MiSP Evolution by Natural Selection / Bacterial Resistance
Teacher Guide, L1 – L3

Introduction

This unit uses the development of resistance to antimicrobials as an example of natural selection. The students should have an introductory understanding of evolution by natural selection before beginning the unit. The activities and questions focus on the role of mutations as a starting point for natural selection. The unit also addresses some of the common misconceptions that students harbor about the concept of natural selection.

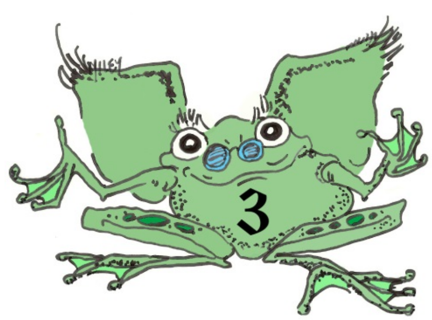
On day 1 review with students their knowledge of evolution and natural selection, and introduce the life cycle of bacteria and the use of antimicrobials. Explain that bacteria are good model organisms for the study of evolution because they have a short generation time, a single-copy genome, and a well-characterized genome. The health issues of microbial resistance should be discussed as well.

On day 2 students will set up an experiment to study the sensitivity of bacteria to a common antimicrobial agent. This experiment will prepare the students to learn about the development of resistance to antimicrobial agents, a process that depends on natural selection.

The antimicrobial used in this unit is triclosan, an agent that is effective against bacteria, fungi, and viruses. It has been used in health care settings at a bactericidal concentration of 0.3% since 1972. Triclosan is now added to many personal care products such as deodorant soaps and hand lotions, typically at 0.1% bacteriostatic concentrations. Triclosan is also used in plastics under the name Microban® (found in cutting boards and toys), and it is used in clothing as Biofresh®. The development of bacterial resistance to triclosan has been studied extensively. It is now known that mutations in at least six unrelated genes can result in resistance to this antimicrobial agent (Levy, 2000).

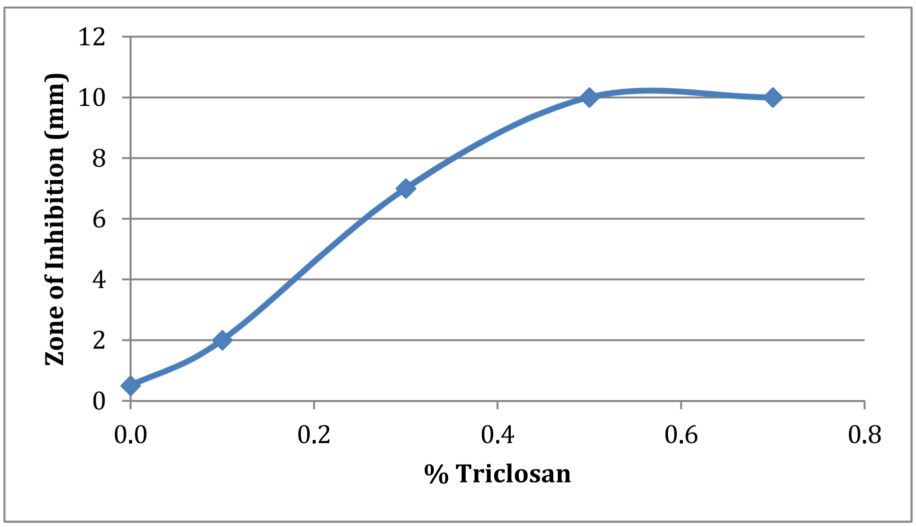
The species of bacteria used in this unit is *Bacillus cereus.* This species is not known to cause disease in humans and is approved for use in laboratories for students of all ages (http://www.science-projects.com/safemicrobes.htm).

The students will plate bacteria in the absence of the antimicrobial agent triclosan and in the presence of disks containing four different concentrations of triclosan: 0.1%, 0.3%, 0.5%, and 0.7%. In this disk-diffusion method, the concentration of the antimicrobial decreases with increased distance from the disk. The bacteria that are spread on individual plates should come from a single colony so they are genetically identical in their resistance to triclosan, with the exception of random mutations during the growth of the colony itself. The colonies that individual students in the class start with may not be identical, again because of random mutations. As the bacteria reproduce on the students' agar plates, additional mutations may occur that make some bacteria more resistant to triclosan. On day 3 the students will measure the zones of inhibition on the plates with triclosan-coated disks. In a circular area close to the disk, all of the bacteria will be killed and the area will be transparent. Cultures containing disks soaked in a higher starting concentration of triclosan should



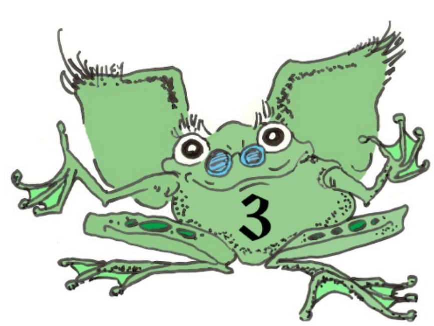
have larger zones of inhibition. On all of the plates, as triclosan diffuses from the disk, the concentration of triclosan decreases and some of the bacteria will be resistant to the lower concentration of triclosan. The area containing some resistant bacteria will be hazy. Questions associated with this exercise prompt the students to recognize that in the "hazy" zone, only some of the bacteria survive. A mutation has occurred in these bacteria that makes them more resistant to the antimicrobial agent. These bacteria have been "selected" by a changing environment.

On day 3 students will also graph the average diameter of the zone of inhibition as the variable dependent on the dose of triclosan. This is known as a dose-response curve. Data from all groups will need to be pooled and averaged for reasonable results. The graph should be relatively linear at lower concentrations, reaching a maximum at higher concentrations, as shown below. Typically, the 0% triclosan (50% ethanol control) will have a small zone of inhibition due to the bacteriostatic properties of ethanol.



On day 4 students will analyze data from an undergraduate student activity (activity adapted from Serafini and Matthews, 2009) in which bacteria from the edge of the zone of inhibition (the hazy area) were re-plated and exposed to disks containing 0.1% triclosan, the concentration found in most consumer products. The zone of inhibition from the subculture was measured and the procedure was repeated. Data from ten plates from each of 15 consecutive subcultures are provided. The original data were modified slightly to facilitate graphing and linear function instruction.

On day 5 an assessment is administered.



Standards

ILST Core Curriculum — Major Understandings:
Standard 4 Living Environment 2.2a, 3.1b, 3.2d
Living Environment Core Curriculum — Major Understandings:
Standard 4 Living Environment 3.1b, 3.1f, 3.1g, 3.1h

Lesson Objectives:

- After completing this unit, students will be able to:
  Recognize that there is genetic variability in the ability of bacteria to grow in the presence of the antimicrobial agent, triclosan
- Recognize that when the environment is changed by the addition of triclosan to the nutrient substrate, the resistant bacteria are better able to survive
- Recognize that repeated exposure to the antimicrobial agent increases the percentage of bacterial cells that are resistant because only these bacteria can survive and reproduce
- Graph the relationship between the dosage of the antimicrobial agent and the zone of inhibition (levels 1-3)
- Graph the curve showing the development of resistance to triclosan in the bacteria (levels 1-3)
- Understand that the relationship between the dosage of antimicrobial and the zone of inhibition has a unit rate of change (levels 2 and 3)
- Recognize that the development of resistance is not linear (levels 2 and 3)
- Express the relationship between dosage and the zone of inhibition as a linear equation (level 3)
- Recognize that the development of resistance cannot be expressed as a linear relationship and that extrapolation from incomplete data leads to erroneous conclusions (level 3).

Day 1 – Natural Selection

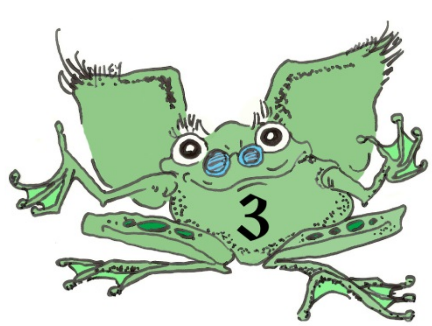
Review the concept of natural selection. The following websites may be helpful:

http://evolution.berkeley.edu

http://serendip.brynmawr.edu/sci_edu/waldron/#evolution

Common misunderstandings that should be reviewed include:

- Natural selection is a process that perfects organisms.
- When the environment changes, the genes of the species change to adapt to the new environment.
- If a particular trait would be advantageous, evolution and natural selection will cause it to



appear.

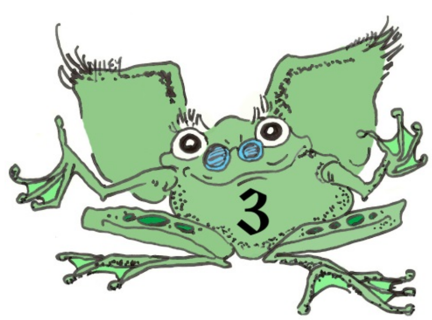
- Introduce the students to basic information about bacteria, bacterial growth, and mutation rates.
- Bacteria reproduce by simple fission. A simulation of a bacterial growth curve incorporating the effects of changing temperature, pH, and NaCl concentration can be found at http://www.foodsafetyfirst.org/fsf_mgsp.html.
- Bacterial division rate varies. Range is from 20 minutes to hours.
- Unlike eukaryotes, bacteria have only one chromosome and therefore only one copy of each gene. Therefore, if a gene mutation occurs, the effect is always expressed.
- Bacteria can exchange genetic material so mutant genes can spread in a population.
- Microorganisms have many important functions. See http://www.actionbioscience.org/evolution/meade_callahan.html.

  Functions include:

  •nutrient recycling
  •health
  •food
  •biodegradation
  •waste water treatment
  •biosynthesis

- Some microorganisms cause diseases, and antibiotics and other antimicrobial agents have been developed to fight these diseases.
- Bacteria can develop resistance to these antimicrobial agents through the process of natural selection.
- The primary reason for the development of antibiotic resistance is mutation with natural selection. Multiple mutations may be required before bacteria become fully resistant to a particular antimicrobial.
- Once resistant genes appear, bacteria have a variety of mechanisms for exchanging those (and other) genes both within and across species. This exchange allows for “accelerated evolution” of bacterial species; it is accelerated in the sense that random mutations that result in antibiotic resistance need not occur in every individual bacterium, or even in every species of pathogen, but can simply be acquired from another organism.

Additional information about bacterial resistance can be found in the “Emerging and Re-Emerging Infectious Diseases” pdf that can be downloaded from: http://science.education.nih.gov/supplements/nih1/diseases/default.htm.



Day 2 – The Antimicrobial Effect of Increasing Concentrations of Triclosan

Lab activity

Students follow Lab Activity: The Antimicrobial Effect of
Increasing Concentrations of Triclosan.

Preparation:

Two days before the experiment, streak several LB agar plates with *Bacillus cereus*. Grow the bacteria at room temperature overnight. *Bacillus cereus* is a soil bacterium that grows at room temperature and does not cause disease.

Prepare enough LB agar plates for your class. You will need five plates per group. LB agar can be obtained from many sources. Mix it with water as directed, boil the suspension, and use an autoclave to sterilize if one is available. Store the suspension in the refrigerator if it is made in advance.

Prepare triclosan solutions (0.1%, 0.3%, 0.5%, and 0.7%). Triclosan can be obtained from Sigma. Dissolve the appropriate amount of triclosan (*x*% = *x* grams/100 ml) in 50% ethanol.

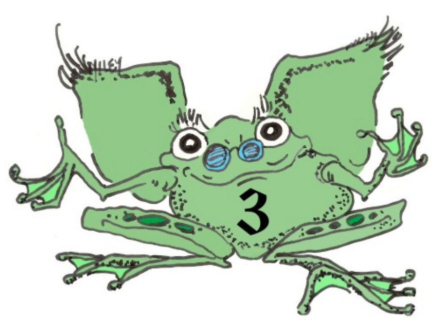
Purchase or prepare sterile disks. Disks can be made by punching holes in heavy filter paper, soaking the resulting disks in 95% ethanol, and allowing them to dry.

The lab procedures state that each group of students will be given sterile filter disks and microfuge tubes containing 50% ethanol and the four concentrations of triclosan. Alternately, you can have disks soaking in the five different solutions and the students can pick up the disks with sterile forceps. The difficulty with this approach is in maintaining consistency in the amount of liquid on the disk. Excess liquid can be removed by touching the disk to a piece of filter paper but this method tends to produce more variable results.

Materials needed:

- sterile forceps (autoclaved or boiled) with pointed end
- sterile swabs
- gloves
- marking pens
- blotting or filter paper
- LB plates (5 per group)
- dilutions of triclosan (aka Irgasan) in microfuge tubes
- 50% ethanol
- sterile filter disks

Students should work in groups. Each group prepares five plates of bacteria. Place a disk soaked



in 50% ethanol on one; this is the control. On remaining plates place a disk soaked in one of the following concentrations of triclosan: 0.1%, 0.3%, 0.5%, and 0.7%. The class should have at least three plates of each concentration. Make sure that the students do not place the disks on the plates when they are too wet.

Grow the plates overnight at room temperature.

Day 3 – The Zone of Inhibition

Students follow Lab Activity: The Antimicrobial Effect of Increasing Concentrations of Triclosan.

Measure the zone of inhibition.
Collect data from all groups and average the data for each level of inhibition.
Draw best-fit line.
It is important to discuss the answers to the science questions in class so the students understand that the bacteria that grow in the hazy area at the edge of the zone of inhibition have developed some resistance to triclosan. This resistance must have been caused by mutation either as the bacteria reproduced in the original colony that was spread on the plates or while the bacteria reproduced on the students' plates. The mutation allowed the resistant bacteria to grow at a higher concentration of triclosan than the original bacteria. Remember to tell the students that multiple mutations may be required before bacteria become fully resistant to a particular antimicrobial.

Day 4 – Development of Resistance

Introduce the problem of bacterial resistance as an example of natural selection.

For background information, see "Emerging and Re-Emerging Infectious Diseases" pdf.

Students follow Evolution by Natural Selection / Bacterial Resistance Worksheet:
Natural Selection of Triclosan-Resistant Bacteria. This activity uses data from an undergraduate laboratory experiment designed to show the development of resistance. Data from ten plates from ten consecutive subcultures are given and averaged. You may want to have the students average the data themselves. The data were manipulated slightly to make the averages easier to graph and analyze.

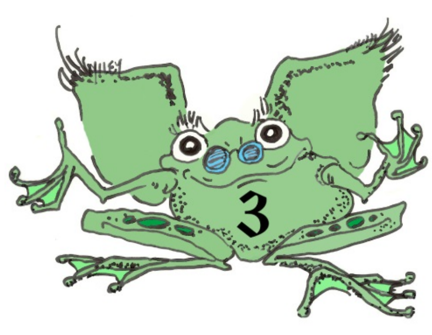
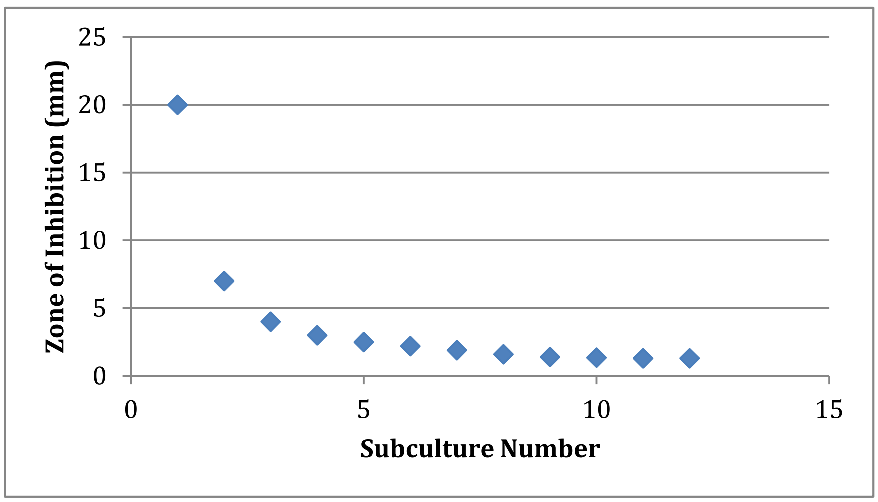
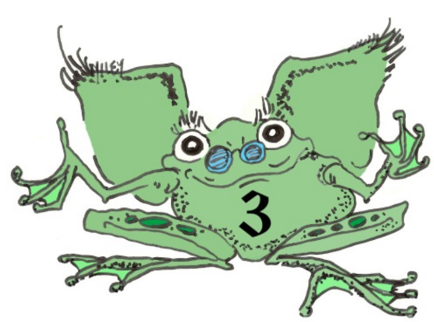


Table 1. Zone of inhibition (mm) and average for ten plates of each subculture

Plate #	Subculture number											
	1	2	3	4	5	6	7	8	9	10	11	12
1	18	7.5	4	3.5	2.8	3	1.8	1.6	1.5	1.6	0.8	1.6
2	21.5	6.5	5	3	3	2.2	2.2	1.8	1.2	1.5	1.1	1.4
3	17	5.5	4.5	2.5	1.7	1.7	2.5	1.5	0.9	1.2	1.5	0.8
4	22.5	8	3	2.5	3.2	2.7	1.7	2	1.1	0.8	1.4	1.9
5	20	7.5	3.5	4	2.5	2.5	1.8	1.3	1.8	1.5	1.3	1.2
6	19	6.5	5.5	3.5	3	2	2.3	0.8	1.6	1.1	1.7	0.9
7	21	5.5	4.5	4.5	2.2	1.7	1.7	1.5	1.4	1.4	1.3	1
8	20.5	7	2.5	3	2.3	2.1	2.1	1.4	1.2	1.3	0.9	1.4
9	20.5	7.5	4	1.5	1.7	1.8	1.4	1.9	1.5	1.4	1.8	1.5
10	20	8.5	3.5	2	2.6	2.3	1.5	2.2	1.8	1.7	1.2	1.3
Avg	**20**	**7**	**4**	**3**	**2.5**	**2.2**	**1.9**	**1.6**	**1.4**	**1.35**	**1.3**	**1.3**



Because the data are not continuous, a line connecting the data points cannot be drawn. However, an examination of the curve and calculation of the change in the zone of inhibition between consecutive subcultures reveals that the relationship between the size of the zone of inhibition and the subculture number is not linear. Questions associated with this activity deal with variability in the original data and probe the students' understanding of genetic variability as a requirement for natural selection. You may want to have your students graph these data as a histogram as well as a line graph. However, to ensure fidelity to the MiSP project, do not <u>eliminate</u> the line graph.



Day 5 – Assessment

Administer the assessment: *MiSP Evolution by Natural Selection / Bacterial Resistance Assessment*.

References

Levy, B. (2000). Antibacterial Household Products: Cause for Concern. Retrieved May 2010 from http://www.cdc.gov/ncidod/eid/vol7no3_supp/levy.htm.

Serafini, A. and D. M. Matthews. (2009). Microbial Resistance to Triclosan: A Case Study in Natural Selection. *American Biology Teacher* 71(9): 536-540.