CONTROL OF BACTERIAL GROWTH

Microbiologists employ a wide range of methodologies to control growth of bacteria. The particular method used depends upon the type of bacteria and the nature of the material upon which control is sought. Thus, control of endospore forming bacteria requires techniques different from that used for other cells, and sterilization of surgical equipment would involve treatments different from those used to prevent infection of a wound.

Antimicrobial agents, i.e., agents that kill or inhibit the growth of microorganisms, are extremely diverse in their physical nature and effects on microorganisms. These agents include chemicals, radiation, and temperature. All useful antimicrobial agents display some degree of **selectivity**, i.e., affecting certain organisms more than others. Selectivity of an agent can often be controlled by the manner in which it is used. For example, extremely high temperatures, potent chemical agents, and ultraviolet light are inimical to all forms of life, yet all of these agents can be used in a way that serve antimicrobial applications. Even **chemotherapeutic agents** such as antibiotics are toxic at high concentrations but can be administered pharmaceutically at lower concentrations to achieve selective anti-microbial activity. Some chemical agents actually kill their bacterial targets, and are said to be **bacteriocidal**. Other types of agents classified as **bacteriostatic** only stop growth of the cells, which clinically is sufficient to allow enough time the body's immune system to launch an adequate defense.

In this exercise you will examine some factors that influence the effectiveness of different antimicrobial agents and the techniques employed to measure their effectiveness.

Summary of exercise

1. You will examine the effects of high temperature on bacterial growth.
2. You will study the antimicrobial activity of ultraviolet light.
3. You will study susceptibility of bacteria to different chemical agents.

I. Effect of temperature on bacterial growth

Heat is the most widely used agent for reducing the number of bacteria in a particular environment. Some bacteria are more resilient to high temperature than others, whereas some actually grow best at high temperature. The effect of temperature often reflects the normal habitat of the organisms in nature. Bacterial ecologists categorize the temperature requirements of bacteria as follows:

Psycrophiles -- grow optimally at temperatures lower than 20°C
Mesophiles -- grow optimally at temperatures between 20 - 40°C.
Thermoduric mesophiles -- can withstand short periods of high temperature but grow optimally in the mesophilic range.
Thermophiles -- grow optimally at temperatures above 40°C.

Supplies

Enterobacter aerogenes (brown cap)
Bacillus stearothermophilus (pink cap)
12 tubes of Tryptic Soy Broth
-- 3 tubes each with brown, green, pink and clear caps
Enterococcus faecalis (green cap)
Your bacterial unknown
Plastic squeeze pipet

Procedure

Preparing a 'quicky' cell suspension of your semester unknown.

1. Transfer your semester unknown to a fresh slant.
2. Using a plastic squeeze pipet, transfer 1 ml of TSB from one of the clear cap tubes to the original slant culture.
3. Squirt the liquid in and out of the pipet enough time to form a turbid cell suspension.
4. You will use this as your cell source for this lab exercise.

Inoculate the cultures for testing the effects of temperature.

1. Using an inoculating loop, aseptically transfer each of the bacterial cultures to 3 tubes of fresh nutrient broth medium with the same cap color. Transfer your unknown into the tubes with clear caps.

Treat the cultures as follows:

2. Place one tube of each culture directly into the 37°C incubator.
3. Place the other two tubes of each culture in the 63°C water bath.
4. After 30 minutes, remove one tube of each culture from the water bath and place it in the 37°C incubator.
5. Leave the remaining tubes at 63°C
6. Incubate all the cultures for 24-48 hours.
7. Check for growth in tubes carefully. Record your results and interpretations in Table 2.

II. UV light as an antimicrobial and mutagenic agent

Ultraviolet light (UV) is electromagnetic radiation that has a wavelength from 100 to 300 nm. UV light is antimicrobial because it damages DNA molecules and the genetic information. When high dosages of UV light are absorbed, damage to the DNA is severe enough to kill the cell. As an antimicrobial agent, UV light is limited by its poor penetration power–it cannot pass through solid objects, including glass and plastic, thus, it is commonly used for killing microorganisms on nonporous surfaces or free-floating in the air (such as in an unused surgical unit), and only under conditions where direct exposure to the eyes or skin is not possible.

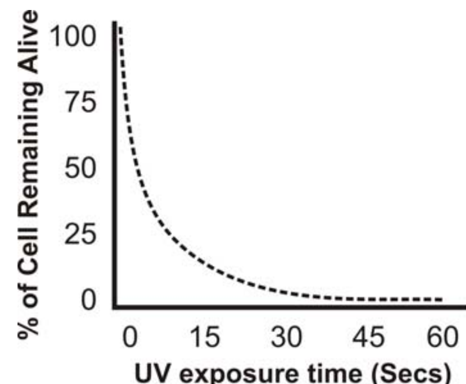


Figure 1. Dose-lethality curve for UV light exposure.

Microbiologists also commonly employ UV light to generate mutant strains of bacteria. At low dosages, the effects

of UV light may be relatively minor, not killing the organism, but creating **mutations**, i.e., changes to the genetic information. However, as shown in Figure 1, lethality of UV light progresses exponentially -- most cells will receive a lethal dose relatively quickly.

In this exercise you will examine how UV light can be used to generate mutant strains of *Serratia marcescens* that lack the normal red pigmentation or display other novel traits. A mutation to a gene controlling pigmentation will result in the formation of a white colony. To achieve bacterial mutation, however, we must prevent cells from repairing damage to DNA. Bacteria possess several "DNA-repair" systems, and one is called **photoreactivation.** Since photoreactivation is light dependent, it can be inactivated by culturing the mutagenized cells in the dark.

Supplies:

- *S. marcescens* (light blue cap)
- sterile cotton swabs
- 5 plates of TSA + 1% glycerol
- 5 pieces of foil

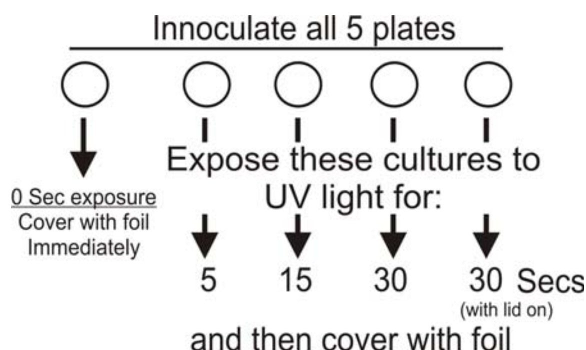


Figure 2. Overview of UV light exposure procedure.

Procedure (Summary of the procedure in Figure 2.)

1. Inoculate the ENTIRE surface of each plate of TSA using a sterile cotton swab as demonstrated in lab. Reinoculate the swab between plates.
2. Wrap one plate in foil. Label it "covered, no exposure" and place this plate directly into your drawer. (This is a control to see how the bacteria grow with no UV exposure.)
3. Label the bottom of the other plates with the following exposure times: 5, 15, and 30 seconds. Label the final plate as "30 sec exposure with lid on" (This is a control to see how well the UV light penetrates the plastic lid.)

*** CAUTION *** THE LAMP OF THE UV LIGHT IS SHIELDED BY A PROTECTIVE VISOR. NEVERTHELESS, DO NOT ATTEMPT TO LOOK DIRECTLY AT THE UV LIGHT.

4. Working at the UV light box, remove the lids from all of the plates (except the onelabeled "covered-30 sec exposure”) and slide them under the UV light.
5. After the appropriate exposure time, withdraw each plate from the UV light box, replace the lid and immediately cover the plate with foil.
6. Place all the plates **in your drawer for about 4 days** – this gives enough time to allow full pigment development.

Results:

7. Compare the bacterial growth on the covered and uncovered plates exposed to UV light for 30 seconds. Record your observations in the results section.
8. Next examine the plates for the presence of white, orange or pink colonies. Record the number of these colonies in Table 1.

III. Susceptibility of Bacteria to Chemical Agents

In the previous exercises you examined the ffect of a single antimicrobial agent against a few selected organisms. Consider the task confronting the clinical microbiologist on a daily basis. Effective antibiotic treatments must be determined as rapidly as possible (for the physical health of the patient) and as cost efficiently as possible (for the financial health of the hospital). This requires testing a wide range of antibiotics against a suspected pathogen. The clinical microbiologist must also recommend an effective dosage.

A. Agar-diffusion (or 'Kirby-Bauer') method

The traditional method used to screen bacteria for antibiotic susceptibility is called the **Kirby-Bauer method**. In this method, the bacteria are swabbed over an agar medium in a plate. Next, small paper disks, each impregnated with a different antibiotic, are aseptically applied to surface of the plate (see Figure 3). During the incubation period, the antibiotics diffuse into the agar medium and, if effective, inhibit the growth of the organism in a zone around the disk.

The results for a particular agent are determined by measuring the diameter of the zone surrounding the disk (see Figure 3). For each type of antibiotic, the zone must be equal to or larger than a certain minimum diameter stated for that antibiotic (Table 1). For example, susceptibility to bacitracin would be indicated when the diameter of the inhibition zone around the disk is EQUAL TO OR GREATER THAN 13 mm. A zone smaller than 13 mm would indicate that bacitracin is not clinically effective against the test organism.

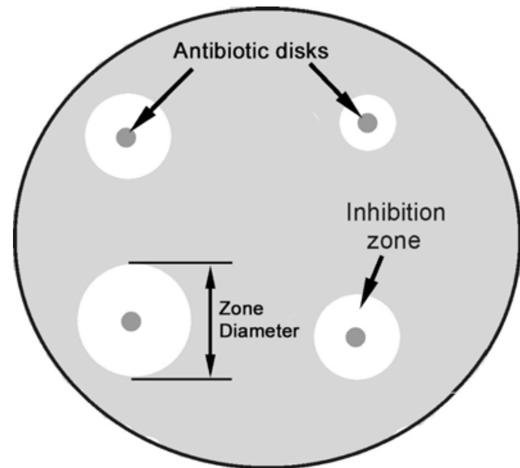


Figure 3. Kirby -Bauer antibiotic sensitivity tests.

Table I. Susceptibility Zones of Selected Antibiotic Agents.

Antibiotic	Mechanism of action	Susceptible Zone diameter	Spectrum
Ampicillin	Cell Wall inhibitor	NA	Gram-positive
Vancomycin (Va-30)	Cell Wall inhibitor	≥ 17	Gram-positive
Polymycin-B (PB-300)	Membrane disruptor	≥ 12	Gram-negative
Ciprofloxacin (Cip-5)	DNA gyrase inhibitor	≥ 21	Gram-negative
Tetracycline (Te-30)	Ribosome inhibitor	≥ 19	Both

B. Minimum Inhibitory Concentration

Often it is necessary to know the specific concentration at which an antibiotic will effectively control growth of a pathogen, what is known as the **minimum inhibitory concentration**. Traditionally, MIC was determined by growing the bacterium in broth cultures containing different concentrations of an antibiotic; the lowest concentration of the antibiotic that inhibited growth was the MIC. Obviously, knowing the minimum inhibitory concentration is valuable information when determining which dosage of antibiotic would be effective.

A more modern and efficient method for determining MIC are **antibiotic gradient strips.** An antibiotic gradient strip contains a particular antibiotic distributed along the length of the strip at different concentrations, as shown to the left. The strip is placed on a plate swabbed confluently with the bacterium. After incubation, the zone of inhibition will overlap the strip at a position that corresponds with the minimum inhibitory concentration (Figure 4), which can be read directly off the strip.

AM
250
225
200
175
150
125
100
85
75
65
55
45
35
25
15
5
2.5
1.0
0.5
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01

Supplies

2 150 cm dia plates of Mueller-Hinton agar (for antibiotic susceptibility)
2 standard size plates of TSA (for antimicrobial test)
4 sterile cotton swabs   Antibiotic test disks
2 antibiotic gradient strips

Organisms to test:
You will test a Gram-positive and a Gram-negative bacterium:

1) One of the organisms will be your **semester unknown** suspended broth medium.

2) For comparison, select a species with the opposite Gram-reaction of your unknown:

Serratia marcescens   Gram-neg (light blue cap)
OR
Enterococcus faecalis   Gram-pos (green cap)

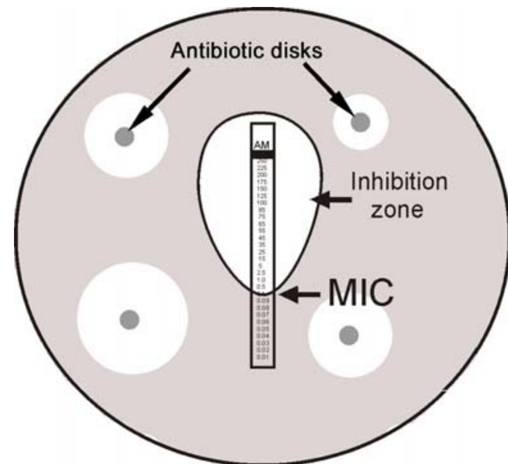


Figure 4. Determining MIC with antibiotic gradient strip

Procedure

A. Testing antibiotic susceptibility (use two 150 cm diameter plates)

1. Identify the bacteria being tested on the bottoms of two plates.
2. Using cotton swabs, inoculate the ENTIRE SURFACE of each plate with the appropriate organism.
3. Lay an Antibiotic Gradient Strip in the center of the plate, as shown in Figure 3.
   ** Do not attempt to move disks or strip after they have touched the medium. **
4. Using flame-sterilized forceps, place 4 different antibiotic susceptibility disks symmetrically on the plate, roughly as shown in Figures 3 and 4.
5. Incubate plates right-side-up at 37°C for 24 - 48 hours.

B. Testing susceptibility to other antimicrobial agents (**use two standard diameter plates** and the same bacteria used for part A.)

You will also test the susceptibility of your test organism to other types of agents. Since these disks will not be marked on their surfaces as to the agent present, you will need to record their contents directly on the petri plate. The agents provided are representative antiseptics and disinfectants commonly used in clinics and laboratories.

1. Label and inoculate **two standard size TSA** with your name, test organism, etc.
2. Divide the bottoms of the plates into 5 sectors (see Figure 5), label sectors for each of the 5 germicidal agents provided on the side bench.
3. Using flame-sterilized forceps, remove a sterile paper disk from the container. Allow one of the chemical agents to soak into the disk by touching the disk edge to the surface of the chemical solution.
4. Apply the disk to the appropriate sector of the plate.
5. Repeat steps 3 and 4 for the other five agents, spacing the disks symmetrically upon the medium (Figure 5).
6. Incubate the plates right-side-up at 37°C for 24 - 48 hours.

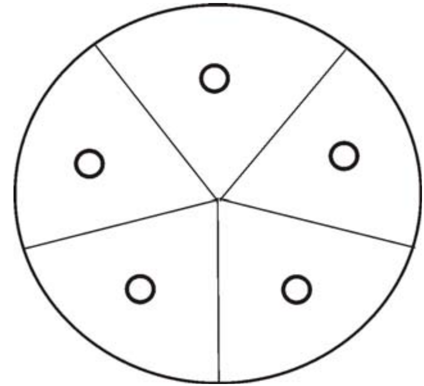


Figure 5. Placement of disks with disinfectants on plate.

Results

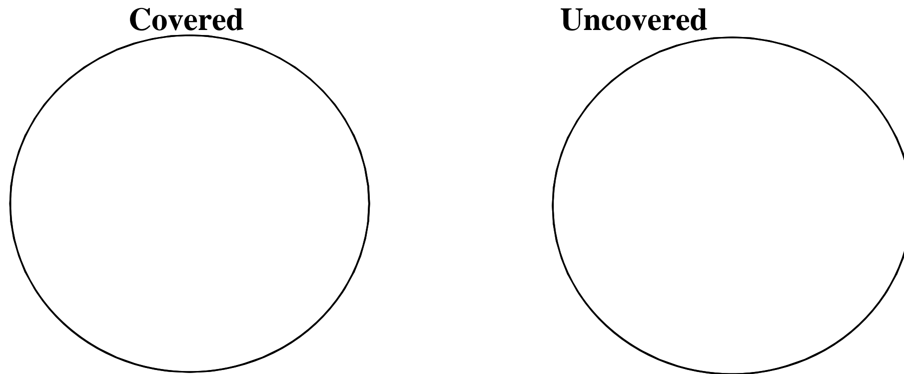
Using the plastic ruler provided in your drawer, measure the zones of inhibition around each disk as shown in Figure 2, and record the point where the inhibition zone crosses the gradient strip. Record the results in Tables 3 and 4; record 'no zone' if the width of the clearing is less than 1 mm. Interpret the susceptibility of the test organisms for each antibiotic by referring to the information in Table I.

Names: ____________________________________

✶✶Indicate where your results did not agree with your expectations, and write a brief explanation of the discrepancy.✶✶

1. Effects of UV light exposure

Record the appearance of the *Serratia* cultures exposed to UV light for 30 seconds in plates covered and uncovered:



Why did UV light not affect the cells in the petri plate that was left covered during the exposure step?

Table 1. Effect of UV light exposure.

	Duration of UV exposure in seconds			
	0 seconds	5 seconds	15 seconds	30 seconds
total number of colonies*				
number of white colonies				

*count colonies or indicate "confluent" growth.

Re-examine Figure 1 and the accompanying text. Do your results support the principles presented there? Explain.

Does a higher of lower exposure time lead to more pigment mutations? Why?

2. Effects of temperature on bacterial growth

Table 2. Effect of temperature on growth and survival of bacteria.

Organism	held at 37°C for entire incubation	Incubated first at 63°C and then at 37°C	Held at 63°C for entire incubation	Ecological description of organism*
E. aerogenes				
E. faecalis				
B. stearothm.				
Your unknown				

record results as: growth = "+" or no growth = "–"
*mesophilic = MS, thermophilic = TP, thermoduric = TD

3. Effects of antibiotics on bacterial growth

Table 3. Antibiotic susceptibilities of tested organisms

Antibiotic on test disk	**Unknown Gram rx: _______**		**Other species Gram Rx: __________ Name: _____________________**	
	Zone diameter (mm)	**Susceptible (y/n)**	**Zone diameter (mm)**	**Susceptible (y/n)**
Name:				

Record 'No zone' if clearing is less than 1 mm in width

Ab on test strip	**MIC read from strip**	**MIC read from strip**
Name:	µg/ml	µg/ml

Do the Kirby-Bauer and MIC test results for your semester unknown agree with the indicated usages (Table 1) of the different antibiotics? Describe any discrepancies.

Does the sensitivity of the bacteria to the antibiotic increase or decrease as the MIC decreases?

Which one the bacteria that you tested was most sensitive to ampicillin? __________________
Explain:

4. Effects of antimicrobial agents on bacterial growth

Table 4. Inhibitory properties of antimicrobial agents.

Name of Antimicrobial Agent	Unknown Gram Rx ______	Other species Gram Rx ______ Name: ______________________
	Zone diameter (mm)	**Zone diameter (mm)**
1.		
2.		
3.		
4.		
5.		

Record 'No zone' if clearing is less than 1 mm in width

Will the inhibition zone be larger or smaller for the bacteria that is most sensitive to the antimicrobial agent? _____________

Which agents(s) selectively inhibited (i.e., had a significantly larger zone diameter for) the:

Gram-positive cells:

Gram-negative cells:

Why does it not make sense to list an agent under both of the above categories?

Also answer this question. (Typed)

In the Kirby-Bauer method, over time the antibiotic diffuses from the disk into the surrounding medium; and for some bacteria the antibiotic may be bacteriocidal, while for others it may be bacteriostatic.

A. Why doesn't the zone of inhibition continuously grow larger as the antibiotic diffuses further and further into the medium?

B. During a Kirby-Bauer test, eventually the antibiotic on the disk will completely dissipate into the medium. When this happens, what will happen to the cells within the inhibition zone if the antibiotic is bacteriocidal and if it is bacteriostatic. Explain.