no oxygen at all (GasPak jarbox or anaerobic chamber), and reduced oxygen at less than 10% (candle jar)--and compare the quality and quantity of growth.

TYPES OF OXYGEN ENVIRONMENTS



In image 1, the **candle jar** on the right has 3-5% CO_2 and 8-10% O_2 (0.3% and 21% in the atmosphere, respectively). This is a handy way to determine if you have an aerobe which is ***microaerophilic***, since they grow optimally under reduced (but present) oxygen conditions as in the candle jar. Many ***microaerophilic*** bacteria will grow poorly at 22% O_2, whereas some will not grow at all (e.g. ***Neisseria gonorrhoea***). Possibly the by-products of aerobic respiration, superoxide radicals and hydrogen peroxide, make it difficult for the ***microaerophiles*** to do well in 22% O_2. Some ***microaerophiles*** are actually capnophilic (requiring elevated CO_2 levels to grow). Strict aerobes may not grow well in a candle jar, depending on the species. The Gram-positive genus ***Bacillus*** and Gram-negative genus ***Pseudomonas*** include aerobic bacillus-shaped bacteria.

Image 1: On the left is a GasPak jar, with a GasPak generator envelope inside. The environment is 0% O_2. On the right is a candle jar with reduced atmospheric O_2 concentrations and CO_2 .



The newer anaerobic system (image 2) consists of a plastic container or box (for the agar plates) and a GasPak paper generating sachet. The sachet contains ascorbic acid and activated carbon which reacts on exposure to air, when removed from the enclosed envelope. Oxygen is rapidly absorbed and CO_2 is produced. When the GasPak paper sachet is placed in a sealed plastic pouch, this reaction will create ideal atmospheric conditions for the growth of anaerobes—anaerobic within 2.5 hours.

Because a GasPak jars and boxes looks the same, whether it has oxygen inside or not, an indicator strip, containing methylene, is included in the jar. Methylene blue is blue when oxidized, colorless when reduced. The carbon within the pouch reacts with free oxygen in the jar, producing 10-15% CO_2.

Image 2: Anaerobic boxes

Quite a few human pathogens are strict **anaerobes**, exemplified by the bacillus-shaped genera---Gram-negative ***Bacteroides***, ***Bacillus*** (***anthracis***), and Gram-positive ***Clostridium*** (***tetani***, ***botulinum***).

Aerotolerants are anaerobes that can grow in the presence of O_2 (compared to the strict anaerobes which would likely die), but they do not use it. And last, but very common, are the **facultative anaerobes** which prefer to use O_2 when present but will grow without it.

Another way to culture and grow anaerobes is the use of reduced media--media without oxygen. **Thioglycollate broth/agar** has a reducing agent in it---the chemical thioglycollate---which binds any free oxygen within the medium. You will also notice that these

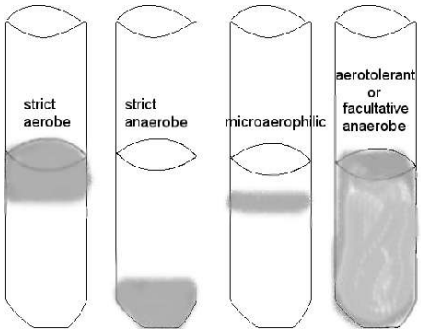
tubes have screw caps, allowing a tight closure, to reduce oxygen entry. However, some oxygen will be in the tube between the cap and the broth and there is no way to get rid of it. So there will be some diffusion of oxygen into the top portion of the broth, and that is where any aerobic bacteria may grow. An indicator, **resazurin**, in the medium will be a light pink in the area of higher oxygen. You can determine whether the bacterium is an anaerobe, facultative anaerobe, or an aerobe by checking out where the organism grows in the column of media. **DO NOT SHAKE IT**!



> Note
>
> Oxygen will permeate the broth when this medium sits around for a while.

Check for the pink color: if so, boil the broth for 5 minutes (removes the oxygen).

growth is indicated by gray area



PROCEDURE

Thioglycollate broth

1. The thioglycollate broth should be either boiled first before inoculation **OR** recently made so that the oxygen content is very low. (Your instructor will tell you if it needs to be boiled).
2. Inoculate a tube of thioglycollate broth with your unknown bacterium: make sure that the loop or needle goes down to the BOTTOM of the broth (do not get **metal holder** in the sterile broth).
3. Incubate at 25 or 37 degrees C as directed.

TSA plates in 3 different oxygen environments

1. Label 3 plates for the table---candle jar, ambient air, and GasPak anaerobic jar.
2. Divide the 3 plates into sections, one for each organism—your unknown, the strict aerobe, the strict anaerobe.
3. Inoculate the section by streaking a straight line or a zigzag (as seen below). HOWEVER, be sure that you inoculate all 3 plates using the same technique.
4. Be sure that the jar has a methylene blue indicator strip (seen above) inside. The methylene blue is blue when oxidized, but colorless when reduced. Before the jar is opened, the strip should be checked to make sure that it is COLORLESS.
5. Incubate at 30 or 37 degrees C

INTERPRETATION: after incubation

TSA plates

Compare the presence/absence of growth, as well as the quantity of growth on the 3 plates. Determine whether aerobic, anaerobic, or facultatively anaerobic.

To the right:

- A is an facultative anaerobe
- B is an aerobe (microaerophilic)
- C is an anaerobe



Thioglycollate broth

Determine WHERE the most amount of growth occurs in the column of liquid---the top, the bottom, top to bottom. DO NOT SHAKE IT! How can you determine if the bacterium is aerobic, anaerobic, or facultatively anaerobic?

Contributors

- Jackie Reynolds, Professor of Biology (Richland College)

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6.2: Temperature, pH, and Osmotic Requirements

Learning Outcomes

- Recognize the cardinal temperatures of growth for bacteria
- State the conditions for classification of psychrophiles, mesophiles, thermophiles, and hyperthermophiles
- Recognize the pH requirements for bacteria
- Recognize the osmotic requirements for bacteria

Environmental Requirements: Temperature

How does temperature affect bacterial growth?

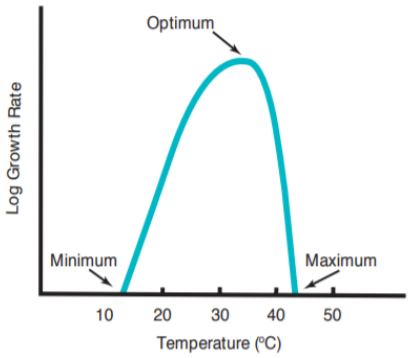
Organisms grow best over a certain temperature range, and this range has restrictions. The **cardinal temperatures** are the range of temperatures over which an organism can grow. Every organism has evolved to live at a particular optimum temperature.

- Minimum: lowest temp where reproduction occurs
- Maximum: highest temp where reproduction occurs
- Optimum: highest rate of reproduction

Organisms are classified based on the temperature ranges they live in:

- Psychrophiles: less than zero
- Psychrotrophs: 0-30°C
- Mesophiles: middle temperatures 15-45°C
- Thermophiles: 40-80°C
- Hyperthermophiles: above 65°C

Microorganisms require a temperature growth range dictated by the heat sensitivity of its cellular components. As a result, microbial growth has a characteristic temperature dependence with distinct **cardinal temperatures**---the minimum, optimum, and maximum, temperatures at which it can grow. The optimum temperature is usually correlated to its natural habitat.



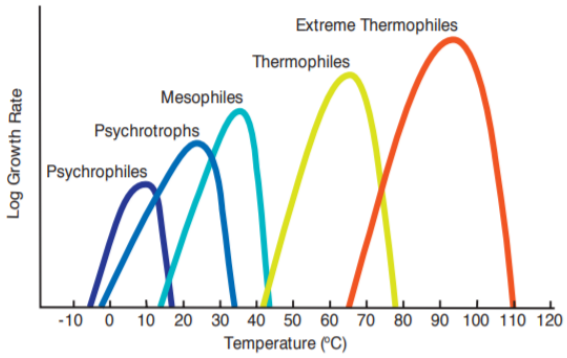


Image 1 and 2: General temperature ranges compared to growth rate

Environmental Requirements: pH

How does pH affect bacterial growth?

Hydrogen ions in a solution = pH. Organisms grow best at a specific pH range based, in part, on the environment they have evolved to live in. If bacteria are outside their optimal pH range their proteins can become denatured. Ranges of pH over which an organism can live place them in groups:

- Acidophiles: below pH 5.5
- Neutrophiles: pH 5.5 -8.5
- Alkaliphiles: pH above 8.5

- The pH is another environmental condition that dictates microbial growth. pH impacts the activities of enzymes and each microbial species has a pH growth range. **Acidophiles** have a growth range between pH 0.0-5.5, **neutrophiles** grow btween 5.5 and 8.5, while **alkalophiles** do best between 8.5-11.5 (or higher). Generally, different microbial genera have characteristic pH optima ranges. The majority of bacteria are neutrophiles while molds and yeasts tend to prefer slightly acidic environments with a pH range of 4-6. Many bacteria produce acids as part of their metabolism, and this can lower the pH of their environment. One excellent example of this are the **Lactic Acid Bacteria (LAB);** a large and diverse group of Gram-positive bacteria that produce lactic acid as the major end product of the fermentation of carbohydrates. The lactic acid can inhibit the growth of pathogenic and food spoilage microorganisms in food. Thus the LAB play a significant role in food fermentation, contributing to a wide variety of fermented products (ie. cheese, yogurt, meat, fish, fruit, vegetable and cereal products). Their breakdown of various carbohydrates, proteins, and lipids contribute to the flavor, texture and nutritional value of the fermented foods.

solution classification	pH	H+ concentration (moles/liter)	common examples	organismal classification
↑	0	10^0	nitric acid	↑
	1	10^-1	stomach acid	
	2	10^-2	lemon juice	
	3	10^-3	vinegar, soda	
	4	10^-4	tomatoes, orange juice	
ACIDIC	5	10^-5	black coffee	acidophiles
	6	10^-6	urine	
NEUTRAL	7	10^-7	pure water	neutrophiles
	8	10^-8	seawater	
ALKALINE	9	10^-9	baking soda	alkaliphiles
	10	10^-10	soap	
	11	10^-11	ammonia	
	12	10^-12	lime water	
	13	10^-13	household bleach	
↓	14	10^-14	drain cleaner	↓

Image 3: pH Classification for microorganisms.

Environmental Requirements: Salinity

How does osmotic pressure affect bacterial growth?

Water is essential to all organisms. The ability to control the movement of water across a membrane is necessary for the survival of all cells. **Osmotic pressure** is the minimum pressure which needs to be applied to a solution to prevent the inward

flow of water across a semi-permeable membrane. The movement of water is controlled by the concentration of solutes contained within the water (usually salt). Bacteria can be classified based upon the salinity they can tolerate:

- Halophiles (prefer NaCl concentrations of 3% or higher)
- Extreme halophiles (prefer NaCl concentrations of 15%-25%)
- Xerophile (prefer low salt concentrations)

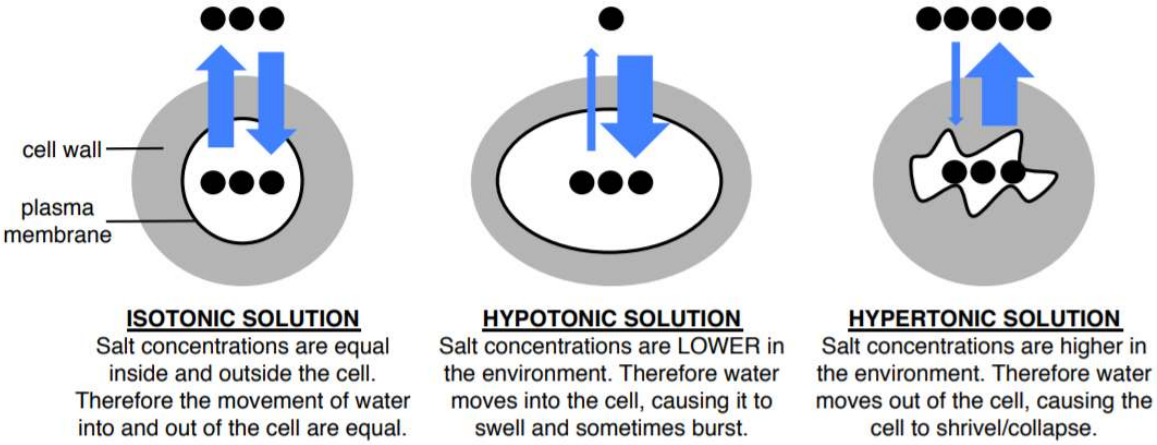


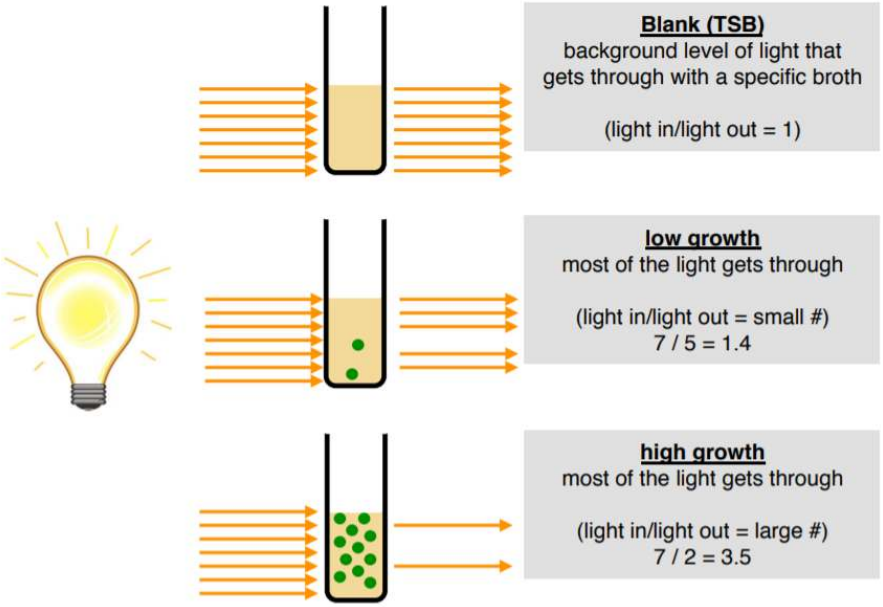
Image 4: Impact of various salt concentrations on movement of water into/out of a cell.

Quantifying Bacterial Growth:

How do we quantify bacterial growth?

Often in microbiology, we need to determine the number of bacterial cells in a broth. We can do this directly through spread plates (Chapter 5: Enumeration of bacteria) or indirectly by assessing the turbidity (cloudiness) of broth tubes. We measure turbidity using a spectrophotometer which gives us a reading of the light absorbance:

- more bacteria = more cloudy = higher absorbance
- less bacteria = less cloudy = lower absorbance



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6.3: Bacterial Growth Dynamics

Learning Outcomes

- Discuss the growth dynamics of a bacterial culture
- Identify phases of bacterial growth curve

Bacterial Growth Dynamics

Bacterial growth refers to an increase in bacterial numbers, not an increase in the size of the individual cells. In most bacteria, growth first involves increase in cell mass and number of ribosomes, then duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of the two chromosomes, septum formation, and cell division. This asexual process of reproduction is called **binary fission** and results in two daughter cells that are genetically identical. This is accomplished by the process of **binary fission,** where a single bacterial cell divides into two.

Watch Video 1: Bacterial Growth



Watch Video 1: Bacterial cell division (00:15). URL: https://youtu.be/gEwzDydciWc

The dynamics of bacterial growth follow a predictable pattern visualized as a **bacterial growth curve.** This growth curve is generated by plotting the increase of cell number versus time. The curve can then be used to determine the **generation time**, the time required for a microbial population to double in cell number. There are typically four growth phases in a closed bacterial culture vessel, like a flask or tube. The phases are **lag, log/exponential**, **stationary, and death** phases.

Lag Phase: immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged (notice the line here is flat, no change in cell number). Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity. The cells are also adapting and adjusting to this media and growth condition, different genes may be turned on to start metabolizing different substrates. There is some repair processes going in, cell is re-synthesizing damaged cell constituents in preparation for binary fission. The length of the lag phase is dependent on a wide variety of factors, including the size of the inoculum; time necessary to recover from physical damage or shock in the transfer; time required for synthesis of essential coenzymes or division factors; and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium, the age of the inoculum (microbe introduced into a culture medium to initiate growth), an “old” culture will probably a lot of dead/aged cells and may take longer to adjust to this medium.

The **Log** (**Exponential) Phase**. The exponential phase of growth is a pattern of balanced growth wherein all the cells are dividing regularly by binary fission, and are growing by geometric progression. The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. The *rate of exponential growth* of a bacterial culture is expressed as **generation time**, also the **doubling time** of the bacterial population. This phase is usually relatively short in the scheme of the entire growth curve. Cells in this phase are most active metabolically, and is preferred for industrial purposes where, for example a product needs to be produced efficiently. Exponentially growing cells are typically at their healthiest and are thus most desirable for studies of their enzymes or other cell components. Because the generation time is constant, a logarithmic plot of growth during the log phase is **a straight line**.

Exponential growth cannot be continued forever in a batch culture. Population growth is limited by one of three factors: 1. exhaustion of available nutrients; 2. accumulation of inhibitory metabolites or end products; 3. exhaustion of space, in this case called a lack of "biological space". Eventually the growth rate slows, the number of microbial deaths balances the number of new cells, and the population stabilizes. This period of equilibrium is called the **Stationary phase**. Bacteria that produce **secondary metabolites**, such as antibiotics, often do so during the stationary phase of the growth cycle (Secondary metabolites are defined as metabolites produced after the active stage of growth).

It is during the stationary phase that spore-forming bacteria have to induce or unmask the activity of dozens of genes that may be involved in sporulation process to prepare for a dormant period. If incubation continues after the population reaches stationary phase, a **death phase** follows, in which the viable cell population declines. During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase. This phase continues until the population is diminished to a tiny fraction of the number of cells in the previous phase or until the population dies out entirely.

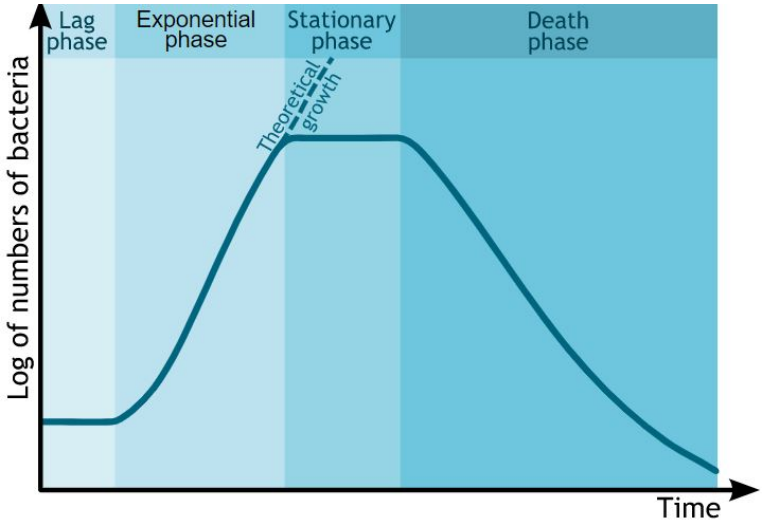


Image 1: Bacterial growth curve showing 4 phases. Image by **Michał Komorniczak** (Poland)**.** https://upload.wikimedia.org/wikiped..._growth_en.svg

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6.4: Bacteriophages

Learning Objectives

- Learn how to culture viruses in a host cell
- Quantitate viruses in a specimen
- Identify viral plaques in a bacterial lawn

Viruses are obligate intracellular parasites that multiple within the host cytoplasm. **Bacteriophages** are viruses which infect bacteria. **PHAGE** (as in phagocytosis) means "to eat", and generally refers to a virus. Viruses multiply within host cells and rely upon the host's metabolic machinery for replication.

Most bacteria have phages that are able to parasitize them. In fact, the ability to be infected with a known phage type is used to identify some strains of bacteria (like ***Staph***), known as **phage typing**. As the virus infects bacterial cells that it has been mixed with, the **lytic infection** destroys the bacteria. The bacteria have been poured into what is called a bacterial lawn on the agar plate. As the surrounding cells are infected and killed by the released viruses, a clear spot on the agar---in the bacterial lawn--develops, called a **plaque**.The plaques can be counted and the number of virus particles or virions in the original specimen, can be quantified as viruses/ ml or **plaque-forming units/ml (PFUs)**.

One common phage is called "**T4"** and it is capable of infecting its host, ***Escherichia coli.*** This type of bacteriophage is called a "coliphage. " Viruses have high specificity for its host, and will only infect a specific bacterium host.

Watch this video animation on T4 phage infecting *E. coli*



Watch Video 1: T4 phage infecting *E. coli.* URL: https://youtu.be/V73nEGXUeBY

Method for estimating the number of bacteriophages

Knowing how to determine the number of microorganisms in a sample is extremely important in microbiology, and requires accurate pipetting, aseptic technique, and calculation skills. Similar to enumeration of bacteria, one has to prepare serial dilutions of a sample and plate these samples out. Although the principles are the same, you will be enumerating viruses instead of bacteria and conserving on materials by using micropipettes and smaller volumes in your dilutions.

The number of viruses in a sample can be determined by direct count using electron microscopy or by determining the number of infectious virus particles using a **plaque assay**. Viruses are obligate intracellular parasites. Therefore, they require a host cell in which to grow. Some viruses lyse the cells in which they have replicated while others appear to cause little cell damage.

A plaque assay can be used to enumerate viruses that lyse their host cells. In a plaque assay the host cells and virus are incubated together for a short time to allow the virus to attach to and enter the host cell. Then the mixture in plated within a semi-solid agar.

This semi-solid agar is poured onto a "bottom agar" that serves to supply adequate nutrients for the host cell. At the end of one cycle of virus replication, a cell infected with a single virus particle will lyse, releasing hundreds of new viruses.

In the semi-solid medium these newly released viruses can only infect neighboring cells; after a second cycle of replication these neighboring cells will be lysed. Those cells that escape infection will continue to grow. After 24 to 48 hours plates that were not infected with a virus will contain a confluent layer of cells (they will resemble the too numerous to count/TNTC plates from bacterial standard/viable plate counts). Those plates that were infected with several hundred viruses may actually appear clear- the viruses will have infected and lysed all of the host cells. Those plates that contain an intermediate number of viruses will have **plaques**, clear or partially clear circular areas in an otherwise turbid background of cellular growth. Each plaque represents the result of one infectious virus, called a **plaque forming unit, or PFU**. Many animal and bacterial viruses can be enumerated using a plaque assay. Bacterial viruses are also referred to as phage; in our lab, we typically use a well-characterized bacterial phage, such as T4, and its host cell, *Escherichia coli*.

General procedure

1. The phage specimen you will use is already diluted to 1/ 1000, and you will dilute further.
2. Bacteria and phage are mixed together in tubes of soft agar. The mix is incubated in the water bath.
3. After incubation the mix is added to the soft agar and poured over the tryptone agar plates.
4. Be SURE to mix the dilutions.
5. Change pipettes between dilutions.
6. Each table will use a different combination of a phage and an ***E. coli*** host.

Detailed procedure with diagram

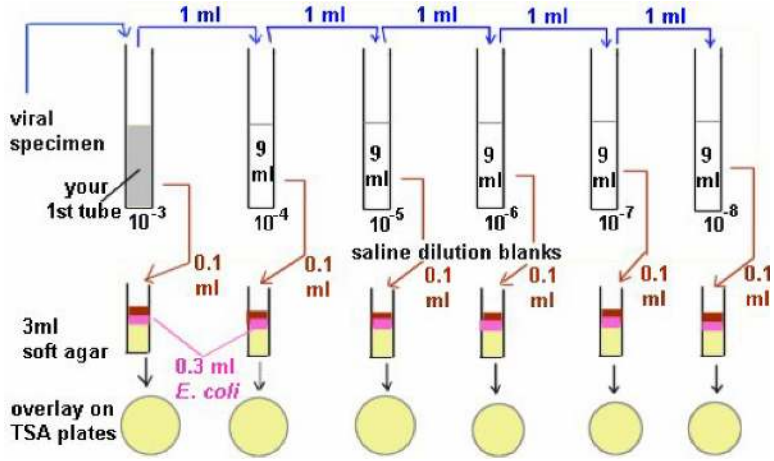


Image 1: Plaque Assay diagram

Set up 5 saline (0.85% NaCl) dilution tubes labeled 10^{-4}, 10^{-5},10^{-6}, 10^{-7}, and 10^{-8}. Into each tube, place 9ml pf into which you will dilute the viral solution. You will be making 1/10 dilutions.

1. Starting with the 10^{-3} dilution of the virus that you picked up (or were given by your instructor), transfer 1 ml to the dilution tube marked 10^{-4} and mix.
2. Make 4 more dilutions out to 10^{-8}.
3. Into 6 microtubes, add 100 microliters of each viral dilution, plus 300 microliters of ***E. coli***. **Let sit at room temperature for 10 minutes while the virus infects the bacteria**. Mix these well.
4. Take the tubes over to the water bath and transfer the entire contents of ***E. coli*** - phage tubes into 6 soft agar tubes, using a sterile plastic transfer pipet. Mix well. **KEEP SOFT AGARS INSIDE OF WATER BATH SO THEY DO NOT SOLIDIFY.**
5. Remove 1 soft agar tube at a time and pour it onto the TSA agar plates, gently rotating the plate **WELL** so as to distribute the phage - bacteria all over the agar.

6. Allow the plates to harden and incubate at 37º C **right side up**.

INTERPRETATION

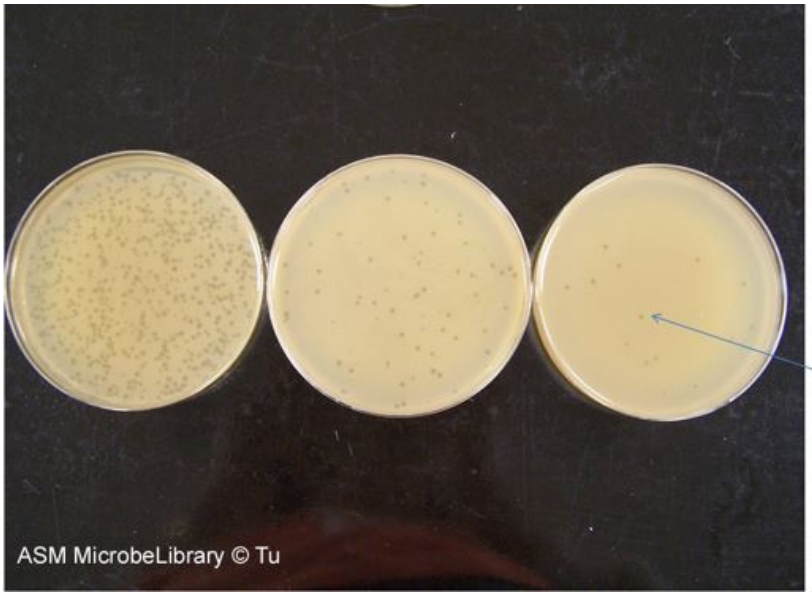


Image 2: Determination of bacteriophage T4 titer by serial dilution and plating on *Escherichia coli* B host. These series of plates show viral plaques--note the round, clear zones on the agar bacterial lawn on each plate (see arrow pointing to one). Image by Anh-Hue T. Tu, Georgia Southwestern State University, Americus, GA.

1. Lay the 6 plates right side up, from lowest dilution towards highest dilution.
2. Pick each plate up, hold it up to the light, and determine which one has between 30 - 300 plaques (you can also use the Quebec colony counters---good backlighting!)
3. Get an accurate count of that plate. Fill in the formula for viral counts.

$$\text{\# viruses/ ml} = \frac{\text{PFUs}}{\text{dilution of tube X amount plated}}$$

4. Calculate the number of viruses per ml. of original specimen.

Watch Video 2: Plaque Assay

In this video, the scientist has already completed the serial dilutions and is demonstrating the mixing of the phage/bacterium solution with the soft top agar and pouring this mixture onto the bottom agar plate.



Watch Video 2: Plaque assay. URL: https://youtu.be/uAlRSB6iCt0

Contributors and Attributions

- Jackie Reynolds, Professor of Biology (Richland College)

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6.5: Lab Procedures- Testing Oxygen requirements

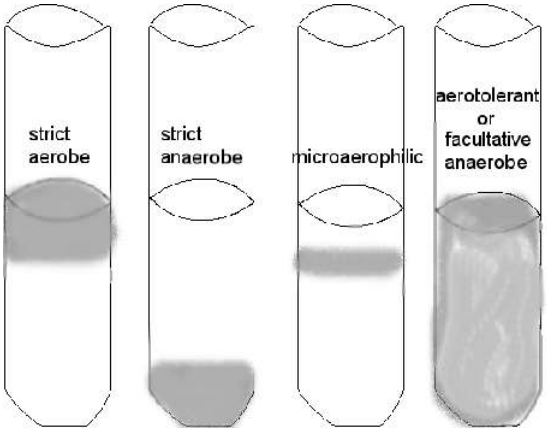
Learning Outcomes

- Inoculating thioglycollate tubes and TSA plates for incubation

Procedure

Thioglycollate broth

1. The thioglycollate broth should be either boiled first before inoculation **OR** recently made so that the oxygen content is very low. (Your instructor will tell you if it needs to be boiled).
2. Inoculate a tube of thioglycollate broth with your unknown bacterium: make sure that the loop or needle goes down to the BOTTOM of the broth (do not get **metal holder** in the sterile broth).
3. Incubate at 25 or 37 degrees C as directed.



Watch Video 1: Deep stab agar slant inoculation



Watch video 1: This procedure is showing you how to inoculate a deep stab agar slant. A similar procedure can be used for inoculating thioglycollate tubes, except that the media is semi-solid agar and there is typically no slant, so you don't have to do that

last zig-zagging streak on the top of the slant. (3:10) Video by Sinclair College eCourse Design & Development. URL: https://youtu.be/p4awvOVGZXA

TSA plates in 3 different oxygen environments

1. Label 3 plates for the table---candle jar, ambient air, and GasPak anaerobic jar.
2. Divide the 3 plates into sections, one for each organism—your unknown, the strict aerobe, the strict anaerobe.
3. Inoculate the section by streaking a straight line or a zigzag (as seen below). HOWEVER, be sure that you inoculate all 3 plates using the same technique.
4. Be sure that the jar has a methylene blue indicator strip (seen above) inside. The methylene blue is blue when oxidized, but colorless when reduced. Before the jar is opened, the strip should be checked to make sure that it is COLORLESS.
5. Incubate at 30 or 37 degrees C

INTERPRETATION: after incubation

TSA plates

Compare the presence/absence of growth, as well as the quantity of growth on the 3 plates. Determine whether aerobic, anaerobic, or facultatively anaerobic.



To the right:

- A is an facultative anaerobe
- B is an aerobe (microaerophilic)
- C is an anaerobe

Thioglycollate broth

Determine WHERE the most amount of growth occurs in the column of liquid---the top, the bottom, top to bottom. DO NOT SHAKE IT! Can you determine if the bacterium is aerobic, anaerobic, or facultatively anaerobic?

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6.6: Lab Procedures- Plaque Assay

Learning Outcomes

- Practice serial dilutions
- Perform a plaque assay
- Determine PFUs

Plaque Assay

Knowing how to determine the number of microorganisms in a sample is extremely important in microbiology, and requires accurate pipetting, aseptic technique, and calculation skills. In this exercise, you will again prepare dilutions and enumerate the microorganisms in a sample. Although the principles are the same, you will be enumerating viruses instead of bacteria and conserving on materials by using micropipettes and smaller volumes in your dilutions.

The number of viruses in a sample can be determined by direct count using electron microscopy or by determining the number of infectious virus particles using a **plaque assay.** Viruses are obligate intracellular parasites. Therefore, they require a host cell in which to grow. Some viruses lyse the cells in which they have replicated while others appear to cause little cell damage. A plaque assay can be used to enumerate viruses that lyse their host cells. In a plaque assay the host cells and virus are incubated together for a short time to allow the virus to attach to and enter the host cell. Then the mixture in plated within a semi-solid agar. This semi-solid agar is poured onto a "bottom agar" that serves to supply adequate nutrients for the host cell. At the end of one cycle of virus replication a cell infected with a single virus particle will lyse, releasing hundreds of new viruses. In the semi-solid medium these newly released viruses can only infect neighboring cells; after a second cycle of replication these neighboring cells will be lysed. Those cells that escape infection will continue to grow. After 24 to 48 hours plates that were not infected with a virus will contain a confluent layer of cells (they will resemble the TNTC plates of the preceding exercise). Those plates that were infected with several hundred viruses may actually appear clear- the viruses will have infected and lysed all of the host cells. Those plates that contain an intermediate number of viruses will have **plaques**, clear or partially clear circular areas in an otherwise turbid background of cellular growth. Each plaque represents the result of one infectious virus, called a **plaque forming unit, or PFU**. Many animal and bacterial viruses can be enumerated using a plaque assay. Bacterial viruses are also referred to as phage; in this exercise you will be using a well-characterized bacterial phage, T4, and its host cell, *Escherichia coli*.

In this exercise you will also be setting up your own dilution tubes and using micropipetters in this laboratory. Your laboratory instructor will go over the proper use of these pipetters and maintenance of aseptic technique.

Materials:

Cultures	*Escherichia coli* strain B culture
Phage sample	T4
Media	4 petri plates containing "bottom agar"
	4 tubes containing 5 mL of "top agar" - these tubes will be in the 50°C water bath
Supplies	Sterile Tryptic Soy broth diluent and 10 sterile microcentrifuge tubes
	Micropipetters and sterile tips

Procedure:

A. Sample Dilution and Plating

1. Prepare 10-fold serial dilutions of the virus through 10^{-6}

a. To do this, first prepare 6 dilution tubes by aseptically pipetting 900 µL of Tryptic Soy broth diluent to each tube - use one pipette tip.

b. Add 100 µL of the T4 phage sample to the first dilution tube, close the cap, and vortex to mix thoroughly. This is a 10^{-1} dilution

(100 µL/100 µL + 900 µL = 100 µL/1,000 µL = 1/10 = 10^{-1}).

c. Use a new pipette tip to transfer 100 µL from the 10^{-1} dilution to the next dilution tube, mix thoroughly, this is your 10^{-2} dilution.

d. Continue making dilutions of the phage until you have used all 6 dilution tubes and have a 10^{-6} dilution of the phage in the 6^{th} tube.

2. Label your remaining 4 sterile tubes 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}, and pipette 100 µL from the appropriate phage dilutions into these labeled tubes (use a new pipette tip for each transfer).

3. Add 100 µL of the *Escherichia coli* culture to each of the 4 labeled tubes. (Use the same pipette tip; add *Escherichia coli* to the tube with the most diluted phage first and work back.)

4. Incubate bacteria with virus for 10 minutes to allow for adsorption (attachment) of the virus to the bacteria.

5. While you are waiting, label the agar plates with your name, lab section, and the virus dilution that will be plated on it: one plate for each 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. Reset the micropipette for 200 µL.

6. After the 10 minutes, get the tubes of top agar from the 50°C water bath; *working quickly*, wipe off the tubes. You may want to get one top agar tube at a time so you won't have to rush; top agar solidifies within a few minutes once it is removed from the 50°C bath.

7. Pipette 200 µL from a labeled tube into a top agar tube, vortex for about 2 seconds, pour onto the appropriately labeled bottom agar plate. Do this for all four labeled tubes.

8. Allow the top agar to solidify (about 5 minutes) before moving the plates. Invert the plates and incubate at 30°C.

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6.7: Results

Plaque Assay

Count the number of plaques

Look at all of your plates prepared last period. Record the number of plaques following the same procedure used in counting bacterial colonies . Calculate the PFU/mL of the original phage sample. Remember, your plated 100 µL (0.1 mL) of the phage dilutions.

1. Record data from your phage plates here:

Total dilution	Plaque Counts
10^{-4}	
10^{-5}	
10^{-6}	
10^{-7}	

PFU/mL of original phage sample:_____

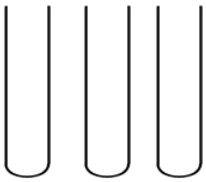
2. Record phage enumeration data for the class here:

Student pairs	Dilution with 30-300 plaques	Number of plaques on plate	PFU/mL in original T4 phage sample.
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			

Class Average:

Testing Oxygen Requirements

Illustrate the growth pattern of each bacterial species inoculated into the tube of thioglycollate agar using the pictures below. Record any other distinguishing characteristics of the growth of each species. Name the classification of the organism based on its oxygen requirements, aerobe, anaerobe, of facultative, in the space below.



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6.8: Review Questions

1. How does a thioglycollate medium work to determine the oxygen requirements of bacteria?
2. You isolated a new bacterium from a local lake. how can you determine it's temperature, salinity, pH, and oxygen requirements?
3. Describe the 4 growth phases in a bacterial growth curve. At which phase is the growth rate equal to the death rate?
4. Bacteriophages are highly specific to their hosts. Explain this statement
5. How do you determine PFUs/mL?

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CHAPTER OVERVIEW

7: Microbial Metabolism

7.1: Introduction to Biochemical Tests Part I

7.2: Introduction to Biochemical Tests Part II

7.3: Lab Procedures- Biochemical Tests

7.4: Results

7.5: Review Questions

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7.1: Introduction to Biochemical Tests Part I

Learning Outcomes

- Observe and interpret the fermentation reactions of representative bacteria in phenol red sugar broths, distinguish between respiration and fermentation, discuss the conditions in which these reactions occur.
- Observe and interpret sugar fermentation and hydrogen sulfide formation in TSI agar slants, discuss the purpose of critical ingredients in TSI agar slants, distinguish between different sugar fermentations, interpret TSI reactions.
- Learn about the role of extracellular enzymes in bacteria, observe the hydrolysis of casein hydrolysis

A. Carbohydrate Fermentation

Fermentation is a metabolic process that some microorganisms use to break down substrates such as glucose and other sugars when O_2 is not available or could not be used by the microorganism. Fermentation includes the reactions of glycolysis (where a single molecule of glucose is broken down into 2 molecules of pyruvate), as well as additional reactions that produce a variety of end products (acids, alcohols, gases). The end products are characteristic of individual bacterial species. Keep in mind, microbes are very versatile, the fermentation substrate *does not* have to be sugars, it can include even unusual compounds like aromatics (benzoate), glycerol (sugar-alcohol), and acetylene (hydrocarbons)!

Much of the original energy in the substrate remains tin the chemical bonds of organic end products, like lactic acid or ethanol. For example, one fermentation waste product is ethanol, its got so much stored energy it can be used in gasoline solutions to be combusted/burned to release that energy stored in its chemical bonds.

Note that fermentation is mainly a mechanism for regenerating NAD+ when respiratory process do not occur. Fermentation also tends to produce waste products that can accumulate in the extracellular environment. By contrast, the waste left over after ATP production by aerobic respiration are limited to CO_2 and H_2O. There can be numerous end products from fermentation, many of which is useful for us, but not necessarily the microbes. We use many fermentation products--as diverse as antibiotics, alcohols, and a variety of foods. Microbes such as yeast and bacteria are genetically engineered to produce valuable fermentation products.

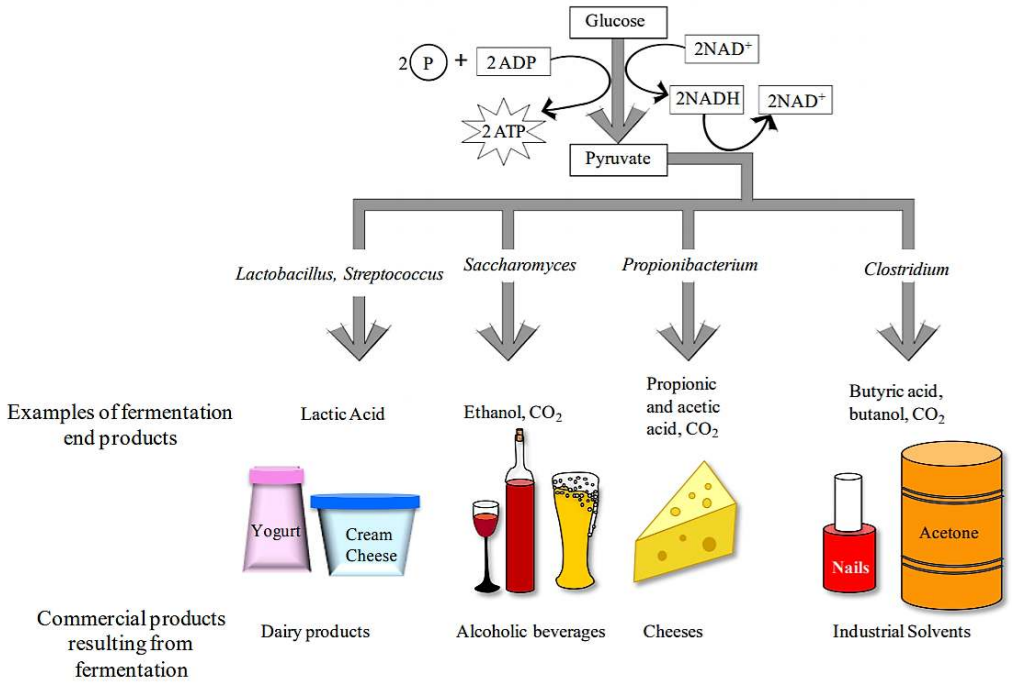


Figure 1: Carbohydrate fermentation

Although the ultimate substrate molecule for fermentation is always glucose, some bacteria use additional chemical reactions to convert other monosaccharides as well as disaccharides into glucose. Therefore bacteria can be differentiated both based on their ability to ferment various carbohydrates, as well as the end products that result from the fermentation process.

The medium used to test carbohydrate fermentation is a nutrient broth that contains a fermentable carbohydrate (usually a monosaccharide or a disaccharide), peptone (amino acids) as well as a pH indicator. The pH of the medium is adjusted to approximately 7.5, so it appears orange/red when using **phenol red pH indicator**. These types of carbohydrate fermentation tubes are therefore called **Phenol red (sugar) broths.** If the carbohydrate in the medium is fermented and acidic end products are formed, a *color change to yellow will result* (see image 1 tubes A and C). Occasionally, bacteria will not ferment the carbohydrate, but instead will break down proteins producing ammonia (NH_3) in the growth medium. In this case, the medium will become more alkaline and appear red (see image 1 tube B).

Some bacteria will produce gases when fermenting a carbohydrate. To detect these gases, a **Durham tube** is used. This is a small inverted glass tube that is placed within the larger glass tube containing the fermentation medium (see image 1). If gases (typically CO_2) are produced during the fermentation process, a bubble will form at the top of the Durham tube (see tube A). If you see a bubble in the Durham tube, the medium will also be acidic. Carbohydrate fermentation media are often used to differentiate members of the family Enterobacteriaceae (e.g., *Escherichia coli*, *Enterobacter aerogenes*) from each other.

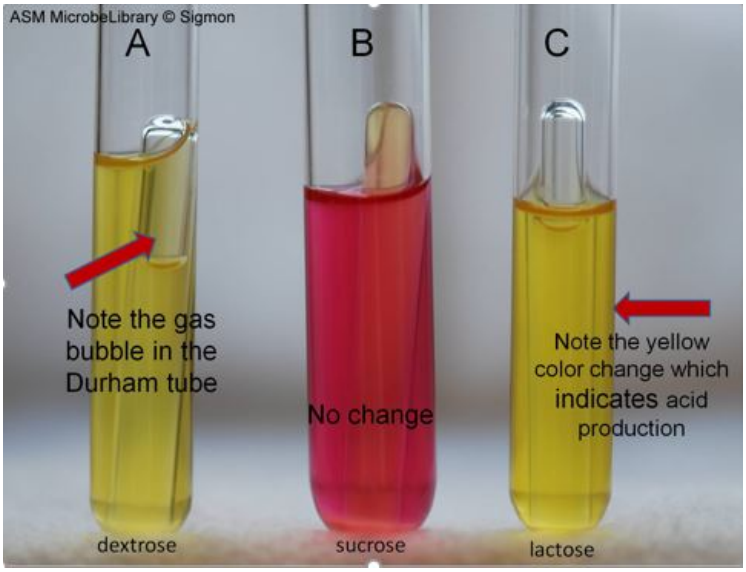


Image 1: Fermentation Reactions Produced by *Escherichia coli* in Phenol Red Sugar Broths Containing Dextrose, Sucrose, and Lactose sugars. Image by Janie Sigmon, York Technical College, Rock Hill, SC.

In many metabolic tests, end products are produced that change the pH of the medium. To measure this pH change, pH indicators (chemicals that change color depending on pH) are included in the medium. Some common pH indicators are phenol red, bromocresol purple, and bromothymol blue. Each pH indicator has a range of pH values over which it changes color (see below).

pH Indicator			
phenol red	< pH 6.8 = yellow	pH 6.8 – 7.4 = red	pH >7.4 = pink/magenta
bromocresol purple	< pH 6.8 = yellow	> 6.8 pH = purple	
bromothymol blue	< pH 6.0 = yellow	pH 6.1 – 7.5 = green	pH >7.5 = blue

Table 1: pH indicators

Watch Video 1: Phenol red sugar broth tests



Watch Video 1: how to perform phenol red sugar tests. Video by Microbial zoo (3:40). URL: https://youtu.be/W8JWInjlXqQ

B. Triple Sugar Iron (TSI) Agar Slants

Triple Sugar Iron (TSI) agar is a medium used for differentiating enteric bacteria. These bacteria typically reside in the gut/intestines of mammals. Some are major bacterial pathogens, such as certain strains of toxigenic *Escherichia coli*, *Salmonella*, *Shigella*, and *Campylobacter* species. The TSI medium can differentiate enterics based on their ability to ferment carbohydrates and reduce sulfur. The TSI medium contains three carbohydrates--glucose, lactose, and sucrose-- and iron ions, sodium thiosulfate, and the pH indicator phenol red. The medium is usually made as a 'slant' agar in a glass tube. Bacteria are inoculated into the slant of medium and into the deep portion (called the butt), where it is anaerobic.

There are 3 reactions possible in the TSI agar. First, if it only ferments glucose, then the slant and the butt will turn yellow due to the production of acidic by-products, but after a few hours, the butt remains yellow but the slant itself *may* will revert back to red as alkaline conditions reappear from the digestion of peptones and the production of ammonium compounds. Second, if lactose or sucrose or both, are fermented, there will be sufficient acid produced to cause both slant and butt to remain yellow. Third, if no carbohydrates are fermented, the slant and butt will remain a red alkaline color.

Gas (CO_2) production from carbohydrate fermentation is noted by the presence of cracks or fissures in the medium. If there is a lot of gas, portions of the medium may even be pushed up the tube (Image 2, middle tube/tube 3, notice small gap/space at bottom of tube).

Another thing TSI agar tests is hydrogen sulfide production because it contains the iron ions and sodium thiosulfate. Some bacteria use sodium thiosulfate in their metabolism and release hydrogen sulfide. The hydrogen sulfide reacts with the iron, yielding **iron sulfide**, which is a black precipitate, the medium will appear black (Image 3 and 4).



Image 2 : Triple sugar iron (TSI) agar was used to grow and differentiate various bacteria. Tube 1 (far left) is the uninoculated control. Tube 2 (second from left) was inoculated with *Pseudomonas aeruginosa* and displays a red slant with no color change in the butt, indicative of a lack of fermentation. Tube 3 (center) was inoculated with *Escherichia coli* and displays a yellow slant and a yellow butt, which indicates glucose and lactose and/or sucrose fermentation. It also exhibits cracks in the agar and lifting of the butt, which is indicative of gas production. Tube 4 (second from right) was inoculated with an unidentified culture and displays a red slant and a yellow butt, which indicates that glucose was fermented with acid production. Tube 5 (far right) was inoculated with Gram-positive *Staphylococcus aureus* and displays a yellow slant and a yellow butt, indicative of glucose and lactose and/or sucrose fermentation. Unlike tube 3, there is no evidence of gas production. All tubes were incubated at 37°C for 24 hours. Image by Clarissa Kaup and J. L. Henriksen, Bellevue University, Bellevue, NE.



Image 3



Image 4



Image 5

Image 3: *Proteus mirabilis* in a triple sugar iron (TSI) slant. This organism ferments only glucose, indicated by the red coloring of the agar. The slant is red due to depletion of glucose and the subsequent digestion of proteins in the agar. There is a large carbon dioxide bubble in the bottom right area of the tube, and the black precipitate indicates hydrogen sulfide was produced. Image by Diane Hartman, Baylor University, Waco, TX.

Image 4: *Proteus vulgaris* in a triple sugar iron (TSI) slant. This organism ferments glucose and sucrose. Acid causes the phenol red indicator in the agar to turn yellow. There is a small carbon dioxide bubble in the bottom right area of the tube. The black precipitate indicates hydrogen sulfide was produced. Image by Diane Hartman, Baylor University, Waco, TX.

Image 5: *Alcaligenes faecalis* in a triple sugar iron (TSI) slant. This organism does not ferment sugars so the medium remains red (no acids are produced in the slant or butt). The slant becomes a deeper shade of red indicating the organism uses the protein in the

medium and produces alkaline waste products. There is no carbon dioxide and no hydrogen sulfide (no black precipitate) production. Image by Diane Hartman, Baylor University, Waco, TX.

Watch Video: how to inoculate & interpret TSI agar slants



Watch Video: how to inoculate & interpret a TSI agar slant. Video by MCCC Microbiology (1:35) URL: https://youtu.be/FuOcN3wB0VM

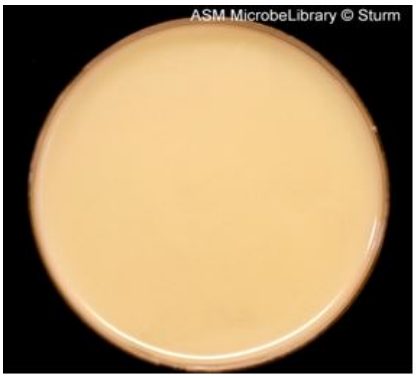
C. Extracellular enzymes

An exoenzyme, or extracellular enzyme, is an enzyme that is secreted by a cell into the environment and functions outside of that cell. Exoenzymes are produced by both prokaryotic and eukaryotic cells. Most often these enzymes are involved in the breakdown of larger macromolecules. The breakdown of these larger macromolecules is critical for allowing their smaller components to pass through the cell membrane and enter into the cell. Bacteria and fungi also produce exoenzymes to digest nutrients in their environment, and these organisms can be used to conduct laboratory assays to identify the presence and function of such exoenzymes. Some pathogenic species also use exoenzymes as virulence factors to assist in their spread.

i. Casein Hydrolysis

Some bacteria secrete extracellular enzymes called proteinases that break down proteins. Milk contains large proteins called **casein**. Some bacteria secrete **caseinases** that break down casein outside of the bacterial cell so the smaller products (e.g., amino acids) can be transported inside the cell and further metabolized.

Milk agar (which contains powdered milk) is used to detect the presence of bacterial caseinases. This medium (Image 6) is cloudy because when milk is mixed with agar, the casein forms a colloid through which light cannot pass. The presence of caseinases can be detected by observing a clearing in the agar around the bacterial growth, which indicates that the caseins have been broken down into transparent end products (amino acids and peptides), which are then taken up by the cells (image 7).



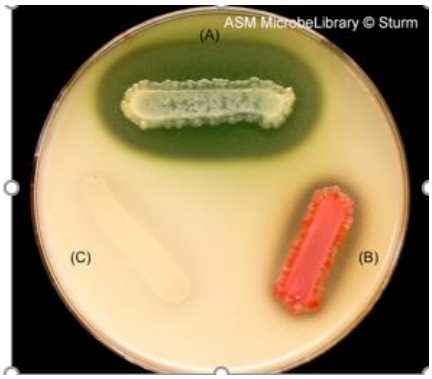
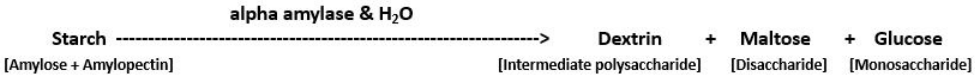


Image 6 (left plate): Milk agar contains skim milk (lactose and casein), peptone, and agar. Many organisms can grow on this medium. This medium is used to detect the production of proteases/caseinases that digest casein to soluble peptides. This results in a clear zone. Soluble peptides can then be absorbed by the cell. Casein is responsible for the white color of milk. When digested by exoenzymes, the white agar turns clear and colorless.

Image 7 (right plate): Milk Agar inoculated with (A) *Pseudomonas aeruginosa*, where casein hydrolysis is indicated by a zone of clearing around the growing colony (green color masking clearing in agar is the diffusable bacterial pigment pyocyanin); (B) *Serratia marcescens*, where casein hydrolysis is indicated by a zone of clearing around the growing colony (red pigment of bacterium is due to prodigiosin production); (C) *Escherichia coli*, no casein hydrolysis, notice there is no clearing zone around the culture streak. Images by Tasha Sturm, Cabrillo College, Aptos, CA.

ii. Starch hydrolysis test

Some bacteria produce exoenzymes called **hydrolases,** which will use water to break apart organic molecules such as the carbohydrate starch. The large polysaccharide molecule starch contains two parts, amylose and amylopectin, these are rapidly hydrolyzed using a hydrolase called **alpha-amylase** to produce smaller molecules: dextrins, maltose, and glucose. Reaction:



To test for the presence of alpha amylase, a **starch hydrolysis test** can be performed. Gram's iodine can be used to indicate the presence of starch, when it contacts starch, it forms a blue to brown complex. If the starch has been broken down/hydrolyzed, then there is a clear area that appears in the medium upon addition of Gram's iodine. This clearing zone indicates the presence of alpha amylase.



Image 8: Starch agar incubated for 24 hours at 37°C with *Bacillus cereus* (left) and *Escherichia coli* (right). After adding iodine, the iodine binds to starch if it is still present in the agar. A clear zone can be seen around the growth of *Bacillus cereus* indicating the production of the exoenzyme amylase, which digests starch to glucose leaving nothing behind in the agar for the iodine to bind. Compare his to *Escherichia coli*, which has no large clearing around the streaked culture area. Image by Tasha Sturm, Cabrillo College, Aptos, CA.

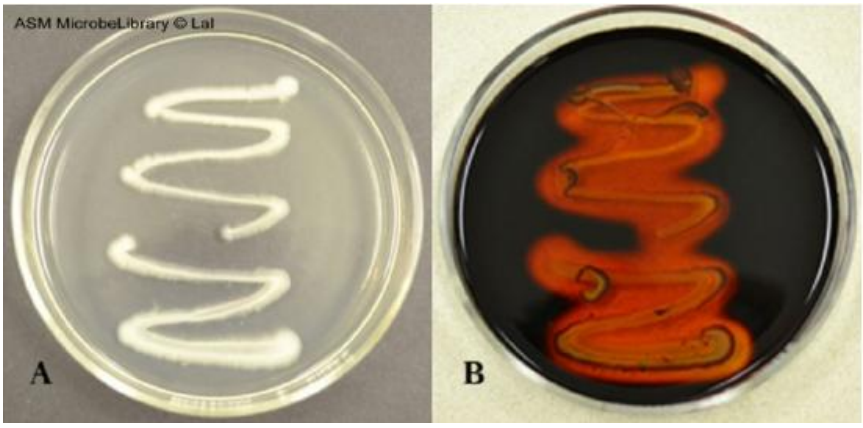


Image 9: Growth of *Bacillus subtilis* on a starch agar plate before the addition of iodine solution (A) and after the addition of iodine solution (B). After the addition of iodine, the clearing surrounding the bacterial growth indicates starch hydrolysis. Image by Archana Lal, Independence Community College, Independence, KS.

Contributors and Attributions

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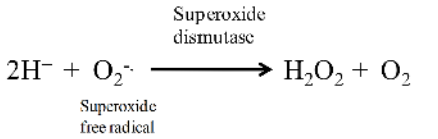
7.2: Introduction to Biochemical Tests Part II

Learning Outcomes

- Observe and interpret the reactions of catalase positive and catalase negative bacteria using catalase reagent, explain the function of the enzyme catalase in cells.
- Observe and interpret the reactions of oxidase positive and oxidase negative bacteria using the oxidase reagent, discuss the role of the enzyme cytochrome c oxidase and respiratory pigment cytochrome c in cell respiration.
- Observe reactions of bacteria in SIM/Sulfur Indole Motility media, describe the purpose of critical ingredients in SIM media, Interpret SIM reactions

A. Catalase Activity

Byproducts of aerobic metabolism include two toxic compounds: superoxide free radicals (O_2^-) and hydrogen peroxide (H_2O_2). These toxic compounds can cause intracellular damage, such as damage to DNA, lipids, and proteins. To remove these compounds, cells produce enzymes to break them down. Cells can convert superoxide free radicals to hydrogen peroxide by using the enzyme **superoxide dismutase (SOD)**; **catalase** breaks down hydrogen peroxide into water and oxygen.



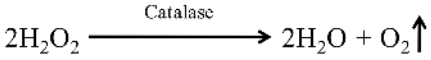


Figure 1: Superoxide dismutase and Catalase reactions

A simple test to determine if bacteria produce catalase is to add hydrogen peroxide to bacteria on an agar slant or to bacteria spread on a slide (image 1). If catalase is present, the hydrogen peroxide will be broken down into water and oxygen gas, resulting in the production of bubbles (+ test). This test does not require any special type of medium, however it should never be performed on organisms that have been grown on blood agar (a medium that contains blood). This is because there is a catalase activity in blood that would produce a false positive result. Most aerobic and facultatively anaerobic organisms produce SOD and catalase (note: some species use peroxidase rather than catalase to break down hydrogen peroxide). Obligate anaerobes lack these enzymes, which is why they cannot survive in an atmosphere containing oxygen. However, some of them have modified versions of these enzymes to deal with any possible exposure to oxygen. The archaeon, *Pyrococcus furiosus*, is an obligate anaerobe that lives on and near hydrothermal vents. Certain segments of these habitats are anoxic and *P. furiosus* occupies these niches. Some anaerobes have a superoxide-reducing system based on a different enzyme, called superoxide reductase (SOR), which reduces O_2^- rather than dismutating it.

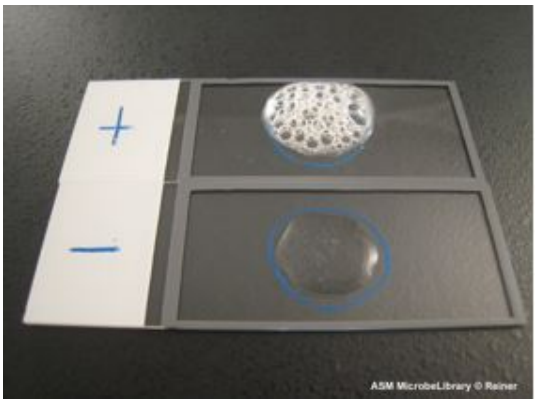


Image 1: Slide catalase test results. Hydrogen peroxide was added directly to the culture on a microscope slide. A positive reaction produced by *Staphylococcus aureus* is indicated by bubbling; a negative reaction produced by *Streptococcus pyogenes* is indicated by lack of bubbling. Image by Karen Reiner, Andrews University, Berrien Springs, MI.

Watch Video 1: Catalase test



Watch video 1: Catalase test. (1:08) Video by MCCC Microbiology. URL:https://youtu.be/r3p4jHjw9o8

B. Oxidase test

Cytochrome oxidase, also known as complex IV, is the terminal, or final, enzyme of the electron transport system/ETS (this does not include ATP synthase). Cytochrome oxidase is a transmembrane molecule found in the mitochondria of eukaryotes and in the cellular space of aerobic prokaryotes. This molecule is a proton pump that plays a vital role in producing energy, in the form of ATP, via the ETS. In the last steps of the energy production process, cytochrome oxidase oxidizes the waste products from the end of the energy making process, converting reactive species, H+ and dioxygen (O_2), to a more stable molecule, water (H_2O).

The **oxidase test** is a key test to differentiate between the families of *Pseudomonadaceae* (ox +) and *Enterobacteriaceae* (ox -), and is useful for speciation and identification of many other bacteria, those that have to use oxygen as the final electron acceptor in aerobic respiration. The enzyme cytochrome oxidase is involved with the reduction of oxygen at the end of the electron transport chain.

A cytochrome c oxidase test utilizes a special reagent called **oxidase reagent,** which is a 1% solution of the chemical tetramethyl-para-phenylenediamine (TMDPD) dihydrochloride. The reduced form of this reagent is colorless, but donates electrons to the cell's cytochrome c oxidase forming an oxidized colored form.

- Oxidase positive bacteria change the color of the reagent from colorless to colored and finally black.

- Oxidase negative bacteria do not contain the cytochrome c oxidase and do not change the color of oxidase reagent from colorless to black.

There are two ways to do the oxidase test, one is using a filter paper (see image 2 below) and the oxidase reagent and the second is doing a 'plate' oxidase test (image 3).



Image 2: Oxidase test on filter paper. A positive oxidase result given by *Pseudomonas aeruginosa* (left) is indicated by a purple color. A negative oxidase result given by *Escherichia coli* (right) is indicated by the lack of color change. Both organisms were rubbed onto a filter that was dipped in oxidase reagent and allowed to dry. Image by Laura Cathcart, University of Maryland, College Park, MD; Sabrina Kramer, University of Maryland, College Park, MD; Patricia Shields, University of Maryland, College Park, MD.

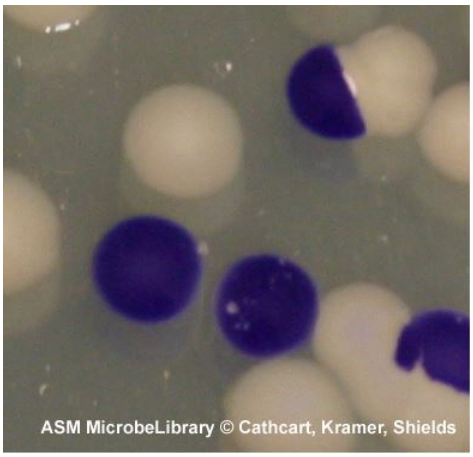


Image 3: Oxidase test on an agar plate with bacterial colonies. This close up view of an agar plate has a mixed culture of oxidase-positive *Vibrio cholerae*, indicated by purple colonies, and oxidase-negative *Escherichia coli*, indicated by lack of color change (they are the white colonies), demonstrate how the plate oxidase test differentiates between the two. In this test, the oxidase reagent was added directly to the colonies which were grown on trypticase soy agar at 37°C for 24 hours. Image by Laura Cathcart, University of Maryland, College Park, MD; Sabrina Kramer, University of Maryland, College Park, MD; Patricia Shields, University of Maryland, College Park, MD.

Oxidase test is most helpful in screening colonies suspected of being one of the *Enterobacteriaceae* (all negative) and in identifying colonies suspected of belonging to other genera such as *Aeromonas*, *Pseudomonas*, *Neisseria*, *Campylobacter, and Pasteurella* (positive).

Watch Video 2: Oxidase Test



Watch Video 2: how to perform an oxidase test. (3:23) Video by URMICRO1. URL: https://youtu.be/7Aa1xO2cC1M

C. Sulfide Indole Motility (SIM) Medium

The **Sulfide-Indole-Motility (SIM)** medium was devised for use as a routine medium in the cultural identification of members of the *Salmonella* and *Shigella* groups, showing hydrogen sulfide production, indole production, and motility in the same tube. These characteristics, along with other biochemical reactions, are of prime importance in the clinical identification of members of the Gram-negative enteric group, many of which are bacterial pathogens. This enteric group is named after the Greek word for intestine - *enteron.*

Sulfide production is tested by the presence of a black precipitate. Microorganisms are capable of producing sulfide and hydrogen sulfide in several ways. One way is when sulfur containing organic compounds, such as the amino acid cysteine, are metabolized, they excrete the sulfide ion (S^{2-}) or hydrogen sulfide (H_2S) as a waste product, which can then combine with the ferrous iron (Fe^{2+}) that is present in the medium to produce ferrous sulfide, which appears as a black precipitate. Bacteria capable of producing the enzyme **cysteine desulfhydrase** are able to remove the sulfhydryl group (- SH) from cysteine to produce sulfide ions (which may combine with hydrogen ions to produce hydrogen sulfide), ammonia and pyruvic acid.

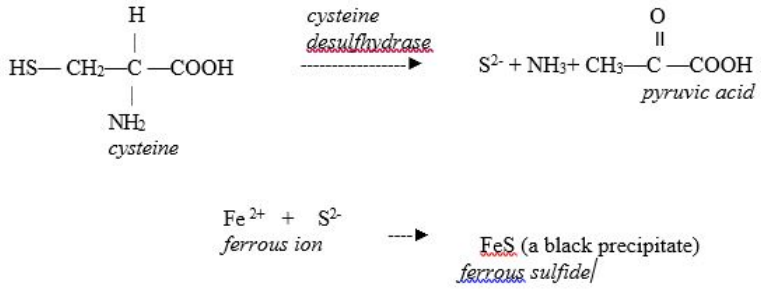


Figure 2: Chemical reaction of cysteine desulfhydrase and formation of ferrous sulfide

Another common way for bacteria to produce hydrogen sulfide is through the use of sulfate (SO_4^{2-}) as a terminal electron acceptor. Anaerobic respiration is the use of an inorganic compound (other than oxygen) as a terminal electron acceptor. You will recall that in aerobic respiration, O_2 is the final electron acceptor, and H_2O is produced as an end product. In anaerobic respiration, SO_4^{2-} would be reduced to H_2S. Anaerobic respiration most commonly involves NO_3^-, SO_3^{2-}, and CO_3^{2-}. When the bacteria

produce hydrogen sulfide, it reacts with ferrous ions present in the culture medium to form the insoluble, black iron/ferrous sulfide precipitate.

Indole production is indicative of the intracellular enzyme tryptophanase. Tryptophanase is an intracellular enzyme, which catalyzes the breakdown of the amino acid tryptophan to pyruvate and indole. The pyruvate is used by the cell as a carbon and energy source; the indole is excreted as a waste product. To test for tryptophanase, a culture is grown in the SIM medium, which contains high levels of tryptophan. After incubation and growth, several drops of Kovac's reagent are added. Kovac's reagent contains para-dimethyl-aminobenzaldehyde, which reacts with indole to produce a reddish pink compound (rosindole). The development of a red ring at the top of the tube is a positive test, see Image 2, tube A and E.

Motility is tested by growth in a semi-solid, soft agar. Motile bacteria can swim through the medium and will show diffuse growth and turbidity away from the line of inoculation.

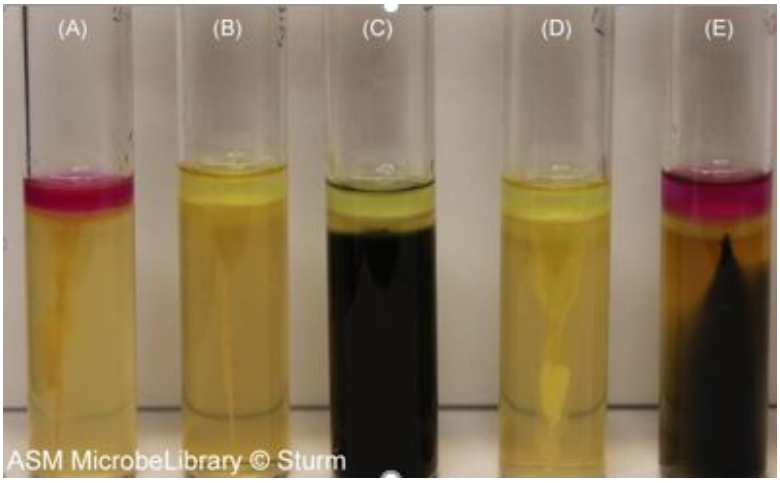


Image 3: Sulfur-indole-motility (SIM) test results from various microbes. From left to right: (A) *Escherichia coli*, (B) *Staphylococcus aureus*, (C) *Salmonella arizonae*, (D) *Enterobacter aerogenes*, and (E) *Proteus vulgaris*. After addition of Kovács reagent, a pink ring at the top of the tube indicates a positive indole result (A and E). Blackening of the media indicates hydrogen sulfide production (C and E). Growth feathering away from the stab line creating a cloudy appearance in the media indicates motility (A, C, D, and E). Growth strictly along the stab line indicates a nonmotile organism (B). Image by Tasha L. Sturm, Cabrillo College, Aptos, CA.

Watch Video 3: SIM medium



Watch Video 3: SIM medium inoculation and interpretation. Video by Dr. Gary Kaiser (4:30)
URL: https://youtu.be/zzBhhVp0qvU

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7.3: Lab Procedures- Biochemical Tests

Make sure you follow aseptic procedures and label everything carefully! Use the inoculation method indicated for each type of medium—these methods may differ. Make sure to thoroughly sterilize your loop or needle between inoculations to ensure that you are only introducing one bacterial species into your medium. If a medium is inoculated with more than one kind of bacterium, a positive result cannot be attributed to a single bacterial species. Be sure to use the correct bacterial species for each test. Follow directions carefully so that you do not waste media.

For comparison purposes, you will be provided with negative controls (media that have not been inoculated) in the next lab when you are analyzing your results.

> **Note**
>
> All media that you inoculate today will be incubated until the next lab, when you will analyze your results .

A. Carbohydrate Fermentation

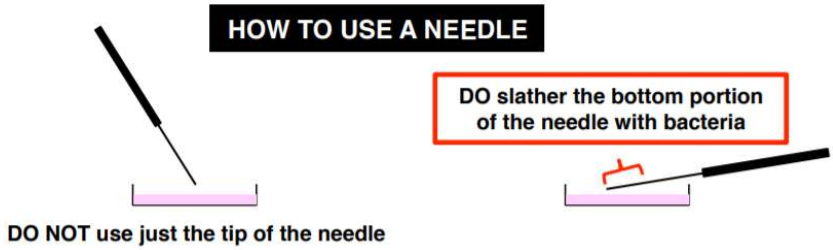
Each student: 1 tube each of the following broths: Lactose + phenol red (green cap), Sucrose + phenol red (yellow cap), Glucose + phenol red (red cap)

Instructions: Choose 1 of the following bacteria: *Proteus vulgaris*, *Escherichia coli*, *Bacillis subtilis*, or *Streptococcus faecalis* (each person at the table should choose a different species)

Inoculate the 3 types of fermentation broth with your chosen bacteria. Prior to inoculating the broths, make note of any small bubbles that might be present in the Durham tubes, so these are not read as evidence of gas formation during fermentation.

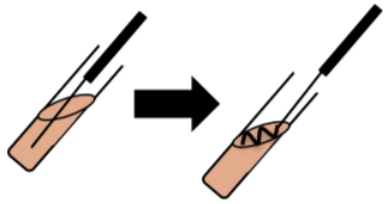
B. Triple sugar iron (TSI) slant/deep

It tests the ability of bacteria to ferment sugars and to produce H_2S, often used to identify Salmonella and Shigella. The medium contains sugars (lactose, sucrose, and glucose) and thiosulfate. Slant/deep allows for aerobic and anaerobic growth conditions.



1. Inoculate your TSI slant/deep with your assigned bacteria using a needle by stabbing fully into the butt ONCE and only ONCE.

2. Then, streak across the slant WITHOUT re-dipping your needle in your plate. KEEP CAPS LOOSE and place it on the rack at the end of the table to be incubated at 37°C



C. Casein Hydrolysis

Each student: 1 milk agar plate

Instructions: Use both these bacteria: *Bacillus subtilis* and *Enterobacter aerogenes*

Divide your milk agar plate into 3 areas using a wax pencil or a sharpie marker. Label the plate to indicate which bacterium will be inoculated into each area. One area is left as a negative control. Using a loop, you will do spot inoculations of each bacterial species in the areas, just as you did on the starch agar plate.

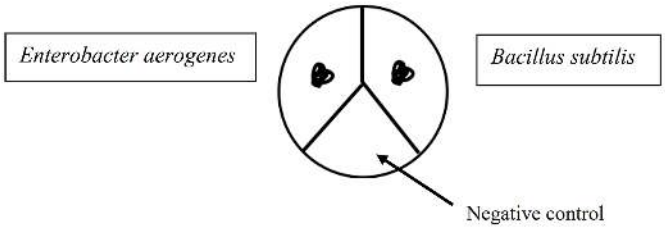


Figure 5.2.2: *Spot inoculation technique for casein hydrolysis*

D. Catalase Activity

Procedure:

1. Transfer a loopful of cells of the species to be tested onto a microscope slide. *Bacillus megaterium* should be used as a positive control and your unknown should be tested.
2. Add a drop of hydrogen peroxide.
3. Look closely for the evolution of bubbles.

E. Oxidase test

Procedure for BD BBL DrySlide: (each pair of students need 1 slide)

1. Open the BD BBL DrySlide Oxidase pouch and remove a slide by grabbing the edge of the slide, do not grab paper slide areas. After removing a slide, fold the top of the pouch over and seal tightly with a self-adhesive sticker (provided).

2. Using a sterile toothpick, pick a portion of the colony to be tested and apply the growth on ONE quadrant of the dry slide. To ensure a proper reaction, spread the inoculum on the slide reaction area to a 3-4mm size. Roll the toothpick over the slide reaction area to transfer the cells.

3. On one oxidase slide, there are 4 quadrants: perform this test on *Bacillus megaterium* as a negative control, *Pseudomonas fluorescens* as a positive control, your unknown, and your partner's unknown. Examine the reaction area for appearance of a dark purple/blue color within 20sec. Disregard color development after 20 sec.

For Gibson Bioscience Swabs: (1 swab per sample used)

1. Open the Gibson Bioscience Swabs package, ensuring that you open only at the end where you can grab the handle of the swab. Touch the swab to a (control(s) or unknown sample) colony on your plate. Examine for purple/blue coloration after 10 sec. Disregard any color development occurring after 60 sec.

F. SIM Tubes

Procedure:

1. Label SIM tubes; one for *Klebsiella aerogenes*, one for *Staphylococcus epidermidis*, one for *Proteus vulgaris*, and one for your unknown.

2. Using your loop, inoculate the tubes with the appropriate cultures.

To inoculate, stab the center of the agar deep continuing down into the agar about 3/4 of the way to the bottom.

3. Incubate at 30°C for 48 hours (your TA will remove from incubator).

4. Following incubation, **FIRST observe results for motility and sulfide production**, **THEN test** each tube for the presence of indole by adding five drops of Kovac's reagent.

Contributions and Attributions

1. Contributed by Nazzy Pakpour & Sharon Horgan Assistant Professor (Biological Sciences) at California State University

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7.4: Results

Record your results for the metabolic tests in the tables on the following pages.

A. Carbohydrate Fermentation

Observe the results of your own carbohydrate fermentation test, as well as the tests done by your table partners. You can compare your inoculated tubes with the negative controls in the front of the class at the instructor's table. Record the results in the table below.

The following convention is used when noting the results of fermentation experiments.

A = acid

G = gas

AG means that both acid and gas are present

If neither acid nor gas is present, you can write "negative"

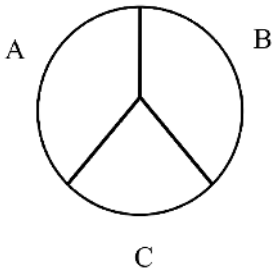
Bacteria	Glucose	Lactose	Sucrose
Proteus vulgaris			
Escherichia coli			
Bacillus subtilis			
Streptococcus faecalis			

B. TSI

Bacterium	Glucose	Lactose	Sucrose	Gas production	H2S production

C. Casein Hydrolysis

Observe your casein plate. It is helpful to hold it up to the light so you can detect the clear zones. Make a drawing of your results the circle below. Indicate the location of the bacterial growth and draw any clear zones that are present. Record your results in the table below.



Bacteria	Clear zone?	Result (+/-)
A. *Enterobacter aerogenes*		
B. *Bacillus subtilis*		
C. Negative control		

D. Catalase Activity

Add a dropper full of H_2O_2 to the surface of the slant. Record your results below.

Bacteria	Presence of bubbles	Result (+/-)
Bacillus megaterium		
Unknown		

E. Oxidase

Bacteria	Color	Oxidase Result (+/-)
Bacillus megaterium		
Pseudomonas fluorescens		

F. SIM Tubes

NOTE: Be sure to look on the surface of the agar as well as within the deep stab for evidence of motility.

A. SIM agar deep tubes

Culture	Motility	Sulfide	Indole
Klebsiella aerogenes			
Staphylococcus epidermidis			
Proteus vulgaris			
Unknown			

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7.5: Review Questions

1. Define the process of fermentation. What are some common metabolic end products produced by different microorganisms during fermentation?

2. What is the purpose of phenol red in a carbohydrate fermentation tube?

3. What is the function of the durham tube in a fermentation tube?

4. What is the purpose of the TSI agar test? What is the purpose of iron and sodium thiosulfate in the TSI agar? what is the pH indicator in TSI agar?

5. How is the SIM medium used to detect motility?

6. What substrates are acted on in SIM medium in order for hydrogen sulfide/H2S to be produced?

7. What does cysteine desulfurase catalyze?

8. What is the component in the SIM tube that makes this medium suitable to detect the production of indole by bacteria?

9. What are exoenzymes? why are they important to bacteria?

10. Why is the enzyme catalase important? Do anaerobic bacteria require catalase? explain your answer.

11. What is the importance of cytochrome oxidase to bacteria that possess it?

12. What is the function of the oxidase reagent in the oxidase test?

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CHAPTER OVERVIEW

8: Bacterial Identification

8.1: Introduction to Bacterial Identification using Culture Media
8.2: Introduction to Bacterial Identification using Enterotube test
8.3: Introduction to Bacterial Identification using Genotypic methods
8.4: Lab Procedures- Enterotube inoculation
8.5: Lab Procedures- PCR and Gel Electrophoresis
8.6: Results
8.7: Review Questions

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8.1: Introduction to Bacterial Identification using Culture Media

Learning Outcomes

- Identify and describe culture media for the growth and identification of bacteria, including examples of selective and/or differential media.

Bacterial Identification with Culture media

One of the common methods to identify bacteria is through the use of specialized media. Back in Module 2, you were introduced to this idea. Here's a recap: In clinical microbiology, it is often important to detect the presence of specific microbes associated with disease or poor sanitation, for this task, selective and/or differential media are used. **Selective media** suppresses the growth of unwanted bacteria and encourage the growth of desired microbes. For example, bismuth sulfite agar can be used to isolate *Salmonella typhi* from feces. Bismuth sulfite inhibits Gram positive bacteria and most Gram negative intestinal bacteria (other than *S. typhi*).

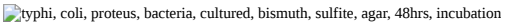


Image 1: Bismuth Sulfite agar

Differential media makes it easier to distinguish colonies of your desired microorganism from other colonies growing on the same plate. Blood agar (which contains red blood cells/RBCs) is a medium often used to identify bacterial species that destroy RBCs. These species, such as *Streptococcus pyogenes*, that causes strep throat, will show a clear ring around their colonies where they have lysed the surrounding blood cells.



Image 2: Normal Upper respiratory flora mixed with *Streptococcus* species. The presence of beta-hemolytic colonies (clear zones around small colonies) indicates the possibility of *Streptococcus pyogenes* infection. Image by Rebecca Buxton, University of Utah, Salt Lake City, UT.

Selective and Differential Media types

A. Mannitol Salt Agar

In some cases, **selective and differential** characteristics are combined into a single medium. If you wanted to isolate the common bacterium, *Staphylococcus aureus* and know that it has a tolerance for high concentrations of sodium chloride and can ferment the

carbohydrate mannitol to form acids, you can use a selective-differential media called **"Mannitol Salt Agar or MSA**" which contains 7.5% sodium chloride, and will discourage the growth of competing microbes and thus *select for* (or favor the growth of) *S. aureus.* MSA is too salty for many microbes. The medium also contains the pH indicator, phenol red, when the pH drops, such as when mannitol in the medium is fermented to acids, it will change color from red to yellow. Thus, the mannitol-fermenting colonies of *S. aureus* are *differentiated from* colonies of bacteria that do not ferment mannitol. Bacteria that can grow at high salt concentration *and* ferment mannitol to acid can be identified on MSA by color change, such as shown on this image 3-*S. aureus* streak in the left half of the plate shows growth of the bacterium and the yellow color indicating mannitol fermentation, while on the right half of the plate, *Streptococcus durans* is not capable of growing on this media (because it cannot tolerate high salt concentration) and thus there are no colonies and the media remains the same color, a pinkish red. If a bacterium is able to grow on MSA (has visible colonies) *but* the medium stays pinkish-red, then it is not capable of fermenting mannitol since the medium doesn't change color.

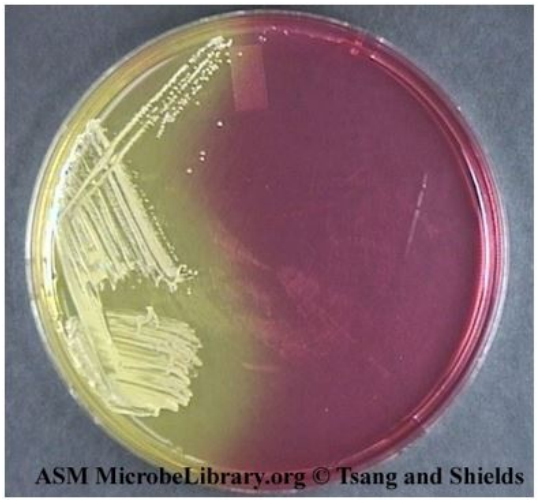


Image 3: Mannitol salt agar inoculated with *Staphylococcus aureus* on the left side of the plate and showing fermentation of mannitol (yellow medium) and inoculated with *Streptococcus durans* on the right side of the plate, which shows no growth (no colonies visible, medium remains reddish pink). Image by Anne Y. Tsang and Patricia Shields, University of Maryland, College Park, MD.

Watch Video 1: Mannitol Salt Agar explained



Watch Video 1: Mannitol Salt Agar explained and showing plate examples. Video by Dr. Gary Kaiser (CCCB). (1:38)
URL: https://youtu.be/kG1_Tf5Vpc0

B. MacConkey agar

MacConkey agar is another example of a Selective & differential medium, it is used to isolate & distinguish between Gram-negative enteric rods. MacConkey agar contains bile salts and crystal violet that inhibit Gram positive bacteria but allow the growth of Gram negative bacteria. In this way, these chemicals act selectively, to inhibit one group but promote the growth of another group.

MacConkey also has the sugar lactose and a pH indicator neutral red it turns pink/red under acid conditions. In this way, it distinguishes between lactose fermenters from non-lactose fermenters.



Image 4: MacConkey agar plate inoculated with the Gram-negative lactose fermenter (pink colonies/streak) *Escherichia coli* and the Gram-negative non-lactose fermenter (off yellow colonies) *Serratia marcescens*. Image by David Miller and Patrick Hanley, Hartwick College, Oneonta, NY)

Watch video 2: MacConkey agar explained



Watch video 2: MacConkey agar explained and showing plate examples. Video by Dr. Gary Kaiser (CCCB). (4:32)
URL: https://youtu.be/yInQ9jApAlU

C. Eosin-Methylene Blue (EMB) agar

Eosin-Methylene Blue (EMB) agar is a selective & differential medium used to isolate Gram-negative enteric bacteria and distinguish between them. EMB contains 2 dyes--Eosin and methylene blue--which act as selective agents to inhibit Gram-positive bacteria but allow for the growth of Gram-negative bacteria.

EMB also contains the sugars lactose and sucrose--and it distinguishes or differentiates between lactose or sucrose fermenters from non-fermenters. The fermenters form colored colonies and the non-fermenters form colorless colonies, similar to the MacConkey agar.

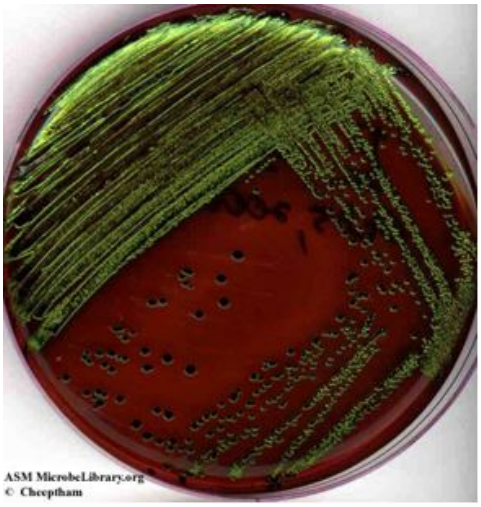


Image 5: Eosin-methylene blue (EMB) agar plate inoculated with *Escherichia coli* (a Gram-negative coliform bacterium) showing good growth of dark blue-black colonies with metallic green sheen indicating vigorous fermentation of lactose and acid production which precipitates the green metallic pigment. Image by Naowarat Cheeptham, Thompson Rivers University, Kamloops, BC, Canada.

D. **Hektoen Enteric (HE) agar**

Hektoen Enteric (HE) agar is a selective & differential medium that is used to isolate and distinguish between the enteric pathogens *Salmonella* and *Shigella*. HE medium contains high concentrations of bile salts as selective agents that inhibit Gram-positive bacteria but also slow the growth of normal intestinal microbiota. HE contains sugars lactose and sucrose, the pH indicator bromothymol blue, sodium thiosulfate and ferric salts. *E. coli* and related coliforms ferment lactose (and sucrose) forming bright orange to salmon pink colonies. *Salmonella and Shigella* are non-lactose fermenters and form green or blue green colonies. *Salmonella* colonies produce Hydrogen sulfide (H_2S) which reacts with the iron in the medium to form iron sulfide (FeS) forming colonies with black centers.

All of the media we discussed are for isolating and differentiating common enteric pathogens that can cause disease in humans.

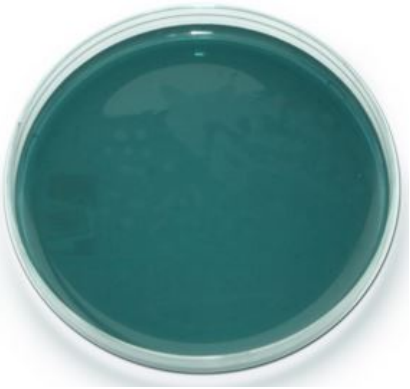


Image 6: Hektoen enteric agar sterile, uninoculated



Image 7: *Salmonella enterica* on Hektoen enteric agar. Note the black center of the transparent colonies, indicating H2S production in the absence of carbohydrate fermentation. All serotypes of *Salmonella* have this appearance on Hektoen enteric agar except for serotype *typhi*, which is a weak H_2S producer. Image by Jan Hudzicki, University of Kansas Medical Center, Kansas City, KS



Image 8: *Enterobacter aerogenes* on Hektoen enteric agar. Note the yellow-orange colonies, indicating the fermentation of at least one of the carbohydrates present in the medium. The lack of black colonies indicates no H_2S production. The orange haze around the colonies is due to the precipitation of the bile salts by the organism. The appearance of *E. aerogenes* on Hektoen enteric agar is typical of most nonpathogenic enteric Gram-negative rods. Image by Jan Hudzicki, University of Kansas Medical Center, Kansas City, KS.

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8.2: Introduction to Bacterial Identification using Enterotube test

Learning Outcomes

- Observe, interpret, and identify bacteria using the Enterotubes/Enteropluri test

Bacterial Identification

The identification of microorganisms is an important part of what many microbiologists do. As you can imagine, clinical microbiologists would need to identify the pathogen that is causing disease in patients. A microbial ecologist is interested in what microbes are contributing to environmental change or they might want to identify new species in the field. Perhaps you are working in a lab and have a contaminant and want to know where it is coming from and what it is. We have already learned some ways to identify microbes, in the last module, you learned about the many biochemical tests available to microbiologists that aid in their identification of bacteria. We'll continue with some of these biochemical tests, focusing on ones that are capable of combining multiple biochemical tests into one, and the use of selective and differential media to isolate and identify bacteria. Then we'll move into bacterial genetics and how we can use molecular methods to identify bacteria.

Enteropluri/Enterotubes

A number of techniques can be used for the identification of specific species and subspecies of ***Enterobacteriaceae*.** Speciation is important because it provides data regarding patterns of susceptibility to antimicrobial agents and changes that occur over a period of time. It is also essential for epidemiological studies such as determination of nosocomial infections and their spread.

In an effort to simplify the speciation of the *Enterobacteriaceae* and reduce the amount of prepared media and incubation space needed by the clinical lab, a number of self-contained **multi-test systems** have been commercially marketed. Some of these multi-test systems have been combined with a computer-prepared manual to provide identification based on the overall **probability of occurrence** for each of the biochemical reactions. In this way, a large number of biochemical tests can economically be performed in a short period of time, and the results can be accurately interpreted with relative ease and assurance.

The EnteroPluri-*Test* /Enterotube is a self-contained, compartmented plastic tube containing 12 different agars (enabling the performance of a total of 15 standard biochemical tests) and an enclosed inoculating wire. After inoculation and incubation, the resulting combination of reactions, together with a Computer Coding and Identification System (CCIS), allows for easy identification. The various biochemical reactions of the EnteroPluri-*Test* and their correct interpretation are discussed below. Although it is designed to identify members of the bacterial family *Enterobacteriaceae*, it will sometimes also identify common biotypes of *Pseudomonas* and other non-fermentative Gram-negative bacilli. It does not identify *Pseudomonas aeruginosa*.

IDENTIFYING MEMBERS OF THE *ENTEROBACTERIACEAE* WITH THE ENTEROPLURI-*TEST*

The EnteroPluri-*Test* contains 12 different agars that can be used to carry out 15 standard biochemical test. Interpret the results of your EnteroPluri-*Test* is based on a coding chart included with the test.

The enterotube is a self-contained, compartmented plastic tube containing twelve different media that allow determination of 15 biochemical reactions (glucose, gas production from glucose, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide (H_2S), indole, adonitol, lactose, arabinose, sorbitol, Voges-Proskauer (VP), dulcitol, phenylalanine deaminase (PA), urea, and citrate). The enclosed inoculating wire allows inoculation of all compartments in one step from one or a few single colonies of your unknown microorganism. The resulting combination of enterotube reactions, together with other metabolism tests can help you to identify unknown organisms.

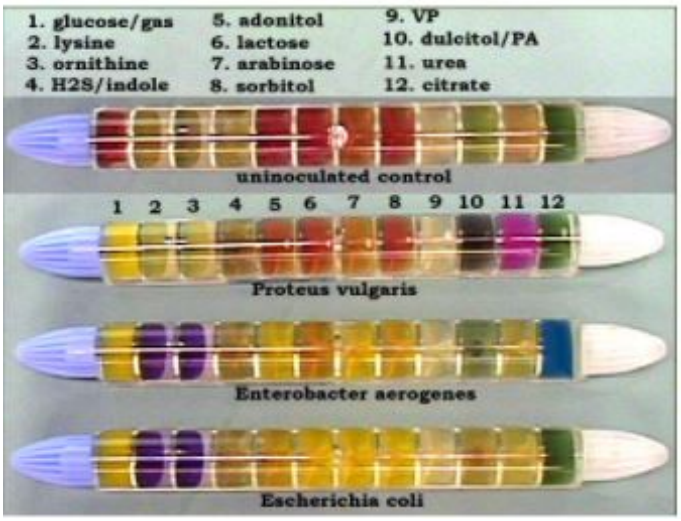


Image 1: Four Enterotubes shown. Uninoculated control tube and 3 tubes inoculated with various bacteria. 24 hr incubation.

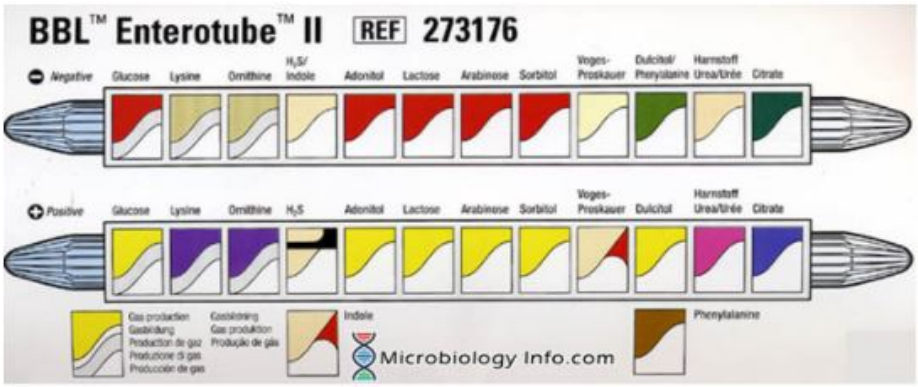


Image 2: Enterotube chambers

Interpretation of Enterotube chamber Results:

A. Fermentation of Sugars

Among the common products of carbohydrate breakdown by microorganisms using fermentative pathways are organic acids (acetic, lactic, etc.), alcohols, and gases (carbon dioxide and hydrogen). The types of product formed, and the proportion of each, depends on the species of microorganism as well as the particular carbohydrate being fermented.

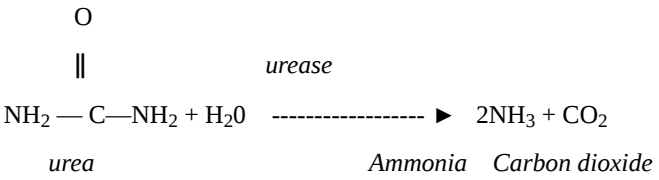
The ability to ferment different sugar compounds will be tested by inoculating a single enterotube. The 5 carbohydrates tested in 1 enterotube are: glucose, adonitol, lactose, arabinose, and sorbitol. The formation of acids can be readily detected by including a pH indicator in the microbial growth medium. Acid production will lower the pH of the medium, resulting in a color change of the indicator, creosol red, from red (alkaline) to yellow (acidic).

End products of bacterial fermentation of ***glucose*** are either acid, or acid and gas. **Gas production** will be indicated by the definite and complete separation of the wax overlay from the surface of the glucose chamber. The glucose chamber medium is covered with wax to provide anaerobic conditions to allow detection of gas formation. Fermentation of ***adonitol, lactose, arabinose,*** and ***sorbitol*** will also result in formation of acidic end products indicated by a change in color of indicator present in the medium from

red to yellow. Any sign of yellow is interpreted as a positive reaction; red should be considered negative. Some strains will give slightly variable reactions, refer to the manufacturer's chart for more details.

B. Urease

Many bacteria are able to use urea as a nitrogen source by splitting it into ammonia and carbon dioxide through the hydrolysis reaction catalyzed by the enzyme urease:



The ammonia reacts in solution to form ammonium carbonate, which results in an increase in the pH of the medium. Urease activity is detected by inoculating a medium containing urea and a pH indicator, phenol red (yellow/beige/light amber at acid pH and red-purple at alkaline pH). Initially the urea media chamber is mostly yellow. After incubation, a red-purple color throughout the medium indicates a rapid urea splitter, a *positive urease* result. No color change, (the agar remains yellow/beige/light amber) is a *negative urease* result. The urease test is included in the enterotube, see Image 1, chamber 11. *Escherichia coli* and *Enterobacter aerogenes* does not normally have the enzyme urease. *Proteus vulgaris* is a rapid urea splitter, as indicated by the bright purple chamber 11.

C. Lysine and Ornithine Decarboxylation

Decarboxylase tests are useful for differentiating bacteria. Microorganisms that have the enzyme decarboxylase can remove the carboxyl group from an amino acid. Certain bacteria can decarboxylate the amino acid lysine using the enzyme lysine decarboxylase, which results in the formation of the alkaline end product, cadaverine. Some bacteria can decarboxylate ornithine (a product of arginine hydrolysis) using the enzyme ornithine decarboxylase (ODC), which results in the formation of the alkaline end product putrescine. The presence of these decarboxylation byproducts, cadaverine and putrescine, are indicated by a change in the color of bromcresol purple, the pH indicator in the enterotube medium, from pale yellow (acidic) to purple (alkaline). The medium is covered with wax to provide anaerobic conditions. Any degree of purple should be interpreted as a positive reaction. The medium remains yellow, a negative reaction, if decarboxylation of lysine or ornithine does not occur. These tests are included in the enterotube

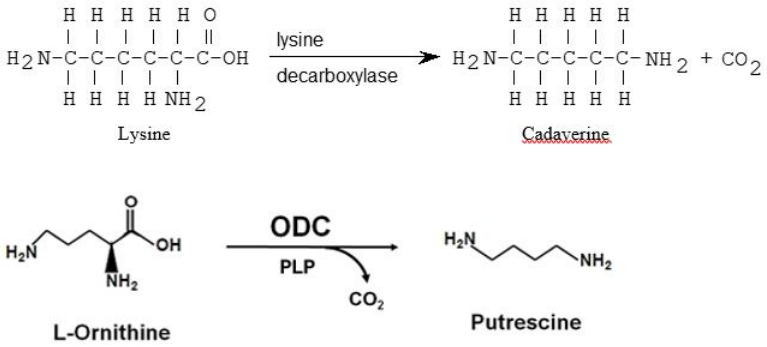


Image 3 Lysine and ornithine decarboxylation reactions. The enzymatic reaction catalyzed by ornithine decarboxylase.The pyridoxal phosphate (PLP)-dependent ODC enzyme catalyzes decarboxylation of ornithine and produces putrescine.

D. Voges-Proskauer

Different bacteria convert dextrose and glucose to pyruvate using different metabolic pathways. Some of these pathways produce unstable acidic products which quickly convert to neutral compounds. Some organisms use the butylene glycol pathway, which produces neutral end products, including acetylmethylcarbinol (acetoin) and 2,3-butanediol. The Voges-Proskauer (VP) test detects organisms that utilize the butylene glycol pathway and produce acetoin during glucose metabolism. After inoculation and incubation of the enterotube, the production of acetoin is detected using Barritt's reagent (potassium hydroxide, and alpha-naphthol) in the VP chamber. The acetoin product is oxidized in the presence of KOH to diacetyl. The diacetyl then reacts to produce a red color. The presence of acetoin, a positive result, is indicated by the development of a red color within 20 minutes, a negative reaction will appear colorless or light amber. **The use of Barritt's reagent in the VP chamber will be performed after all the results are read for the other chambers in the enterotube.**

E. Citrate Utilization

This test detects those organisms which are capable of utilizing citrate, in the form of its sodium salt, as the sole source of carbon. Organisms capable of utilizing citrate produce alkaline metabolites which change the color of the indicator from green (acidic) to deep blue (alkaline). Any degree of blue should be considered positive. Certain microorganisms will not always produce the ideal "strong" positive color change. Lighter shades of the same basic color should be considered positive here. The citrate utilization is tested in the enterotube.

How to Inoculate & Interpret an Enterotube

Watch video 1: how to inoculate an Enterotube with your bacterial sample



Watch Video 1: how to inoculate an enterotube performed in Microbiology labs at NC State. (5:08) URL: https://youtu.be/3pmaDdZPLJg

Watch Video 2: how to interpret Enterotube results



Watch Video 2: how to interpret Enterotube results. Video by Professor B (5:23). URL: https://youtu.be/CwxvUq4lTZ4

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8.3: Introduction to Bacterial Identification using Genotypic methods

Learning Outcomes

- Discuss the characterization of microbes based on phenotypic and genotypic methods
- Discuss how PCR is used to identify bacterial species. Describe the process of PCR.
- Explain the theory of PCR, its purpose, and applications
- Discuss how to visualize an agarose gel
- Interpret a DNA gel

Bacterial identification and characterization

Phenotypic methods

Throughout this semester, we have learned about many methods to characterize and identify bacteria. These methods include characterizing cell shape (cellular morphology), identifying Gram status or specialized cellular features through staining, growth requirements (oxygen, pH, temperature, etc), appearance of colonies (colony morphology), and through biochemical reactions (enterotubes, selective and/or differential media types, etc.). All of these are primarily **Phenotypic** methods of analysis and characterization, that is, the results are a product of the *expression* of their genes.

- **Cellular morphology:** cell shape--through microscopy
- **Staining characteristics:** Gram status, cell structures such as flagella, endospores---microscopy
- **Growth characteristics:** culturing requirements such as oxygen, osmotic pressure, temperature, colony morphology---- culturing techniques
- **Biochemical characteristics:** biochemical tests such as enterotubes, oxidase, catalase tests, selective/differential media.

Genotypic methods

The last method used for the identification of bacteria is **Genetic analysis** through the use of nucleic acid probes or other molecular techniques.

The application of molecular techniques for detecting and identifying pathogens is widely used. In particular, in surveillance studies these methods provide reliable epidemiological data for tracing the source of human infections, such as a foodborne illness outbreak. A wide range of molecular techniques (including pulsed field gel electrophoresis, multilocus sequence typing, random amplified polymorphism deoxyribonucleic acid, repetitive extragenic palindromic, deoxyribonucleic acid sequencing, multiplex polymerase chain reaction and many more) have been used for detecting, speciating, typing, classifying and/or characterizing pathogens of great significance to humans.

The advent of the “molecular biology age” has provided a plethora of tools and techniques for the detection, identification, characterization, and typing of bacteria for a range of clinical and research purposes. Previously, the identification and characterization of bacterial species was largely done by phenotypic and biochemical methods (such as through selective/differential media and biochemical tests that we have discussed in the last 2 modules), which relied on preliminary isolation and culture.

While these methods continue to hold place in certain settings, molecular-based techniques have provided unprecedented insights into bacterial identification and typing. To name a few examples, genotypic methods have enabled the identification of a large diversity of previously unknown taxa, the characterization of uncultivable bacteria, and facilitated metagenomics studies on large and diverse bacterial communities. Both clinical and research setting have provided in depth insights into bacterial virulence, pathogenesis, antibiotic resistance, and epidemiological typing, as well as identification of novel, emerging, and re-emerging

species. In addition, the widespread use and availability of molecular tools for bacterial genotyping has resulted in high throughput analysis, more sensitive and discriminatory results, and rapid turn-around-times, which are only likely to get better with automated tools and data analysis pipelines.

Most molecular methods for bacterial identification are based on some variation of DNA analysis, either amplification or sequencing based. These methods range from relatively simple DNA amplification-based approaches (PCR, real-time PCR, RAPD-PCR) towards more complex methods based on restriction fragment analysis, targeted gene and whole-genome sequencing, and mass spectrometry. In addition to this, approaches based on unique protein signatures such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and similar variations have also been explored.

Polymerase Chain Reaction

Many of these molecular techniques utilize the **polymerase chain reaction or PCR.** A technique you have probably heard of in other classes. Polymerase chain reaction (PCR) enables researchers to produce millions of copies of a specific DNA sequence in approximately two hours. This automated process bypasses the need to use bacteria for amplifying DNA. During the course of a bacterial infection, the rapid identification of the causative agent(s) is necessary for the determination of effective treatment options, thus molecular methods such as PCR is a popular option due to its speed. You may already know that for detection of the SARS-CoV2 virus, RT-PCR is widely used.

PCR allows DNA amplification so that specific segments of DNA can be copied numerous times, and then separated and analyzed by gel electrophoresis. The presence of a specific fragment of amplified DNA can be used to either identify an organisms or a particular characteristic such as antibiotic resistance. We often use the **16S rRNA gene** for bacterial identification purposes because it is present in all bacteria, it is highly conserved sequences (large regions of nucleotide similarity) which are interspersed with variable regions that are genus- or species-specific. Bacteria can be identified by nucleotide sequence analysis of the 16S rRNA PCR product and comparing it to a database with known sequences.

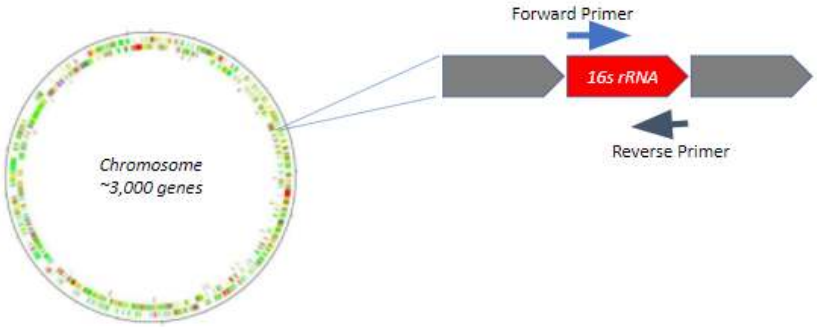


Figure 1: Schematic PCR primers binding to the 16S rRNA gene for amplification from a bacterial chromosome.

In a PCR reaction, the is a series of steps that occur. Usually the dsDNA is denatured to ssDNA. At 55-58degC, a pair of synthesized oligonucleotide primers anneal to the ssDNA that flank the sequence of interest. At 72degC, a thermostable DNA polymerase will replicate the ssDNA to dsDNA sequences. The cycle repeats itself 20-40x to amplify the DNA.

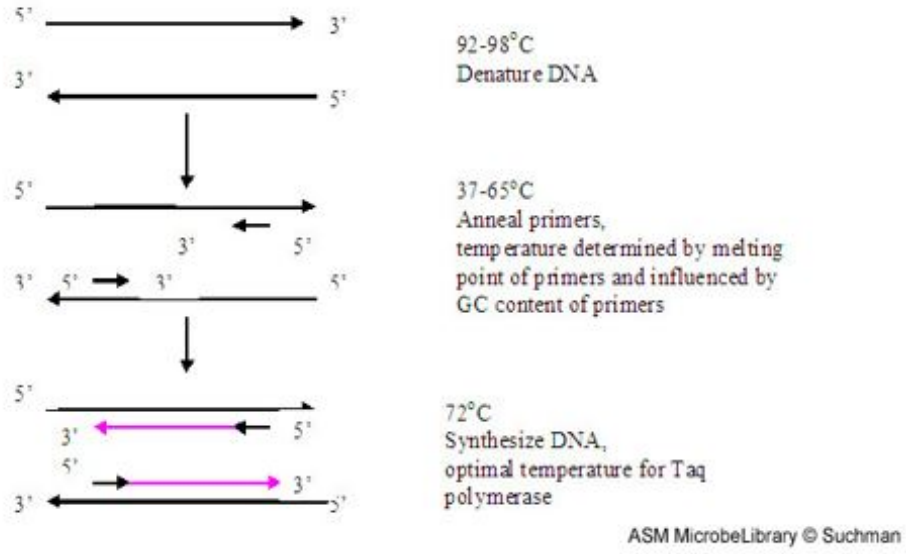


Figure 2: PCR reaction. Image by Erica Suchman, Colorado State University, Ft. Collins, CO.

The sequence of events in the PCR is as follows. The temperature is raised to 92-98°C, causing the DNA strands to separate. Two primer sequences of approximately 20 nucleotides each are annealed to opposite strands of DNA. (RNA requires an initial reverse transcription step to create a double-stranded cDNA template.) The temperature is raised to the optimum for a polymerase from a thermophylic bacterium; usually *Thermus aquaticus (Taq)* is used at 72°C. Replication continues from the 3' OH of the primers, producing two copies of the DNA. The temperature is again raised to 92-98°C, causing the DNA strands to separate. Then the temperature is lowered to allow new primers to attach to each of the four strands created in the previous reaction. The temperature used during the annealing of primers must be optimized for each individual primer set. The *Taq* polymerase fortunately is stable during the DNA melting step and is able to begin a new cycle of synthesis. The process is repeated for 20 to 40 cycles so that additional copies arise exponentially, i.e., in a chain reaction.

Watch Video 1: how to set up a PCR reaction



Watch Video 1: how to set up a PCR reaction. Video by Hands-on DNA. (4:38) URL:https://youtu.be/95qOSslefMM

Visualizing PCR products

After amplification, the PCR product, sometimes called an amplicon, is visualized and analyzed on an agarose gel and is abundant enough to be detected with an ethidium bromide or SYBR safe stain (see image below). The amplicon is compared to known sized molecular markers for production of bands of the correct size.

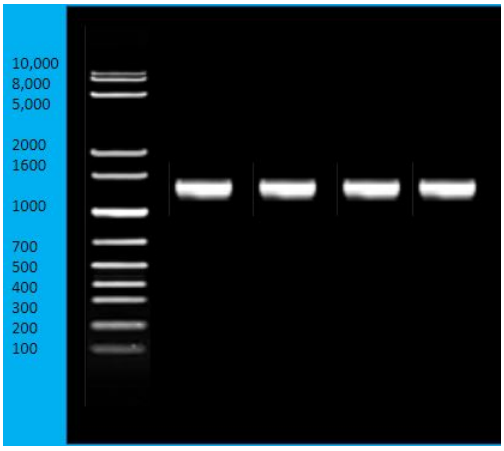


Image 1: Agarose gel electrophoresis of 16S rRNA PCR products. Note the 4 bands at the 1600 bp marker.

The 16S rRNA gene is frequently a target for those who are interested in identifying the genera or species of a bacterium. If you amplify the entire gene (~1500 bp), that is more than sufficient for a sequencing results that will tell you the identity of the bacterium.

Watch video 2: how to run an agarose gel



Watch video 2: how to load and run an agarose gel. Video by Bio-Rad Explorer. (4:06) URL: https://youtu.be/uAttNVEEEwY

Targeting other genes

In some cases, you don't need to identify the genus or species of the bacterium, but rather some other characteristics, such as antibiotic resistance. In which case, you design your primers to target a specific **antibiotic gene.** In the gel below, the *mec*A gene for methicillin resistance was targeted.

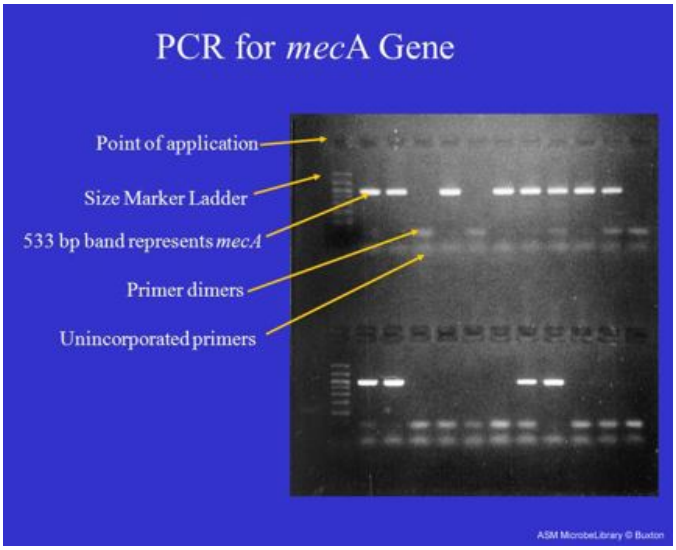


Image 2: Ethidium bromide-stained agarose mini-gel visualizing the products of multiple PCR reactions amplifying a portion of the *mec*A gene that encodes for methicillin resistance in *Staphylococcus aureus* ("MRSA"). Distinct 533 base-pair bands are clearly visible in the samples containing the resistance gene. Less distinct bands, indicating smaller primer dimers and unincorporated primers, are also visible . (Labeled view) (Rebecca Buxton, University of Utah, Salt Lake City, UT)

Watch video 3: how to interpret a DNA gel



Watch video 3: how to interpret a DNA gel. Video by Nicole Lantz. (2:30) URL: https://youtu.be/eDmaBtxym30

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8.4: Lab Procedures- Enterotube inoculation

Learning Outcome

- Learn how to inoculate an enterotube

Enterotube Inoculation Procedure:

1. Take one enterotube, record your initials, and unknown number on the label.

2. Remove both caps. The tip of the inoculating wire is under the white cap, it is already sterile**. Do not heat sterilize this wire.** Using your NA plate of your unknown culture, pick a well isolated colony directly with the tip of the enterotube inoculating wire (Figure 1). A visible amount of inoculum should be seen at the tip and side of the wire. Avoid touching agar with wire.

3. Inoculate enterotube by first twisting wire, then withdrawing wire through all 12 compartments applying a turning motion (Figure 2). Reinsert wire (without sterilizing) into enterotube, using a turning motion through all 12 compartments, until the notch of the wire is aligned with the opening of the tube (Figure 3). The tip of the wire should be seen in the citrate compartment. **Break wire at the notch** by bending. The portion of the wire remaining in the tube maintains anaerobic conditions necessary for the true fermentation of glucose, production of gas, and decarboxylation of lysine and ornithine.

4. With the broken off part of the wire, punch holes through the foil covering the air inlets of the last 8 compartments (adonitol, lactose, arabinose, sorbitol, Voges-Proskauer, dulcitol/PA, urea, and citrate) in order to support aerobic growth in these compartments (Figure 4). Replace both caps.

5. Incubate at 30ºC for 18-24hrs with enterotube lying on its flat surface or in an upright position. Allow air circulation between incubated tubes.

6. Interpret and record all reactions with exception of Voges-Proskauer. All other tests must be read before the Voges-Proskauer test is performed as the reagents added for these tests may alter the remainder of the enterotube reactions. **The Dulcitol/PA and H_2S/Indole compartment results will not be used since they are not reliable if read after 2 days.** After all reactions are recorded, follow directions below for completion of VP test.

Voges-Proskauer Test Reagent Addition:

1. Place enterotube horizontally on the bench with glucose compartment pointing downward. By using a toothpick, punch a hole about 3-4mm in diameter in the plastic film above the VP chamber.

2. Add two drops of 40% KOH solution and three drops of 5% alpha-naphthol. A positive test is indicated by the development of a red color within 20 minutes. Do not wait longer than 20 minutes before reading the results!

Watch this video 1 on Enterotube Inoculation



Watch Video 1: Enterotube inoculation performed in our Microbiology laboratories at NC State. URL:https://youtu.be/3pmaDdZPLJg

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8.5: Lab Procedures- PCR and Gel Electrophoresis

Learning Outcomes

- Perform a colony PCR
- Run an agarose gel on PCR products

Colony PCR (For 16S rRNA Sequence Analysis)

Polymerase chain reaction (PCR) is molecular technique used to amplify specific regions of DNA for applications such as sequencing and genetic analysis. Typically, there is a limited amount of DNA in the sample to study and amplification is required. PCR is carried out in a test tube with the DNA template, primers specific for the region that is desired, DNA polymerase, and reagents that stabilize the reaction. Once the reaction is put together, it will go into a thermocycler (PCR machine) that will create the conditions for DNA replication to occur. Each round of PCR requires three steps, denaturation, annealing, and elongation, each of which doubles the amount of DNA template present in the reaction. By repeating this process multiple times, usually 30, this will amplify the DNA exponentially.

PCR bead method

Materials:

- 27F primer (20uM stock)
- 1492R primer (20uM stock)
- GE Illustra PuReTaq Ready to go PCR bead and tube
- Sterile nuclease-free deionized water (molecular grade)
- T-Streak plate with bacterial isolate
- Micropipettors and tips (P10, P100)

Procedures

Adapted from “GE Illustra PuRe Taq Ready to go PCR beads” guide

1. Obtain PCR bead tubes, which contain Taq polymerase (heat resistant enzyme) and other necessary reagents. Using a sharpie, label the top of the tubes with PCR reaction number assigned in class. Make sure not to accidentally rub this off when handling the tube and double check when you put the tube into the PCR machine that your labeling is still visible.
2. Add 25 µL of Master mix (contains molecular grade water + 16S rRNA primers) into the PCR bead tube. The bead will start to dissolve and slightly effervesce.
3. As you dispense the Master mix, insert the micropipette tip into the mix so that you actually *see* the small volume go directly into the mix.
4. Using a micropipette tip, carefully touch the colony on the streak plate. A small, visible dab of cells that barely fill the very end of the pipette tip will provide enough DNA template for the reaction.
5. Dip pipette tip into reaction mix and gently swirl for 5-10 seconds to dislodge cells. Cap the tubes. Avoid forming bubbles.
6. Transfer tubes to thermal cycler.
7. Select appropriate program† to start cycling (about 2 hours).
8. Once cycling is complete, remove tubes and incubate on ice. Follow your instructor’s instructions about storage, and follow up protocols to quality test the PCR products and prepare them for sequencing.

***Protocol adapted from “puRe Taq Ready-To-Go PCR Beads” guide*

16S rRNA Primers:

Forward Primer (27F)

5’ – AGA GTT TGA TCC TGG CTC AG – 3’

Reverse Primer (1492R)

5’ – ACG GCT ACC TTG TTA CGA CTT – 3’

PCR Cycle Protocol:

1. 94^{o}C for 10 min
2. 94^{o}C 30 sec – Denaturation step
3. 58^{o}C 30 sec - Annealing step
4. 72^{o}C 1 min 50 sec (1 min per kb of DNA template) – Elongation step
5. Repeat Steps 2-4 30X
6. 72^{o}C for 10min – Final extension step

Agarose Gel Electrophoresis

For visualizing and analysis, we will have to "run" the PCR products out on an agarose gel. Invitrogen’s E-gel system will be used. This system is a complete buffer-less system for agarose gel electrophoresis. There is a pre-cast agarose gel (E-gel) that is a self-contained gel that includes electrodes packaged inside a dry, disposable, UV-transparent cassette. The gel contains either Sybr-safe or ethidium bromide for visualization of DNA. The E-gel runs in a single device that is both a base and a power supply, called the E-gel Powerbase.

Protocol and images below is adapted from Invitrogen’s E-gel Technical Guide.

Materials

- DNA sample (from PCR reaction)
- 1KB Molecular weight markers
- Loading dye Mix

General guidelines

- Run gels stored at room temperature
- Keep samples uniform and load deionized water into empty wells
- Load gel within 15 mins of opening the pouch
- E-gel can only be used once

Procedure

Sample preparation and Loading gel:

Prepare your DNA samples by adding deionized water to the required amount of DNA to bring the total sample volume to 20ul.

1. The Lab Instructor will add the 1Kb Ladder to the gel.
2. Add 4ul of PCR reaction to new microcentrifuge tube.
3. Add 16ul of Loading dye Mix to this microcentrifuge tube.
4. **Once you set up the E-gel powerbase (below)**, load the entire 20ul volume to the correct gel well. Make sure to note which gel well you loaded your sample into.

Setting up the E-gel Powerbase:

1. Plug the Powerbase into an electrical outlet using the adaptor plug.

2. Open the package containing the gel and insert the gel (with the come in place) into the apparatus right edge first. Press firmly at the top and bottom to seat the gel in the base. You should hear a snap when it is in place. The Invitrogen logo should be located at the bottom of the base, close to the positive pole. See diagram below. A steady, red light, indicates the E-gel is correctly inserted (Ready Mode).

PCR Clean-Up

The PCR clean-up process is performed using a commercial product. Depending on the availability of the different commercial kits, your TA will determine and provide the kit to use in lab. Directions will be provided with the kit.

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8.6: Results

Genotypic Analysis

16S rRNA PCR Results

After a 16SrRNA colony PCR on your unknown, a DNA sequencing reaction on the PCR products, and analysis of the sequence via BLAST, what is the closest genus of our unknown organism?

Phenotypic Analysis

Culturing Results

Explain any culturing results here. Did you use any selective and/or differential media types to determine characteristics of your unknown?

Biochemical Tests

Explain any biochemical tests results here. Tests could include enterotube results, carbohydrate fermentation, casein hydrolysis, oxidase, catalase, tests etc.

Does the phenotypic results align with your genotypic results?

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8.7: Review Questions

1. Explain how a selective and differential media works.
2. MSA/mannitol salt agar is selective for what bacteria? how does the medium select for this population?
3. A MacConkey plate inoculated with an unknown bacterium does not grow. What can this tell you?
4. How does HE/Hektoen agar work to select and differentiate bacterial groups?
5. What is the advantage to a multi-test system like the Enterotubes?
6. Why is a combination of both phenotypic and genotypic methods ideal for identifying and characterizing a bacterium?

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