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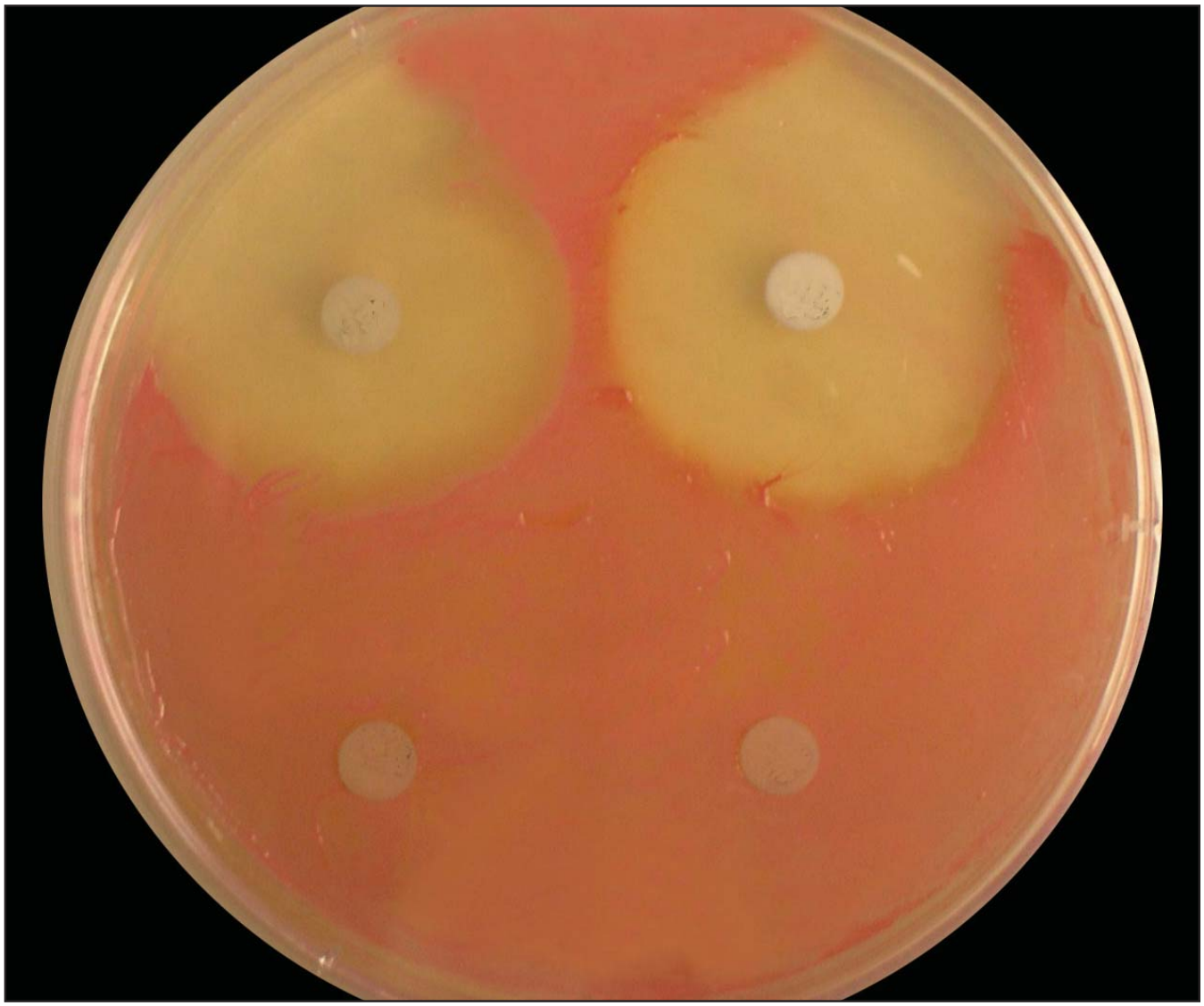
Antibiotics in Action

TEACHER'S MANUAL WITH STUDENT GUIDE



by Priya and Shiladitya DasSarma

CAROLINA
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Antibiotics in Action

Teacher's Manual

Photocopy Masters

About the authors

Priya and Shil DasSarma have studied *Halobacterium* sp. NRC-1 for over 20 years. They are enthusiastic about making these safe, convenient microbes accessible to educators and students at all levels. Visit their website at http://www.carolina.com/life_science/halobacteria for more information, ideas, and contact information.

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Antibiotics in Action

Overview

Using the materials in this kit, students grow microbial cultures of *Halobacterium* sp. NRC-1 and then test them for sensitivity to the antibiotics ampicillin, bacitracin, novobiocin, and tetracycline. This activity introduces students to the concept of antibiotics and to antibiograms (antibiotic susceptibility patterns of microbes). This lab is a safe way to examine how antibiotics affect microbes and to learn about a key method used in working with antibiotics and microbes.

This Teacher's Manual contains laboratory preparation and presentation instructions, lists of additional sources of information, a glossary, and a reproducible blackline master Student Guide. The Student Guide contains background information about antibiotics, step-by-step sterile technique and laboratory procedures, and discussion questions for assessment and review. The materials supplied with this kit are sufficient for 32 students working in pairs.

Objectives

- To provide students with experience handling microorganisms.
- To give students a better understanding of antibiotics and the advantages and disadvantages of their use.
- To observe and discuss a microbial example of natural selection and biological evolution.
- To familiarize students with the complexity of the interactions between science, technology, and society.

Materials

The materials supplied with this kit are sufficient for 32 students working in pairs. The materials are supplied for use with the exercises in this kit only. Carolina Biological Supply Company disclaims any other uses of these materials.

Included in the kit

3 *Halobacterium* sp. NRC-1 liquid cultures
4 135-mL bottles of *Halobacterium* agar
20 petri plates
20 sterile, cotton-tipped applicator sticks
20 sterile dropping pipets
ampicillin (10 μg) impregnated disks
bacitracin (2 international units) impregnated disks
novobiocin (5 μg) impregnated disks
tetracycline (30 μg) impregnated disks
control disks (no antibiotics added)
8 forceps
8 rulers

Needed, but not supplied

airtight containers (e.g., Tupperware® containers or Ziploc® bags)
disinfectant (e.g., 70% ethanol or bleach)
incubators, 37°C or 42°C
indelible markers
Bunsen burner (optional)
gloves (optional)

If the plates are incubated at 42°C, results may be obtained within one week, and if at 37°C, in one to two weeks.

Note: In the interest of keeping the cost of this kit low, cotton-tipped applicator sticks are used instead of spreaders to create bacterial lawns. If you wish to have students use spreaders (to follow more closely the procedures used in working microbiology labs), order Carolina's item 70-3414 (500 presterilized, disposable Lazy-L-Spreaders™). Smaller quantities are also available.

Background

Antibiotics and Man

The term **antibiotic** was originally coined to describe chemicals produced by microorganisms with the capacity to kill or to inhibit the growth of other microorganisms. Today the definition also includes **antimicrobial compounds**, which are produced not in nature but in the laboratory. There are three kinds of antibiotics in use in the medical field today:

1. Natural antibiotics such as penicillin, which are produced by natural agents such as fungi.
2. Semi-synthetic antibiotics such as ampicillin, which are chemically-modified variants of natural antibiotics, altered to enhance their efficacy, lower their side effects, or alter the range of microbes that are susceptible to them.
3. Synthetic antibiotics such as the sulfonamides, which are designed and produced in the laboratory.

Antibiotics work in different ways. They can be **bacteriostatic**, in that they inhibit microbial cell replication and thus give the host a chance to activate its immune response and clear the infection. Antibiotics can also be **bactericidal**, in that they cause the microbes to die. The spectrum of activity of each antibiotic reflects the range of microorganisms that are susceptible to the antibiotic. Antibiotics can be specific to one or a select few species, or they can be general, **broad range antibiotics**, meaning they affect a wide range of microbes. The susceptibility of microbes to antibiotics depends upon their genetic characteristics and (in a patient) the site of infection. A successful antibiotic should have as much **selective toxicity** as possible, i.e., it should destroy the **pathogen** (the disease-causing microbe) yet not harm the host.

There are no completely selective antibiotics; all of them have some effect on the host. However, the widely used penicillin G comes close; it is selective for inhibiting bacterial peptidoglycan cell wall synthesis, a process absent in mammalian cells. However, some people are allergic to penicillin and cannot use penicillin G.

It is important to note that antibiotics, which prevent the growth or reproduction of bacteria within or on an organism, are distinct from antiseptics, which are only used externally to discourage the growth of microorganisms, and from disinfectants, which are substances used to prevent the growth or reproduction of microbes on inanimate objects.

The human body contains numerically more microbial cells than human cells, and if a microbe ends up in the wrong location, there is potential for disease. Our first line of defense is our skin, which prevents many microbes from entering our bodies. Similarly, our mucous membranes act as traps for microbes and provide our immune system a chance to destroy them. If microbes get past these first lines of defense, additional immune defenses come into play to eliminate the microbes from our body. In fact, most of the time we have been infected, we don't even realize it! However, if our defenses alone aren't effective, we make our way to a physician and look for a quick cure…

A physician considers several factors before deciding on a course of action. These include

- the patient's condition. What is ailing the patient, and where? Also, what condition is the patient in (e.g., general health, age, genetic risk factors, previous drug interactions, allergies, etc.)?
- the causative agent for the ailment. Gram staining, culturing, and other testing can identify the causative agent.
- the microbe's sensitivity, if any. Knowing the microbe's drug sensitivity enables the physician to use the best drug as well as the proper dosage. Giving the proper dosage of the drug means using the correct amount of drug over a long enough period of time such that the pathogen is cleared from the patient's body. Determining the proper dose is aided by knowing the **minimum inhibitory concentration** (MIC) and/or the **minimum bactericidal concentration** (MBC) of an antibiotic with respect to the pathogen. The minimum inhibitory concentration of an antimicrobial agent is the lowest concentration at which visible microbial growth is inhibited. The minimum bactericidal concentration is the lowest concentration of antibiotic needed for it to be lethal within a defined period for 99% of the isolated pathogen tested.

Chemotherapy, the use of chemical compounds (including herbal remedies) to treat diseases has been in use since ancient times. However, antibiotics were first discovered in the 1930s through the famous accident of Sir Alexander Fleming, in which the area around a contaminating mold (later named *Penicillum notatum*) on an old petri plate was found to be free of the staphylococcus bacteria Fleming was growing. Further investigation into the phenomenon led to the discovery of penicillin and, later, other antimicrobial agents. Initially, crude extracts were used, but in time, the pure antimicrobial compounds were isolated and identified. These "miracle drugs" were used

effectively to treat many ailments. With the aid of antibiotics, patients who otherwise would have succumbed to sepsis (a toxic condition that can result when bacteria or bacterial toxins spread beyond the focus of the initial infection) were often able to make a full recovery.

Antibiotics have gained widespread use, as well as, some would argue, overuse. In agriculture, livestock animals, some used for human consumption, are often fed low doses of antibiotics prophylactically. Initially, it was believed that the general health and growth of the animals would increase, because the antibiotics would inhibit harmful microbes that would otherwise stunt the growth of the animal. The question of risk versus benefit for these practices has since been hotly debated.

Because of the prolonged use of most antibiotics, some microbial strains are no longer susceptible to these miracle drugs. Why is this? One mechanism is through natural selection of spontaneously occurring **resistant** strains that can develop from an originally antibiotic-sensitive strain. These spontaneously occurring resistant strains arise through a natural mutation process. This mechanism of developing drug resistance may take a while, because resistant strains may rarely arise, or a patient's immune system may eliminate the mutated sensitive strain before it has a chance to multiply. Another mechanism of acquiring antibiotic resistance is through transfer from another microbe of a **plasmid** containing an antibiotic-resistance gene. Plasmids are circular, extrachromosomal pieces of DNA that can be transferred from microorganism to microorganism. Plasmids containing antibiotic resistance genes may also be referred to as **resistance factors**, or R factors. In an environment in which antibiotics are present, a microorganism acquiring resistance to the antibiotic via either of these mechanisms has a selective advantage over those that do not, and becomes the predominant strain. Because of the prevalence of antibiotic use in hospitals, many "hospital strains" of microbes have become resistant to one or more antibiotics. This creates a major problem for health care providers and their patients.

Because of the appearance of new infectious agents and dangerous, antibiotic resistant strains, studying the basis of antibiotic resistance and development of new antibiotics are extremely important. Academic researchers and pharmaceutical companies are seeking more and novel antibiotics by using modern tools, including synthetic chemistry processes, computational drug design, and **metagenomics**. Metagenomics is the study of genomes from mixed populations of organisms gleaned directly from the environment, and is being used to discover novel genes and proteins.

Measuring Antibiotic Susceptibility Using Antibiograms

In the course of the search for new antibiotics, scientists use multiple tools and techniques. One of these tools is the **antibiogram**, an assay used to determine a microbe's sensitivity to antibiotics. Antibiograms are also used in the health care and agricultural fields. In the agricultural field, they are used to examine strains of infectious agents found on and in animal patients as well as in food products. In medicine, they are often used to determine the best drug to treat a patient who has an infection.

In the clinical setting, the **disk diffusion test** (also called the Kirby-Bauer test) is used to create the antibiograms. The test requires that certain conditions be held constant [e.g., young cultures (2–5 hours old) must be used at particular concentrations or at a particular optical density, and must be incubated for a specific length of time, etc.]. To set up the test, cultures are plated onto agar plates that will support the growth of the culture. Antibiotic-impregnated disks are then placed on the agar surface. As the microbes grow to create a lawn, their growth is inhibited to varying degrees by the different antibiotics diffusing from each of the various disks. As a result, in areas around some of the antibiotic disks, **zones of clearing** can be observed. The size of these zones depends on several conditions, including

1. the microbe's sensitivity to the antibiotic.
2. the rate of antibiotic diffusion through the medium.
3. the rate of microbial growth.
4. the type of culture medium.
5. the growth conditions (e.g., temperature).
6. the concentration of microbes in the inoculation medium.
7. the age of the culture.

Which antibiotic disks the zones of clearing are around, and the size of these zones (which can indicate the MIC), are critical in determining which antibiotic and what concentration should be used for treatment. However, as discussed above, in order to obtain accurate and clinically useful information from this test, specific and multiple conditions are standardized and carefully controlled.

In this laboratory exercise, we will create an antibiogram using the halophilic microbe, *Halobacterium* sp. NRC-1. This organism requires a high-salt environment to thrive and is different from most of the microbes encountered in a doctor's office or hospital. Its genome has been fully sequenced and studied, and it is not known to cause any human diseases. Not only that, it will lyse at salinities lower than 2.5 M NaCl, even in seawater.

Halobacterium sp. NRC-1 in the Classroom

Halobacterium sp. NRC-1 is an ideal microbe with which to study antibiotic action. There is no risk of passing on to a human pathogen any antibiotic resistance that may arise during the experiment, as could occur if working with a strain (however nonpathogenic) that grows under the same conditions as many pathogens. This is because *Halobacterium* sp. NRC-1 will not grow in the same medium as most of the more commonly encountered microbes that are pathogenic to humans, and because most human pathogens cannot grow on the hypersaline (high salt) medium required by *Halobacterium* sp. NRC-1.

Due to the nature of the microbe and the media used in this exercise, gloves are not necessary. However, you may opt to have your students wear gloves in order to simulate sterile technique.

We will use ampicillin, bacitracin, novobiocin, and tetracycline to examine the effects of various antibiotics on *Halobacterium* sp. NRC-1. The method of action of each antibiotic is as follows:

- Ampicillin penetrates Gram-positive bacteria, inhibiting the final stage of bacterial cell wall synthesis, leading to cell lysis.
- Bacitracin interferes with the dephosphorylation of the C55-isoprenyl pyrophosphate (which carries the building blocks of the peptidoglycan bacterial cell wall) outside of the inner membrane.
- Novobiocin inhibits DNA gyrase and interferes with DNA replication in bacteria.
- Tetracycline inhibits protein synthesis; it binds to the 16S part of the 30S ribosomal subunit and prevents the amino-acyl tRNA from binding to the A site of the ribosome.

Teacher Preparation and Presentation

You will need to

- read the entire Teacher's Manual and Student Guide and familiarize yourself with the information and instructions given to students.
- photocopy the Student Guide for each student or team of students.
- decide how and when to share the various components of the Student Guide with the class. This includes the Background information, Lab 1 worksheets, Lab 2 worksheets, and Discussion Questions.
- prepare media plates (see below).
- set up and supply 16 workstations. Assign each station three of the four antibiotics to test. Make sure each station also has one control disk.

Preparing Media Plates

1. To melt the medium, slightly loosen the cap(s) and place the bottle(s) of medium in a pot of water. Bring the water to a boil. Make sure the water level is even with the level of the medium in the bottle(s). Leave the bottle(s) in the boiling water until the medium has completely melted. This will take approximately 30 minutes.
2. Allow the medium to cool to 55°C either by allowing the pot of water to cool to that temperature or by letting the bottle(s) sit for several minutes at room temperature. The bottle(s) should feel comfortably hot to the touch when around 55°C.
3. Disinfect the work surface by wiping it with a disinfectant such as 70% ethanol or bleach. Wash your hands thoroughly. Unpack the petri dishes, being careful not to disturb their sterility. Align the sterile plates along the edge of a clean, level tabletop away from any draft or breeze.
4. Remove the cap and flame the mouth of a bottle of medium. Lift the lid of the petri dish just enough to pour in the molten medium. Carefully, pour to a depth of about 5 mm per plate. Replace the lid immediately to prevent contamination.

5. Repeat Step 4 with the other bottle(s) of medium.
6. Let the plates stand undisturbed until they solidify (about one hour). Let the plates sit out until any condensation on the lid evaporates. **Note:** *It is important that the plates be allowed to dry.* If the plates are too wet, the antibiotic will diffuse to the point that the zones of clearing will be too big to measure. Excess water will also lyse the *Halobacterium*. You can check for excess moisture by "pre-incubating" the plates at 37°C or 42°C for a few hours; if there is still a condensate present, slightly tilt the lids and allow the humidity to escape. However, do not let the plates get too dry, or the salt will crystallize.

   Plates may be stored upside down in airtight containers in the refrigerator. Always store plates in airtight containers, even during the incubation period, as they will easily dry out and salt crystals will form otherwise.
7. Dispose of the empty bottles in an autoclave disposal bag, or soak them overnight in a 10% solution of bleach before discarding.

For a review of sterile technique, and for the lab procedure, refer to the Student Guide.

Disposal of Cultures

Dispose of microbial cultures in a safe manner. This usually involves either autoclaving all labware that comes in contact with cultures or soaking it in a 10% bleach solution before disposal.

Further Investigations

You may wish to have students use the extra plates included in the kit for research projects. For example, students might test the effect of various substances (such as antiseptics) by placing them on filter paper disks that can be laid onto a freshly plated *Halobacterium* lawn. If nearby growth is inhibited or killed (indicated by a zone of clearing around the disk) the substance is toxic to the microbes. If a zone of clearing does not appear, it indicates that the microbes are tolerant of the substance, if not utilizing it. If the disk is overrun with *Halobacterium*, then the microbes are attracted to the substance and, being motile, move toward it. Be sure to use a control disk, to illustrate how the microbes respond to a disk that has nothing on it (negative control).

Expected Results

Disk	Effect on Microbe Growth
Control disk	no effect
Ampicillin	no effect
Bacitracin	inhibits growth well
Novobiocin	inhibits growth well
Tetracycline	little or no effect on microbe growth

Resources

Note: *At the time of this printing, all Web sites in the listing below were active.*

General mechanisms of antibiotics:

Amábile-Cuevas, Carlos. March–April 2003. New Antibiotics and New Resistance. *American Scientist.* 91(2): 138–149.

Baron, Samuel (Ed). 1996. *Medical Microbiology*, 4th ed. University of Texas Medical Branch, Galveston, TX.
Medical Microbiology text on the NIH Web site:
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Books&cmd=search&doptcmdl=TOCView&term=antibiotic+mechanism+AND+mmed%5Bbook%5D

Boyle, V. James, Marilyn E. Fancher, and Richard W. Ross, Jr. March 1973. Rapid, modified Kirby-Bauer susceptibility test with single, high-concentration antimicrobial disks. *Antimicrobial Agents and Chemotherapy.* 3(3): 418–424.

Bacitracin effect on *Halobacterium*:

Mescher, M.F., and J.L. Strominger. August 1976. Structural (shape-maintaining) role of the cell surface glycoprotein of *Halobacterium salinarium. Proceedings of the National Academy of Sciences (USA).* 73(8): 2687–2691.

Novobiocin effect on *Halobacterium*:

Sioud M., G. Baldacci, A.M. de Recondo, and P. Forterre. 1988. Novobiocin induces positive supercoiling of small plasmids from halophilic archaebacteria *in vivo. Nucleic Acids Research.* 16(4): 1379–1391.

Halobacterium sp. NRC-1: The NEW Model Microbe. ©Carolina Biological Supply Company.

This Web site provides information, descriptions, and testimonials supporting Carolina's *Halobacterium* kits and products. It provides a link to the haloarchaea experts in the DasSarma Lab Group, as well as links to related research and scholarly articles.

http://www.carolina.com/life_science/halobacteria/index.asp

Foodborne Pathogenic Microorganisms and Natural Toxins Handbook. The U.S. Food and Drug Administration and the Center for Food Safety and Applied Nutrition.

This is the Home page of the "Bad Bug Book," which examines foodborne bacteria, viruses, parasites, and natural toxins. Links to other government publications, agencies, and resources are also accessible from this site.

http://www.cfsan.fda.gov/~mow/intro.html

The HaloEd Project. ©UMBI/COMB, University of Maryland.

This Web site, dedicated to biotechnology education, provides access to a great deal of information and research about *Halobacterium* sp. NRC-1.

http://halo.umbi.umd.edu/~haloed/

The DasSarma Team would be happy to discuss projects with students and educators and can be reached by email:
dassarma@comcast.net

Related Products

Following is a list of related kits available from Carolina Biological Supply Company. For pricing and culture information, please refer to the most recent *Carolina™ Science* catalog, call toll free 800-334-5551, or visit Carolina Biological Supply online at www.carolina.com.

RN-15-4665	Epidemic Simulation Kit (Classroom Kit)
RN-15-4665R	Epidemic Simulation Kit (Demonstration Kit)
RN-15-4680	Glo Germ™ Kit
RN-15-4770	Introduction to Life in an Extreme Environment
RN-15-4771	Basic Microbiology Skills Part 1
RN-15-4772	Basic Microbiology Skills Part 2
RN-15-4773	Extremely Easy DNA Extraction Kit

Glossary

Antibiogram antibiotic susceptibility pattern of a microorganism.

Antibiotic (*anti-* = against; *-biotic* = having a mode of life) a chemical with the capacity to kill or inhibit the growth of microorganisms. Originally limited to chemicals produced by microbes, but now also includes those synthesized by chemists.

Antimicrobial compound a synthesized chemical that acts to inhibit microbial growth or to kill the microbe.

Bactericidal causing microbial cell death and/or cell lysis.

Bacteriostatic inhibiting microbial cell division without killing the microbe.

Broad range antibiotic (broad spectrum antibiotic) an antibiotic that is active against a wide range of disease-causing bacteria.

Disk diffusion test (Kirby-Bauer test) a test for bacteriostatic activity of an antibiotic. Uses an antibiotic-infused paper disk on an inoculated petri plate to create zones of clearing, which indicate antibiotic susceptibility.

Metagenomics the science that involves looking at all the DNA in a particular population or environment and identifying novel enzymes, genes, and processes. It allows you to find novel genes and proteins from organisms without having to culture the organism.

Minimum bactericidal concentration (MBC) the lowest concentration of antibiotic needed to be lethal within a given time frame for 99% of the isolated pathogen tested. Unit of measure: μg/mL.

Minimum inhibitory concentration (MIC) the lowest concentration of antibiotic that will still inhibit the growth of the pathogen being tested. Unit of measure: μg/mL.

Pathogen a disease-causing agent.

Plasmid circular, extrachromosomal pieces of DNA having the capability of replicating in cells.

Resistance factor (R factor) bacterial plasmids that carry genes conferring resistance to antibiotics and which are sometimes transferable to other bacteria.

Resistant capable of growth in the presence of a substance (antibiotic) or under a certain condition.

Selective toxicity the ability of a chemical to affect a pathogen's growth or to kill it without damaging the host's cells.

Zones of clearing (zones of inhibition) in the disk diffusion test, the area around antibiotic-infused disks where no cell growth occurs.

Name _____

Date _____

Antibiotics in Action

Background

Antibiotics and Man

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A physician considers several factors before deciding on a course of action. These include

- the patient's condition. What is ailing the patient, and where? Also, what condition is the patient in (e.g., general health, age, genetic risk factors, previous drug interactions, allergies, etc.)?
- the causative agent for the ailment. Gram staining, culturing, and other testing can identify the causative agent.

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Because of the prolonged use of most antibiotics, some microbial strains are no longer susceptible to these miracle drugs. Why is this? One mechanism is through natural selection of spontaneously occurring **resistant** strains that can develop from an originally antibiotic-sensitive strain. These spontaneously occurring resistant strains arise through a natural mutation process. This mechanism of developing drug resistance may take a while, because resistant strains may rarely arise, or a patient's immune system may eliminate the mutated sensitive strain before it has a chance to multiply. Another mechanism of acquiring antibiotic resistance is through transfer from another microbe of a **plasmid** containing an antibiotic-resistance gene. Plasmids are circular, extrachromosomal pieces of DNA that can be transferred from microorganism to microorganism. Plasmids containing antibiotic resistance genes may also be referred to as **resistance factors**, or R factors. In an environment in which antibiotics are present, a microorganism acquiring resistance to the antibiotic via either of these mechanisms has a selective advantage over those that do not, and becomes the predominant strain. Because of the prevalence of antibiotic use in hospitals, many "hospital strains" of microbes have become resistant to one or more antibiotics. This creates a major problem for health care providers and their patients.

Because of the appearance of new infectious agents and dangerous, antibiotic resistant strains, studying the basis of antibiotic resistance and development of new antibiotics are extremely important. Academic researchers and pharmaceutical companies are seeking more and novel antibiotics by using modern tools, including synthetic chemistry processes, computational drug design, and **metagenomics**. Metagenomics is the study of genomes from mixed populations of organisms gleaned directly from the environment, and is being used to discover novel genes and proteins.

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Measuring Antibiotic Susceptibility Using Antibiograms

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In the clinical setting, the **disk diffusion test** (also called the Kirby-Bauer test) is used to create the antibiograms. The test requires that certain conditions be held constant [e.g., young cultures (2–5 hours old) must be used at particular concentrations or at a particular optical density, and must be incubated for a specific length of time, etc.]. To set up the test, cultures are plated onto agar plates that will support the growth of the culture. Antibiotic-impregnated disks are then placed on the agar surface. As the microbes grow to create a lawn, their growth is inhibited to varying degrees by the different antibiotics diffusing from each of the various disks. As a result, in areas around some of the antibiotic disks, **zones of clearing** can be observed. The size of these zones depends on several conditions, including

1. the microbe's sensitivity to the antibiotic.
2. the rate of antibiotic diffusion through the medium.
3. the rate of microbial growth.
4. the type of culture medium.
5. the growth conditions (e.g., temperature).
6. the concentration of microbes in the inoculation medium.
7. the age of the culture.

Which antibiotic disks the zones of clearing are around, and the size of these zones (which can indicate the MIC), are critical in determining which antibiotic and what concentration should be used for treatment. However, as discussed above, in order to obtain accurate and clinically useful information from this test, specific and multiple conditions are standardized and carefully controlled.

In this laboratory exercise, we will create an antibiogram using the halophilic microbe, *Halobacterium* sp. NRC-1. This organism requires a high-salt environment to thrive and is different from most of the microbes encountered in a doctor's office or hospital. Its genome has been fully sequenced and studied, and it is not known to cause any human diseases. Not only that, it will lyse at salinities lower than 2.5 M NaCl, even in seawater.

Halobacterium sp. NRC-1 in the Classroom

Halobacterium sp. NRC-1 is an ideal microbe with which to study antibiotic action. There is no risk of passing on to a human pathogen any antibiotic resistance that may arise during the experiment, as could occur if working with a strain (however nonpathogenic) that grows under the same conditions as many pathogens. This is because *Halobacterium* sp. NRC-1 will not grow in the same medium as most of the more commonly encountered microbes that are pathogenic to humans, and because most human pathogens cannot grow on the hypersaline (high salt) medium required by *Halobacterium* sp. NRC-1.

You will study the effects of the antibiotics ampicillin, bacitracin, novobiocin, and tetracycline on *Halobacterium* sp. NRC-1.

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Sterile Technique

1. Wipe your work area with a disinfectant such as alcohol or bleach, and then wash your hands with soap and water.
2. Put on gloves (if provided by your instructor).
3. Light the Bunsen burner. The openings or "lips" of test tubes and flasks must be flamed during the transfer of media or cultures. Heating the container directs air convection currents upward and away from the opening, momentarily preventing airborne contaminants from entering. It is essential to perform the transfer quickly, before the opening cools.
4. Hold the test tube at a 45° angle in your left hand if you are right-handed, or in your right hand if you are left-handed. Use your other hand to hold the transferring instrument (in this case, a pipet). Use the little finger of the hand not holding the test tube to remove and hold the cap of the culture container. Flame the top of the test tube. Take out the inoculum (the culture to be transferred), reflame the opening of the culture container, and replace the cap.
5. When working with petri plates, always hold the lid over the plate to prevent contaminants from landing on the surface of the agar. Always replace the lid as soon as possible. Realize that the lid is designed to fit loosely to allow diffusion of air around the edges, while minimizing the possibility of contamination. When you lift the lid, hold it directly over the plate. Never place the lid on the bench top.
6. Turn off the burner.
7. Once again, wipe your work area with a disinfectant, and then wash your hands thoroughly.

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Lab 1: Establishing a Lawn of *Halobacterium* sp. NRC-1

Procedure

1. Turn over the petri plate in order to label the bottom of the plate as follows:
   - **a.** Divide the plate into quarters using an indelible marker and a ruler.
   - **b.** On the outer edge of one segment, write "CT." In this quarter of the plate, you will place your control disk. On the outer edge of the other three segments, write the name or abbreviation for the three antibiotics you will be testing.
   - **c.** In small letters along the edge of the plate, write your initials, station number, or team name (as directed by your teacher), and the current date.
2. Turn the plate over. Follow these steps to plate out a lawn of *Halobacterium* sp. NRC-1:
   - **a.** Remove the cap of the starter culture. Flame the lip of the container. (If no Bunsen burner is available, you may, for the purposes of this lab, omit the flaming step.)
   - **b.** Aspirate several drops of culture up into a sterile dropping pipet and replace the lid on the starter culture.
   - **c.** Lift the lid of the petri plate. Hold the lid over the plate while you work. Pipet 5 or 6 drops (~250 μL) onto the center of the plate.
   - **d.** With a sterile swab, spread the starter culture evenly across the surface of the agar. It is critical that the culture be spread evenly, and that no areas of the plate are left uncovered.
   - **e.** Replace the cover of the petri plate. Dispose of the applicator stick as directed by your teacher.
3. Use forceps to gently press the appropriate disk onto the agar surface in the center of each quarter of the plate. According to the markings on the edge of the plate (see Step 1b), place one control disk and three different antibiotic-infused disks. Make sure each disk is securely contacting the agar and will not fall off. Do not invert the plate.
4. Allow the plate to sit out overnight. (On the following day, you or your teacher must place the plate into an airtight container and then into an incubator.)

Data Collection and Analysis

1. Describe the appearance of the starter culture of *Halobacterium* sp. NRC-1.

_____

_____

_____

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2. What is the purpose of the control disk in this experiment?

3. Draw a schematic of your plate (as seen from above). Label each section clearly.

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Lab 2: Lab Analysis

Procedure

1. After the appropriate length of time has passed, closely observe your plate. Look for the antibiotic susceptibility pattern of *Halobacterium* sp. NRC-1:
   - If there is growth right up to the antibiotic disk, *Halobacterium* sp. NRC-1 is **resistant** to the antibiotic.
   - If there is a zone of clearing around the antibiotic disk, *Halobacterium* sp. NRC-1 is **sensitive** to the antibiotic.
2. The zone of clearing may be devoid of colonies. On the other hand, small, "pinpoint" colonies may have arisen. These are colonies of resistant spontaneous mutants. Count the number of pinpoint colonies around each antibiotic disk, if any.
3. Measure the diameter of the zone of clearing. Remember to subtract the diameter of the antibiotic disk.

Data Collection and Analysis

1. Draw a schematic of your plate as seen from above. Label each section clearly.

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2. Complete the table below based upon your observations of your plate.

Disk Used	**Appearance on Plate** Is there growth right up to the antibiotic disk, or is there a zone of clearing around the disk?	**Diameter of Cleared Area** (Remember to subtract the disk diameter.)	**Number of Pinpoint Colonies**	Is *Halobacterium* sp. NRC-1 **resistant** or **susceptible** to the antibiotic?

3. Using data gathered by your classmates, complete the tables on the following pages.

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Name _____

Date _____

Ampicillin

Initials, Station Number, or Team Name	**Appearance on Plate** Is there growth right up to the antibiotic disk, or is there a zone of clearing around the disk?	**Diameter of Cleared Area** (Remember to subtract the disk diameter.)	**Number of Pinpoint Colonies**	Is *Halobacterium* sp. NRC-1 **resistant** or **susceptible** to the antibiotic?

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Bacitracin

Initials, Station Number, or Team Name	**Appearance on Plate** Is there growth right up to the antibiotic disk, or is there a zone of clearing around the disk?	**Diameter of Cleared Area** (Remember to subtract the disk diameter.)	**Number of Pinpoint Colonies**	Is *Halobacterium* sp. NRC-1 **resistant** or **susceptible** to the antibiotic?

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Antibiotics in Action

Worksheet

Name _____

Date _____

Novobiocin

Initials, Station Number, or Team Name	**Appearance on Plate** Is there growth right up to the antibiotic disk, or is there a zone of clearing around the disk?	**Diameter of Cleared Area** (Remember to subtract the disk diameter.)	**Number of Pinpoint Colonies**	Is *Halobacterium* sp. NRC-1 **resistant** or **susceptible** to the antibiotic?

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Name _____

Date _____

Tetracycline

Initials, Station Number, or Team Name	**Appearance on Plate** Is there growth right up to the antibiotic disk, or is there a zone of clearing around the disk?	**Diameter of Cleared Area** (Remember to subtract the disk diameter.)	**Number of Pinpoint Colonies**	Is *Halobacterium* sp. NRC-1 **resistant** or **susceptible** to the antibiotic?

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Name _____

Date _____

Discussion Questions

1. Using what you know about the response of *Halobacterium* sp. NRC-1 to several antibiotics, explain the order in which you would use the four antibiotics tested, if you suspected *Halobacterium* sp. NRC-1 to be a pathogen. Support your answer with data from your classroom results.

2. a. Were the numbers of spontaneous resistant colonies the same for all the antibiotic disks?

   b. Why?

3. a. For each antibiotic disk type, were the numbers of spontaneous resistant colonies the same for all the plates in your class?

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b. Why do you think this is so?

4. How do you think this type of information regarding the occurrence of spontaneous antibiotic resistant mutants should figure into the use of antibiotics in a medical setting? (This is in part a bioethics question and should not be seen as having an absolute answer.)

5. Livestock farmers often give their animals prophylactic doses of antibiotics in order to ward off or prevent infection. What do you think of this practice, and what impact do you think this has on the microbes found in the food supply? You may wish to research this issue further before answering, in order to give a more complete answer.

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