

Experimental Evolution of Antibiotic Resistance in Bacteria

AMY C. KRIST SASHA A. SHOWSH

Evolution is typically measured as a change in allele or genotype frequencies over one or more generations. Consequently, evolution is difficult to show experimentally in a semester-long lab course because most organisms have longer generation times than fifteen weeks. Therefore, we designed an experiment to demonstrate and study evolution using bacteria as a model system.

Bacteria are ideal organisms for studying evolution because they reproduce quickly and asexually, they permit the study of large sample sizes, they are easy to propagate, and it is easy to manipulate their environment (Elena & Lenski, 2003). In this simple experiment, the common soil bacterium, *Bacillus thuringiensis*, evolves resistance to the antibiotic streptomycin. Evolution of antibiotic resistance can be observed over a few weeks due to the short generation time of *B. thuringiensis*, which can divide every 45 minutes; the natural occurrence of random spontaneous mutations that occur during cell division; and experimental conditions that allow natural selection to occur. Thus, mutation and natural selection, the higher rate of reproduction or survival of organisms possessing favorable heritable traits, are the mechanisms of evolutionary change in this experiment.

The antibiotic streptomycin prevents bacterial growth and division by binding to the bacterial ribosome and inhibiting protein synthesis. Two different spontaneous mutations may cause *B. thuringiensis* to become resistant to streptomycin. One mutation alters the shape of the ribosome so that the mutated ribosome is still capable of synthesizing protein but streptomycin is unable to bind to and inactivate the altered ribosome. Another mutation alters the shape of a protein in the cell membrane that is involved in transporting the antibiotic into the cell. This mutation results in lower permeability of the cell membrane to streptomycin. Bacteria that possess either of these mutations are able to grow and divide in the presence of streptomycin.

There is a high probability that these mutations will occur in the experiment because of a combination of the frequency of spontaneous mutations, the number of cell divisions that occur in a short period of time, and the sheer number of bacteria present on each experimental plate. In bacteria, spontaneous mutations in a gene occur at an average frequency of one in every 10^9 generations. When this mutation rate is applied to the millions of cells on each Petri plate that will each experience over 16 million cell divisions, this means that most students will observe mutations.

AMY C. KRIST is a Research Scientist, Department of Zoology and Physiology, University of Wyoming, Laramie, WY 82701; e-mail: krist@uwyo.edu. SASHA A. SHOWSH is Associate Professor, Department of Biology, University of Wisconsin – Eau Claire, Eau Claire, WI 54702; e-mail: showhsa@uwec.edu.

Hence, due to the intrinsic properties of bacteria and some simple manipulations, the students are able to observe a bacterial strain that is initially susceptible to the antibiotic evolve into one that is resistant. This experiment allows students to observe evolution in action and it illustrates how easily pathogenic bacteria can evolve into resistant forms that are much more difficult to treat. Since we are currently experiencing a major crisis in public health with the emergence of antibiotic resistant strains of bacteria (reviewed in Cohen, 2000) the subject matter of this laboratory experiment is highly relevant and, in our experience, very interesting to freshman college students. In addition, the topic provides the class an opportunity to explore the crisis of antibiotic resistance in greater depth (e.g., through articles, film, discussion).

The major goals of this experiment are to observe evolution in real time, to understand mutation and natural selection and how they cause the bacteria to evolve, and to provide opportunities for the students to use evolutionary terminology. In the following sections, we will address the specific methods for setting up and performing the lab. In addition, we will address the results that are expected, the meaning of the results, how we assess students' understanding of the lab, and how we meet the goals of the experiment.

Instructor Preparation & Materials

We designed the experiment to be completed in three consecutive laboratory periods. In the course that we teach, these are in three consecutive weeks. However, the time required could be condensed considerably if labs are scheduled to meet more than once a week. In the first and second lab periods, the students are actively performing the experiment and in the third period, they are examining and interpreting the results. We also reserve some time in the third period to discuss a short film about antibiotic resistance and have the students complete a writing exercise about the experiment. Details are provided below.

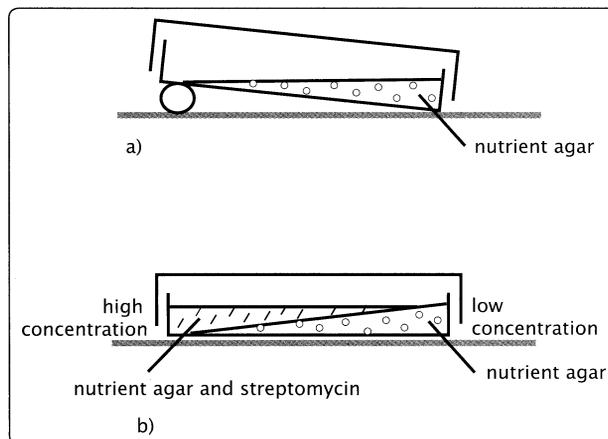
Materials

- Petri plates containing appropriate growth medium (one plate per group)
- Petri plates containing appropriate growth medium with streptomycin (two per group; one for the first lab period and one for the second)
- gradient plates (one plate per group)
- loops (one per group)
- Bunsen burners
- culture of *B. thuringiensis*, slant or plate (one per every two to four groups). The bacteria can be obtained from the American Type Culture Collection (<http://www.atcc.org>).
- incubator set at 37° C

Preparation of Materials

1. Prepare plates containing growth medium with agar. Using aseptic techniques, pour sterilized growth medium containing agar into the appropriate number of Petri plates (save the plastic sleeves after opening). We used Todd Hewitt Broth (THB) with agar but any complex medium (e.g., Nutrient Broth, Luria-Bertani) is appropriate. Allow the plates to cool and solidify. These plates can be prepared up to two weeks in advance. Store in the refrigerator (4° C) in plastic sleeves to minimize drying. These plates will be referred to as THB plates.
2. Prepare plates containing streptomycin. Add filter-sterilized streptomycin stock (50 mg/ml) to sterilized growth medium tempered to 50° C to obtain a final concentration of 250 µg streptomycin/ml. Using aseptic techniques, pour the solution into the appropriate number of Petri plates. Allow the plates to cool and solidify. These plates can be prepared up to one week in advance. Store in the refrigerator (4° C) in Petri plate sleeves to minimize drying. These plates will be referred to as Strep plates.
3. Prepare gradient plates. To make these plates, pour approximately half of the contents of a typical plate of growth medium with agar (approximately 15 ml). Allow the plates to cool and solidify on a slant. To produce the slant, we let the plates solidify while tipped up on 10 ml pipets (Figure 1a). This part of the gradient can be prepared a week in advance and stored as previously described. Twenty-four to thirty-six hours prior to class, make the growth medium with streptomycin (as described in Step 2; 250 µg/ml) and pour the medium on top of the gradient plate, being careful not to cover the entire surface (you want one end that contains only growth medium with agar; Figure 1b). Do not make the plates in advance of thirty-six hours because the streptomycin will diffuse through the agar and destroy the antibiotic gradient. After cooling, refrigerate as described previously to slow the rate of diffusion. The gradient plate is named for the antibiotic gradient that is created on the plate; after the antibiotic has diffused somewhat, the antibiotic concentration is essentially 0 µg/ml at one end of the plate and increases to 250 µg/ml at the other end.

Figure 1. Preparation of an antibiotic gradient. a) Approximately 15 ml of nutrient agar is poured into a Petri plate, and allowed to solidify on a slant. We produce a slant by tipping the plate up on a 10 ml pipet. b) Twenty-four to thirty-six hours prior to use, the nutrient agar and streptomycin mixture is poured on top of the slant of nutrient agar. The entire surface is not covered so that one end of the plate contains only nutrient agar. High and low concentration refer to the concentration of streptomycin.



37° C. After incubation, the positive and negative control plates are removed and stored in the refrigerator until the last class period.

is given one THB plate, one Strep plate and one gradient plate. The students transfer *B. thuringiensis* from a culture to the THB plate (the positive control), and the Strep plate (the negative control). The students then spread the bacteria on the gradient plate, starting from the end of the plate that does not contain streptomycin and spreading the cells toward the end that contains 250 µg/ml streptomycin. It is on the gradient plate where “natural” selection favors cells with streptomycin resistance because resistant cells have higher relative fitness and thus can divide and form colonies. Relative fitness is the survival and reproduction of an individual compared to the average fitness of the population.

All of the plates are incubated, agar side up, for 24 hours at

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Procedure

First Lab Period

At the beginning of the first lab period, the students are instructed about proper handling of non-pathogenic bacteria, aseptic techniques, and the procedures used for transferring bacterial cells to Petri plates. In this lab, each group of students

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After the students have set up the experiment, they make predictions about what they expect to observe on each of the plates after incubation. For the gradient plate, we ask them what kind of results they expect after the initial incubation and after the re-streaking (described later). This entire process takes about one hour. We use the rest of the class to discuss an article on the problem of antibiotic resistance (Levy, 1998). This article discusses the scope and causes of the problem, the mechanisms of antibiotic resistance, and how antibiotic resistance evolves.

Between First & Second Lab Periods

The gradient plates are streaked at least one more time to facilitate selection. This re-streaking is accomplished by repeatedly using the loop to spread the bacteria from the end of the gradient plate with no antibiotic to the end with 250 µg/ml streptomycin. If resistant colonies are not observed after the initial re-streak, the process can be repeated. After each re-streak the plates are incubated for 24 to 48 hours at 37° C. This re-streaking process increases the frequency of antibiotic resistant cells in two ways. First, the streaking physically separates colonies of resistant bacteria so that each resistant cell can develop into a new colony. Second, physically separating bacteria growing on the part of the plate with no streptomycin provides further opportunity for the cells to divide and potentially mutate into resistant cells. When the plate is re-streaked, these new mutated cells can be moved to the streptomycin gradient and grow into resistant colonies. Since our class meets only once a week, the instructors re-streak the plates outside of class. However, it is a simple process and could be done by the students.

Second Lab Period

In the second lab period, the students observe the three plates that they streaked the first week. By this time the gradient plate has been re-streaked one or two times. If resistant colonies are present (colonies growing on the end of the gradient plate with a high concentration of streptomycin), the students remove the resistant colonies from the gradient plate, streak them onto a new Strep plate, and incubate them for 24 to 48 hours at 37° C. The students are then instructed to make predictions about the results they expect from this second streptomycin plate. These activities typically take only about 20 minutes. In our class, these activities are performed at the beginning of the lab period and the rest of the period is spent on a separate lab exercise.

Third Lab Period

In the third lab period the students observe all of their plates and discuss the results. Specifically, the students are instructed to compare the positive control (THB plate), the negative control (Strep plate from the first lab period), and the Strep plate with the resistant colonies (Strep from the second lab period). Working in groups, the students discuss possible explanations for the observed differences among all the plates, and the process that occurred on the gradient plate. After we discuss these explanations as a class, the students watch a 20-

Figure 2. The appearance of a THB plate (containing agar and Todd Hewitt Broth as the nutrient medium) after the plate was streaked with the initial culture of *B. thuringiensis* and incubated at 37° C for 24 hours. This plate is the positive control and demonstrates that the bacterial culture grows very well on the growth medium.



Figure 3. The appearance of a Strep plate (containing agar, Todd Hewitt Broth, and streptomycin) after the plate was streaked with the initial culture of *B. thuringiensis* and incubated at 37° C for 24 hours. This plate is the negative control and demonstrates that the initial bacterial culture does not grow in the presence of streptomycin.



Figure 4. The appearance of the gradient plate after the plate was streaked once with the initial culture of *B. thuringiensis* and incubated at 37° C for 24 hours. The only growth on the plate is at the top where there is no streptomycin.

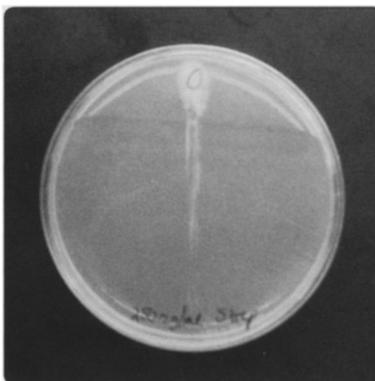


Figure 5. The appearance of the gradient plate after the plate was re-streaked twice and incubated at 37° C for 24 hours between each streaking. The colonies growing towards the bottom of the plate are resistant to streptomycin.



minute segment from a recent film describing the evolution of a strain of *Mycobacterium tuberculosis* that is resistant to multiple drugs (excerpt from "Evolution: The Evolutionary Arms Race," 2001). After the film, the students work independently on an in-class writing assignment. For the first part of this assignment, the students are asked to use scientific terminology to explain why the exercise demonstrates evolution and natural selection, and how natural selection occurs in the experiment. We also ask the students to describe an analogy between the story portrayed in the film and our experiment. This writing exercise is the primary way that we assess the students' understanding of what occurred in the experiment and whether they can apply this understanding to a novel situation (the one presented in the film).

Results/Discussion

After incubating the THB plate (positive control) and the Strep plate (negative control) the students should observe that the THB plate has thousands of colonies of *B. thuringiensis* and the Strep plate has none (Figures 2 and 3). These plates

illustrate that *B. thuringiensis* grows very well on the growth medium (positive control), but does not grow on the growth medium containing streptomycin (the negative control; see Figure 3). The difference in growth between the two plates demonstrates that the starting culture of *B. thuringiensis* is susceptible to the antibiotic streptomycin.

The first time that bacterial cells are transferred to the gradient plate, there is usually abundant growth on the end of the plate with no streptomycin and little to no growth on the gradient (and no growth on the 250 µg/ml end of the gradient; Figure 4). However, after re-streaking once or twice, there are usually multiple (2-50) colonies forming on the gradient (Figure 5). In a class with eight groups of students, there is usually one group that has a gradient plate without resistant colonies. We use this result to emphasize the random nature of mutation.

For the majority of students who had resistant colonies growing on their gradient plate, the transfer of resistant colonies from the gradient plate to a second Strep plate results in the growth of thousands of resistant colonies (Figure 6). This prolific growth is in striking contrast to the complete lack of growth on the first Strep plate (the negative control; see Figure 3). This difference demonstrates that *B. thuringiensis* evolved resistance to streptomycin over the course of the experiment.

This simple experiment enables students to observe evolution. They observe the processes of mutation and selection occurring on their own gradient plates and when they compare the negative control (first Strep plate) to the second Strep plate, they observe that these processes brought about an evolutionary change in the resistant bacteria.

We use several methods to achieve the major goals of this experiment. First, we consistently use the language of evolution in the context of the experiment. For example, when we discuss the process that occurred on the gradient plate, we explain that differences in relative fitness result in natural selection. Thus, when streptomycin is present, the mutated bacteria have higher relative fitness, they are “selected,” and as a result, evolution occurs because the mutation for antibiotic resistance is heritable. Second, in small group discussions, we provide the students the opportunity to practice using the terminology while working together to determine what occurred during the experiment. Third, we have the students write about the exercise (described earlier). After the writing exercise, we have a large group discussion in which we review the processes and stress the ease with which evolution occurs.

This experiment is valuable to the students for several reasons. First, the experiment allows students to observe evolution occurring in real time. This is a rare opportunity since in most natural circumstances it is impossible to observe evolution. Second, the experiment illustrates the operation of two mechanisms of evolution, mutation and selection, and how they bring about evolutionary change. This is important because many college students hold major misconceptions about what evolution is, and about the concepts central to

Figure 6. The appearance of the second Strep plate after it was streaked with cells from antibiotic resistant colonies on the gradient plate and incubated at 37° C for 24 hours. The abundant growth of streptomycin resistant colonies on this plate contrasts sharply with the complete absence of growth on the first Strep plate.



understanding evolution (Alters & Nelson, 2002). Third, the experiment provides a relevant context in which to discuss a major public health crisis, the widespread evolution of antibiotic resistance among pathogens. Finally, the experiment illustrates how rapidly and easily evolution can occur; evolution does not always operate slowly, and natural selection is the inevitable outcome of organisms possessing different relative fitness.

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