

Mutation and Selection: An Exploration of Antibiotic Resistance in *Serratia marcescens*

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This laboratory module gives students hands-on experience with mutation and selection in bacteria. An initial classwide project prepares students to design separate group projects. During the first two weeks students observe *de novo* streptomycin-resistance mutations in *Serratia marcescens* and compare classwide mutation patterns with those found in Luria and Delbruck's classical experiment showing that mutations are spontaneous rather than induced. In the second and third weeks students design and implement their own experiments related to antibiotic resistance. Topics such as bacterial growth, use of controls, serial dilutions, sterile technique and the difference between mutation rate and mutation frequency are addressed.

Keywords: mutation, selection, antibiotic resistance, *Serratia marcescens*, inquiry laboratory, streptomycin, microbes, invertebrates, Winogradsky columns, bacteria, protists, ecological community

Introduction

Often the key to success in biology labs is giving the ownership of a research project to the students and allowing them to design and analyze their own experiments. The challenge is finding that balance between providing structure and giving enough freedom to students who may have just learnt the steps of experimental design. This three-week laboratory module aims to give students hands-on experience with mutation and selection in bacteria using the topic of antibiotic resistance. This lab was designed as part of Cornell University's new Investigative Laboratory which is a stand-alone two credit laboratory course required of freshmen biology majors. It has been used in this course for three years as a three week laboratory module (three hours of lab each week taught by graduate student teaching assistants) supported by three one-hour lectures. Although used at the freshman level at Cornell (a highly prepared group of freshmen, many with Advance Placement credits from high school), this module could also potentially be used in an upper level microbiology, evolution or cell biology course. During the first two weeks of this laboratory module students observe the pattern of *de novo* streptomycin-resistance mutations in *Serratia marcescens* and compare classwide mutation patterns with those found by Luria and Delbruck in their classical experiment showing that mutations are spontaneous rather than induced. Related topics such as bacterial growth, posi-

tive and negative controls and the difference between mutation rate and mutation frequency are also addressed. The first two weeks give students the necessary skills to prepare them for the second and third weeks in which the students design and implement their own experiments related to antibiotic resistance. Students will have semi-guided experiments, in which they can choose from several variables we provide them, but they have the freedom to form their hypotheses, come up with predictions, identify confounding factors, design an experiment and analyze their data. This laboratory unit could easily be restructured for use in upper level laboratory classes where groups could be allowed more freedom in their experimental design than is possible in a large introductory biology course. Technical skills learned in this lab module include pipetting, preparing serial dilutions, using a spectrophotometer and becoming familiar with sterile technique. Students also practice scientific communication skills by writing a scientific paper or presenting a poster on their group's experiment. A complete list of learning objectives is given in the student outline so that students are aware of learning objectives for the laboratory module. Students enjoy this module because it teaches them basic technical skills and provides a balance between initial structure followed by guided freedom in designing their own experiments.

Student Outline

Laboratory Synopsis

The goal of this module is to give you hands-on experience observing mutation and selection in bacteria, using antibiotics as a selective agent. In week one you will set up a class-wide experiment to observe naturally occurring streptomycin-resistance mutations in *Serratia*. You will also work with your lab group to design a follow-up experiment to further examine mutation and selection in *Serratia*. In week two you will collect data for the class wide experiment and set up your small group experiment; in week three you will obtain results from your small group experiment and consider how best to present your results.

Laboratory Objectives

After completing this laboratory module you should be able to:

Conceptual

1. Explain how natural selection works and analyze selective pressures and other variables (e.g. population size, mutation rate) that can influence the evolution of antibiotic resistance.
2. Describe Luria and Delbrück's experiment and explain why it suggested that mutations are spontaneous rather than induced.
3. Graphically represent the typical phases of bacterial growth and estimate the number of cells in an exponentially growing culture when given the number of starting cells and the number of generations.
4. Convert bacterial culture optical density (OD) measurements into estimated cell concentrations when given the necessary conversion equation.
5. Apply the concept of serial dilutions to calculate expected concentrations of cells in a given dilution and determine the dilution necessary to obtain a desired concentration of cells.
6. Determine appropriate controls for mutation and selection experiments with bacteria.
7. Describe how mutations affect the genotype and phenotype of bacteria.
8. Explain why observed mutant frequency among replicate cultures cannot be used to directly estimate the mutation rate for a given trait. Identify variables that could affect mutant frequency and/or mutation rate.
9. Evaluate experimental design, recognize experimental constraints, and interpret and compare experimental results for experiments on mutation and selection in bacteria.
10. Compare aminoglycoside and beta-lactam antibiotics, their modes of action and their relative effects on gram-positive and gram-negative bacteria.
11. Describe horizontal transfer of antibiotic resistance and contrast this resistance mechanism with the process of new mutation followed by selection for resistance.
12. Interpret graphs showing the response of a patient's bacterial population to an antibiotic treatment and generate plausible hypotheses explaining bacterial population responses.
13. Present experimental results in a scientific paper or poster.

Procedural

1. Pipet properly and determine and execute appropriate serial dilutions
2. Employ sterile technique when working with bacterial cultures.
3. Set up and use a spectrophotometer to measure optical density of a bacterial culture.

Questions to Prepare You for This Laboratory:

Week One

1. For a typical bacterial population, what are the four phases of growth?
2. If you have a starting population of 50 cells, how many cells will you have after 10 generations of exponential growth? After 15?

3. If you have a culture with 10^6 cells/ml, how could you use serial dilutions to obtain a solution with 10^3 cells/ml?
4. For Luria and Delbrück's experiment, what type of results would have supported the induced mutation model?
5. Where do streptomycin and penicillin originally come from?
6. How do aminoglycoside and beta-lactam antibiotics differ in their modes of action?

Week Two

1. Why does the Poisson distribution describe the distribution of the number of new mutation that occurs within each replicate culture, but **not** the distribution of mutants in the final generation?
2. In the equation $m = -\ln P_0$, m is the average number of mutational events over the time period/number of generations examined (i.e. the time the bacteria were growing in a replicate culture). What do you need to do to calculate a mutation rate once you know m ?
3. What is the difference between mutation rate and frequency of mutants?
4. If you look at 10 replicate *Serratia* cultures plated on antibiotic plates, how do you know whether it was induced or spontaneous mutation? Think about the frequency of mutants!
5. What is the relationship between sensitivity to an antibiotic and the diameter of the zone of inhibition in a disc diffusion experiment?
6. On a dose response curve, what causes the flat regions at low concentrations and high concentration?

Introduction

Evolution by Natural and Artificial Selection

Evolution by natural selection is a key concept in biology. Because one can easily grow bacteria in large numbers and for many generations over a relatively short period of time, we can observe evolution in action by experimentally selecting for naturally occurring mutations in lab. Specifically, we will use an antibiotic, streptomycin, as our agent of selection so that we can investigate the evolution of antibiotic resistance.

Evolution can be defined as change in allele frequencies within a population over time. When individuals with certain heritable traits survive and reproduce better than individuals with other heritable traits, a population evolves due to natural selection. In this lab module, we will consider bacterial populations of billions of individual cells and observe naturally occurring variations in a cell's ability to grow in the presence of an antibiotic. The antibiotic will provide a selective pressure that favors individuals with traits that allow them to survive in the presence of the antibiotic.

Strictly speaking, we will be observing artificial selection because we will artificially select for a trait that interests us, just as plant breeders can artificially select for heritable traits they wish to encourage within a population by only allowing plants with the desired traits to reproduce. It is an interesting question as to whether human use of antibiotics over the past 70 years, and the effects of this on worldwide bacterial populations, is a case of natural or artificial selection. If one considers it to be artificial selection, it is an unusual case in that it is the bacteria with the undesirable trait of antibiotic resistance that have increased in frequency.

Bacterial Growth

It is relatively easy to study mutation and selection within bacterial populations because bacteria grow fast! Note that when we refer to bacterial growth we mean growth of a population of bacteria, not of individual cells. Bacterial populations increase in size due to the reproduction of individual bacteria via binary fission. Since this process produces two bacteria from one 'parent' cell, a single bacterium (zero generation) produces two bacteria after one generation, which can then produce four bacteria in the second generation, which, in turn, can produce eight bacteria in the third generation.

The simple case of growth from a single initial parent cell is shown in Fig. 1. In the first generation produced, there are two cells called daughter cells (the parent cell effectively ceases to exist after the division since it split in two to make the two daughter cells). If a mutational event occurs in one of the two complementary DNA strands in the parent cell's DNA double helix, a daughter cell may differ from the parent cell in some way (indicated in figure by change in cell color, although a mutation could affect any trait). As the cells continue to grow and divide in the culture, the offspring of the original mutant cell will inherit the 'mutant' DNA form and may be called 'mutants' since they are indistinguishable from a cell with the same *de novo* mutation. New mutational events are rare, but once a mutation has occurred, inheritance of the mutant allele by the cell's offspring is not rare; in fact, it is the expected outcome.

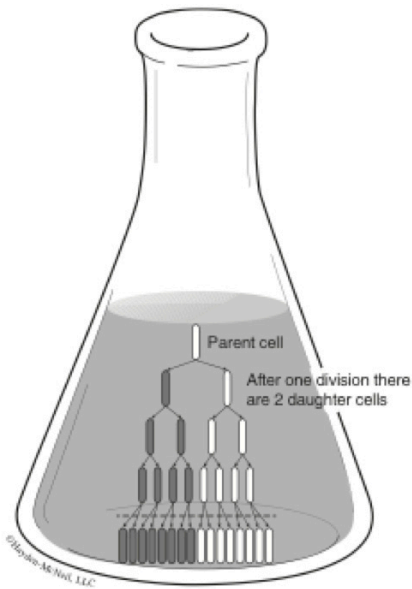


Figure 1. Growth of a bacterial culture starting from a single cell.

Bacterial growth is **exponential** since each cell splits in two to make twice as many cells in the next generation. Thus, after n generations, a single bacterial cell can produce 2^n 'offspring'. In cases where the starting population (N_0) contains more than a single cell, the potential final cell number or population size (N) after n generations of growth can be expressed mathematically as:

$$N = N_0 2^n \quad (\text{Equation 1})$$

Semi-logarithmic (or semi-log) plots are often used to show exponential growth, with time on the x-axis and the log of the number of viable cells on the y-axis. Use of a logarithmic scale on the y-axis allows one to show a large range of values (for example, if one uses \log_{10} , each graph interval will show one power of ten since $\log_{10} 10 = 1$ and $\log_{10} 100 = 2$). Pure exponential growth, when plotted thus, will give a straight line as shown in the exponential growth phase of Fig. 2. Of course, bacterial populations cannot grow exponentially forever; if they could, a single bacterial cell could produce a population of cells that would exceed the weight of the earth in less than a week.

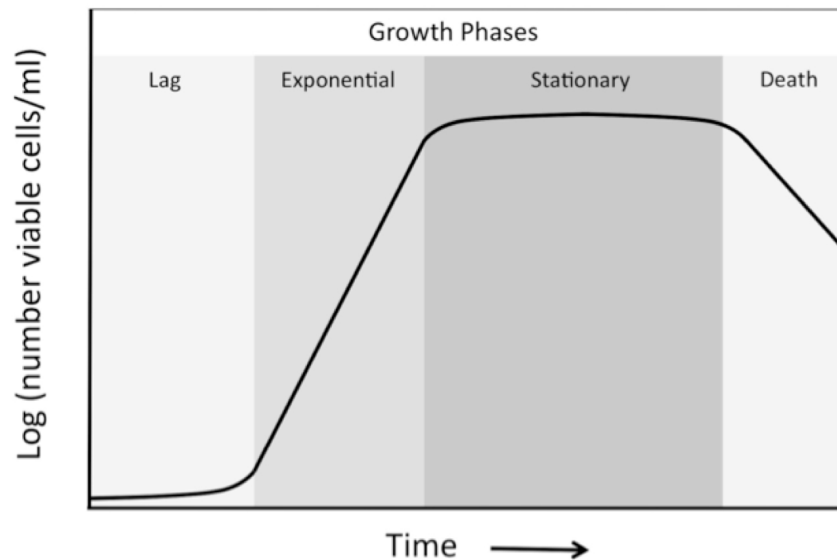


Figure 2. Growth of a bacterial population typically shows four phases. Note that the y-axis uses a logarithmic scale. On this type of semilog plot, exponential growth appears as a straight line.

An exponentially growing bacterial population will eventually run out of some necessary nutrient or secrete enough metabolic by-products and/or toxins to slow and then stop growth. During this **stationary phase**, the population size is stable, although some cells may still reproduce as others die. At some point, nutrient limitation and build-up of metabolic by-products and toxins cause the number of deaths to exceed the number of new cells produced and the population will enter the **death phase** (Todar, 2008). Bacteria also typically show a **lag phase** when a new culture is started during which time new enzymes are made or the cells otherwise adjust to the new medium (a **culture** is a population of bacteria grown in specially prepared nutrient medium – a solid or liquid substance used for growing microorganism). See Maloy (1994) for additional information on microbial genetics.

Luria and Delbrück's Evidence that Mutations Occur Spontaneously

Back in the 1930's, microbiologists knew that if a growing bacterial culture were exposed to an antibiotic or a **bacteriophage** (a virus that attacks bacteria), the cells would seem to all die; but if left for a while, bacterial growth would eventually re-appear (without any new bacteria being introduced). Some argued that the presence of the antibiotic or bacteriophage somehow caused or **induced** new mutations that conferred resistance to appear in a few surviving cells. Others argued that resistant mutations occurred **spontaneously** and that these resistant mutants were the only ones to survive the exposure to the antibiotic or bacteriophage. In either case, it took time for the few surviving resistant cells to start dividing and complete enough generations of growth to re-establish a visible resistant bacterial population (Johnson, 1995).

Salvador Luria and Max Delbrück (1943) came up with a clever way to test these two mutation models. They inoculated many small replicate cultures with only a few (50 to 500) *Escherichia coli* cells from a culture that was known to be sensitive to bacteriophage (i.e. the bacteriophage could kill it). They allowed these cultures to grow until the stationary phase was reached at a population size of 10^7 to 10^9 bacteria. Each of the replicate cultures was then assayed for resistant cells by plating the culture on agar plates covered with the bacteriophage. In the presence of the bacteriophage, only resistant bacteria were able to divide, producing visible colonies within a day or two. Each colony represented the presence of one initially resistant cell.

Luria and Delbrück determined the mean number of colonies produced by replicate cultures and compared this mean with the **variance** in the number of colonies produced (variance is a measure of the amount of variation around the mean). They predicted that there should be a relatively low variance in the number of resistant colonies observed from replicate cultures if mutations were induced by exposure to the bacteriophage. However, there should be a high variance in replicate culture colony numbers (variance \gg mean) if mutations occurred spontaneously during the population's growth in culture prior to exposure to the bacteriophage (Fig. 3).

In summary, according to the **induced mutation model**, one should expect a low rate of mutation, or conversion, to the resistant phenotype following exposure to the bacteriophage in the generation plated. There would, of course, be some slight variation around the mean due to random factors that might affect this process. However, if mutations occurred **spontaneously** during the culture growth **prior** to the bacteriophage exposure, there should be much more variation in the number of cells showing the mutant phenotype due to the relative amplification of a mutation. A mutation occurring early in the culture's exponential growth would have time to replicate and produce many more copies of resistant cells as compared to a mutation occurring late in the culture's growth.

In Luria and Delbrück's actual experiment, 20 replicate cultures were started from 50 - 500 founder cells and bacteria grew for at least 15 generations. Luria and Delbrück (1943) found that in every experiment the fluctuation of the numbers of resistant bacteria was tremendously higher than could be accounted for by the sampling errors. They concluded that resistance was due to spontaneous mutation that occurred throughout a culture's growth independently of any action of the virus. The results of this 'fluctuation test' became the first strong evidence for the existence of spontaneous mutation.

Figure 3 (opposite page). Predicted outcomes for replicate cultures under the Induced Mutation and Spontaneous Mutation Models. In this simplified depiction, each replicate culture is started from a single sensitive cell. Four rounds of cell division produce 16 cells in each replicate culture (only five replicate cultures are shown). Each replicate culture's final generation (cells below the dashed line) is plated and exposed to bacteriophage. Resistant cells are shown in dark gray. In the induced model, mutations only occur in the generation exposed to the bacteriophage. In the spontaneous mutation model, mutations can occur at any time as the culture grows. Under the Spontaneous Mutation model, resistant cells either 1) acquire their resistance via a new mutational event or 2) inherit a resistant allele from a resistant 'parent' cell. Through inheritance, a replicate culture that has a single mutation occur early during its growth will have many more cells expressing the mutant phenotype in the final generation than will a replicate culture in which a mutational event occurs late in the culture's growth.

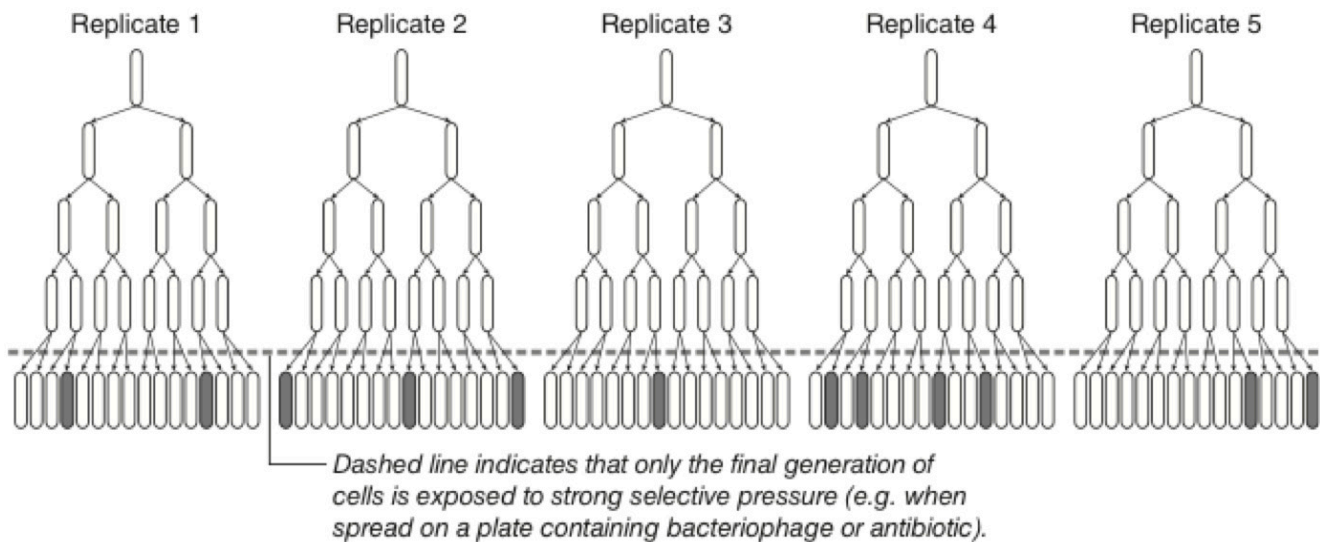
Mutations, Frequency of Mutants

All the information necessary to make and run a bacterial cell can be found in its DNA. Before a bacterium divides, it must replicate its DNA so that each daughter cell will have a complete copy of this information. In the absence of any mutagen or damaging radiation, most mutations (changes from the parental genome) are due to errors in the replication process. Unfortunately, it is difficult to directly measure this mutation rate.

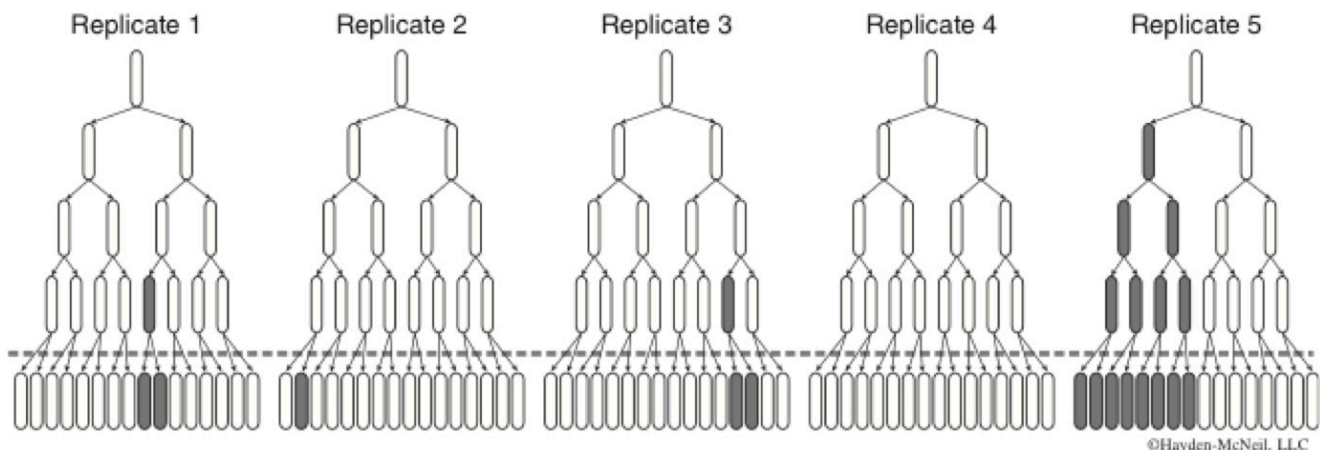
The **frequency** of resistant cells at the end of many generations of growth does **not** directly measure the mutation rate for that trait because many of the resistant mutants will have inherited their mutation rather than directly experiencing a mutational event (Rosche and Foster, 2000). For example, in Figure 3's replicate 5 example, a single mutation in the first generation (one mutation in 15 divisions) leads to an observed mutant/bacteriophage resistant cell frequency of 50% in the fourth generation since eight of the 16 cells have inherited the resistant allele from the original mutant. In this lab module, we will perform a variation of Luria and Delbrück's experiment to observe the frequency of resistant mutants for mutations that confer resistance to the antibiotic streptomycin. More information on frequency of mutants versus mutation rate can be found in the section **Using Luria-Delbrück's Fluctuation Test to Estimate Mutation Rate**.

Choose one of the spontaneous mutation model replicates. What is the frequency of resistant 'mutants' in the final generation? How many mutational events occurred?

Induced Mutation Model



Spontaneous Mutation Model



Antibiotics

This term originally referred solely to substances produced by one microorganism that inhibited the growth of other microorganisms. However, many antibiotics are now produced synthetically, including man-made variations. Different types of antibiotics act by different mechanisms against different groups of bacteria to inhibit growth.

Aminoglycoside Antibiotics

Streptomycin is an aminoglycoside antibiotic that was first identified from soil bacteria (Kingston, 2004). These soil bacteria secrete streptomycin, in part, to try to inhibit the growth of competing bacteria. Streptomycin was widely used to treat tuberculosis, but is rarely used today due to its fairly severe side effects. As the name, aminoglycoside, suggests, this class of antibiotics comprises molecules that contain amino groups bonded to sugars via glycosidic bonds (see Fig. 4, left side). Kanamycin and neomycin also belong to this group of antibiotics.

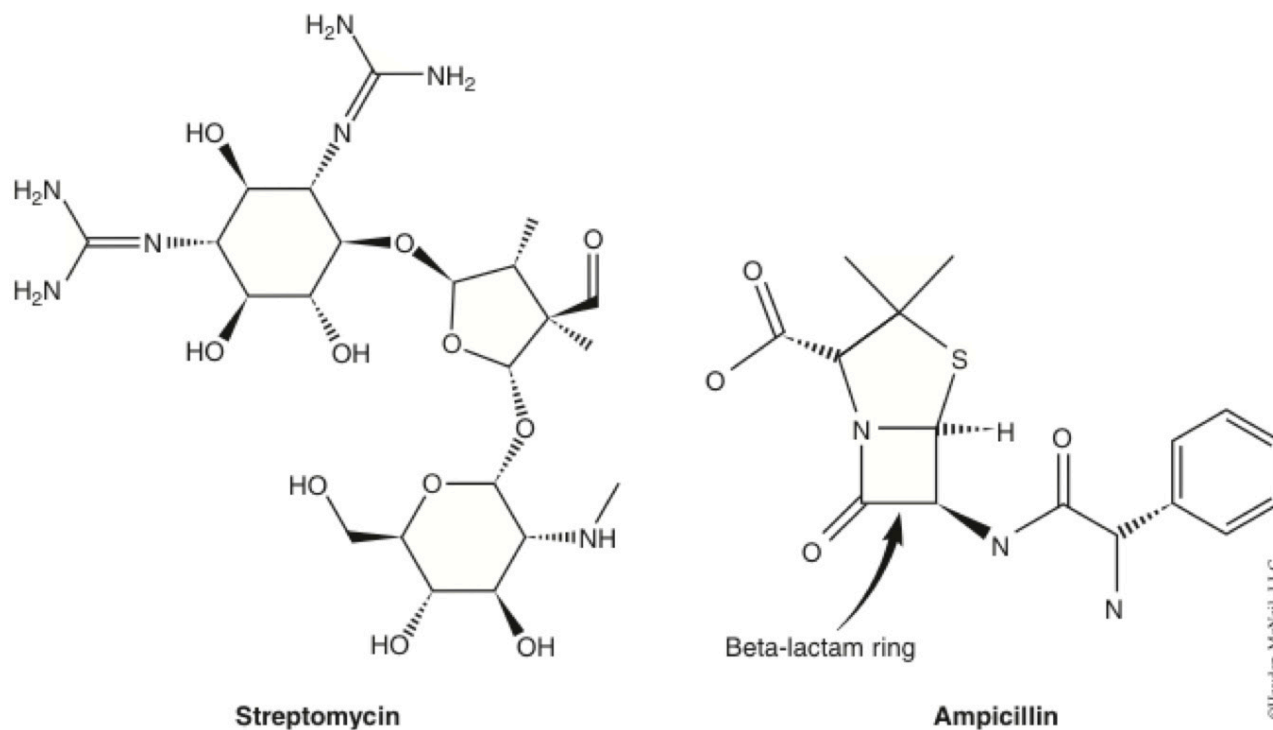


Figure 4. Streptomycin (left) and Ampicillin (right). Streptomycin is an aminoglycoside antibiotic whereas ampicillin is a beta-lactam antibiotic.

Streptomycin inhibits bacterial growth because it inhibits **translation**. Recall that while the DNA carries the information, this information must be first **transcribed** into RNA before it can be used (see an introductory biology textbook such as Sadava et al., 2011 for additional information on translation and transcription). For protein coding genes, a ribosome then uses messenger RNA (mRNA) to direct protein synthesis in a process called **translation** (Fig. 5). Specifically, streptomycin binds to the 30S ribosomal unit and prevents it from attaching to the 50S ribosomal unit during the initiation of translation (Mayer, 2010). One common mutation that confers streptomycin resistance is a single base pair change that causes a single amino acid change in one of the 30S ribosomal proteins that prevents streptomycin binding (Paulander et al., 2009). If streptomycin cannot bind to the ribosomal subunit, it cannot affect translation and the cell will be 'resistant' to streptomycin (able to grow in the presence of streptomycin if the change in the ribosome does not seriously damage ribosome function). For information on antibiotic resistance and its impact on human health, see Rosenblatt-Farrell (2009), Stearns and Ebert (2001) and many other sources.

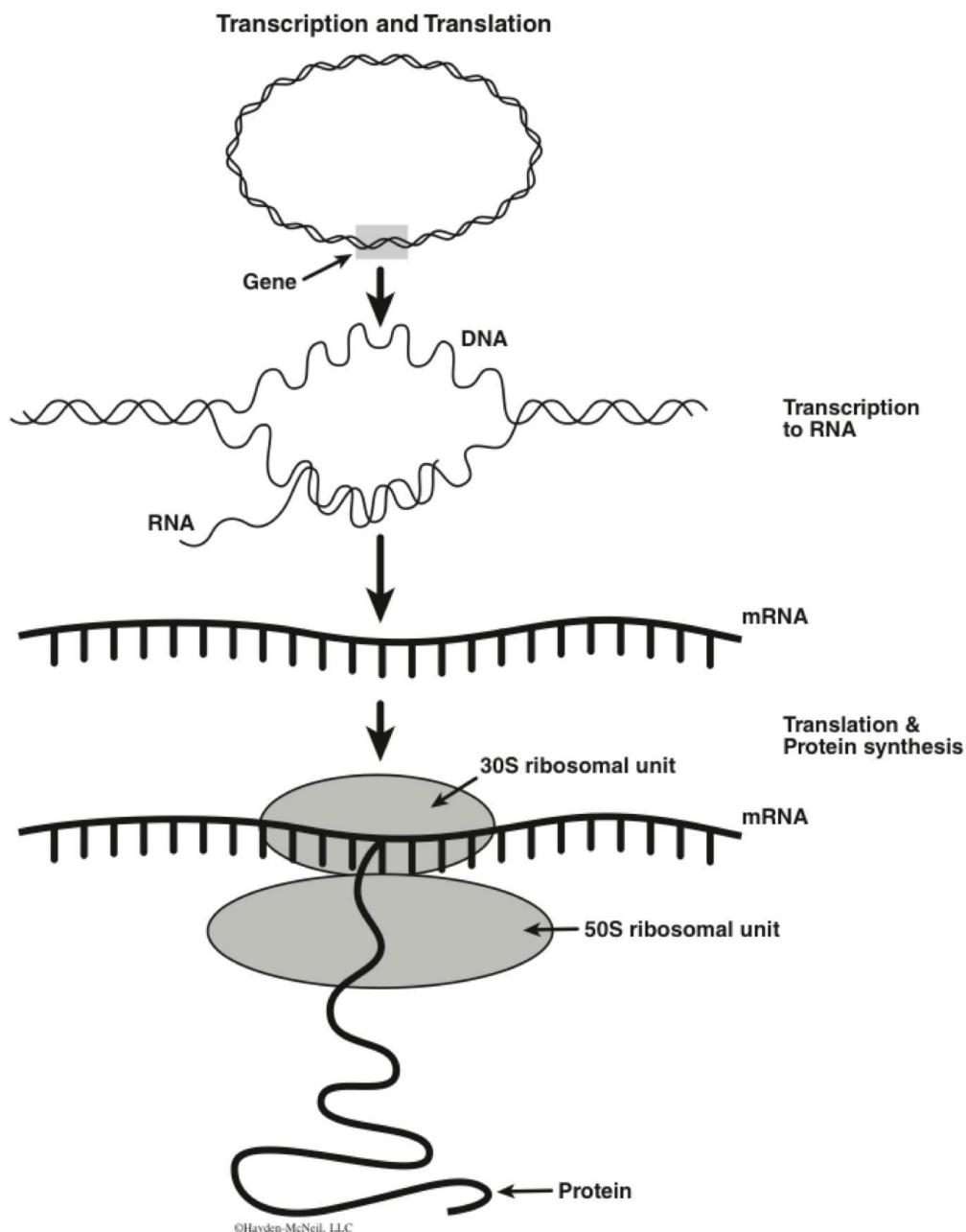


Figure 5. The “Central Dogma” of molecular biology. Information is coded in DNA, which is replicated prior to cell division so that each daughter cell receives a complete copy. Messenger RNA is made during a process called transcription, and is then used by the ribosome to synthesize proteins in a process called translation. Ribosomal RNA (rRNA) is also coded for by DNA but does not undergo translation. Instead, the rRNA directly combines with ribosomal proteins to form a ribosomal subunit.

Beta-lactam Antibiotics

Another important group of antibiotics with a completely different mechanism are the beta-lactam antibiotics. Penicillin is a beta-lactam antibiotic that was isolated from fungi, and ampicillin is a synthetic molecule similar to penicillin. These antibiotics are named for the beta-lactam ring in their structure (Fig. 4, right). Beta-lactam antibiotics affect a bacterial enzyme, transpeptidase, which is essential to bacterial cell wall synthesis.

Gram-positive bacteria have a thick layer of peptidoglycan in their outer wall. Gram-negative bacteria have a thinner peptidoglycan layer surrounded by an outer membrane that contains many lipopolysaccharides (Madigan et al., 2009). Bacteria are designated as gram-positive or gram-negative due to their different appearance following a gram stain (gram-positive bacteria

appear darker). *Serratia* has gram-negative type cell walls. Differences in the cell wall structure of these different types of bacteria can affect sensitivity and resistance to some types of antibiotics. For instance, beta-lactam antibiotics tend to be more effective against gram-positive bacteria. Sensitivity or resistance to this class of antibiotics is affected by cell wall structure (Fig. 6) and by the presence or absence of beta-lactamase, an enzyme that breaks the beta-lactam ring of the antibiotic, thus destroying its efficacy (Lewis et al., 2002). Some of you may use the beta-lactam antibiotic ampicillin in your group experiment.

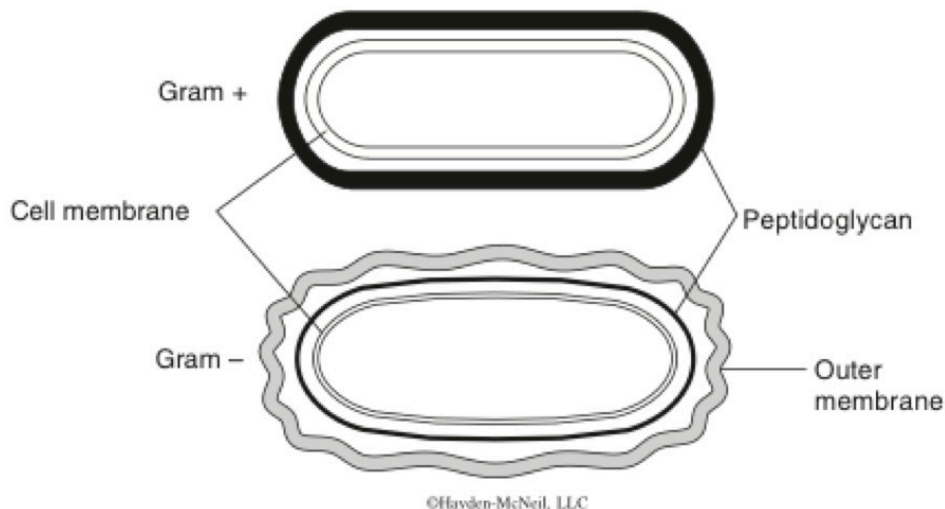


Figure 6. Gram-positive (top) and Gram-negative (bottom) bacterial walls.

Our Model Organism - Serratia marcescens

Serratia marcescens is a species of gram-negative bacteria commonly found in soil and water and sometimes present in the human gut. Most strains can produce a bright red pigment called prodigiosin. We will use *Serratia* for our experiment because it grows easily in the lab, produces several visually distinguishable color mutants (Morrison 1966; Mody et al., 1990), and is normally non-pathogenic. In the 1950's, the U.S. army thought *Serratia* posed no health threat at all and so used it in a bioterrorism test (Tansey, 2004). Unfortunately, it turns out that *Serratia* can cause infections if it is introduced internally (i.e. during surgery or by injection), especially in immune-compromised individuals (Kurz et al. 2003). For this reason, we will take *Serratia* seriously and practice sterile technique and preventative hand washing.

A Technical Note – Serial Dilutions

Bacteria grow so fast that estimating their numbers and getting a population or sample size within a certain range is difficult. For example, if you have a culture with 10^8 cells per ml, you would have 10^7 cells in 0.1 ml (equal to 100 μ l) and 10^6 cells in 10 μ l. Thus, to sample about 100 cells, you would have to take only 0.001 μ l from the original culture. Unfortunately, you cannot even buy a micropipettor that allows you to transfer this small a volume. Thus, you must dilute the culture to get fewer cells per ml before sampling. Culture samples are commonly diluted using a technique called serial dilutions, a technique also used to dilute other mixtures.

You are familiar with the concept of dilution if you have ever bought concentrated orange juice or lemonade, since you mix, or dilute, the concentrate with water to make the juice before drinking it. A serial dilution just repeats this process by further diluting the first dilution. You would be doing a serial dilution if you took your orange juice made up from the concentrate and water and then diluted it further by adding just a teaspoon of the juice to a cup of water. In a 1:10 dilution, one part of your starting mixture or sample is added to 9 parts of your liquid solvent to make a total volume of 10 parts. The resulting 1:10 dilution has one-tenth the concentration of your starting mixture or sample. If you need to dilute by a lot, you could add one part (e.g. 1 ml) of your 1:10 dilution to a volume of 9 parts (e.g. 9 ml) of fluid to make a 1:10 dilution of your 1:10 dilution. Since this dilution is one-tenth the concentration of the 1:10 dilution, it is a 1:100 dilution of your original starting mixture and is 1/100th as concentrated as your starting sample. You could continue this process further to get even more dilute mixtures or samples (Fig. 7).

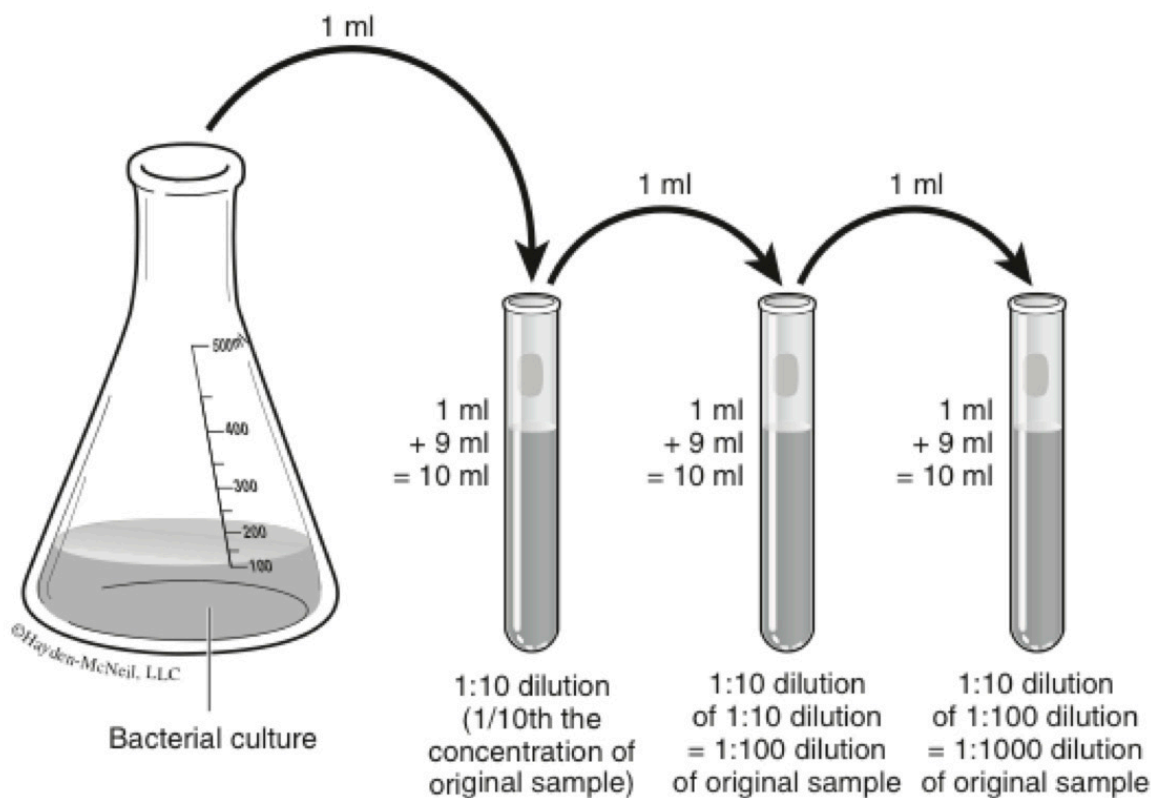


Figure 7. Serial Dilution of an initial sample or culture to obtain solutions that are 1/10th, 1/100th and 1/1000th the concentration of the initial sample (1:10, 1:100 and 1:1000 dilutions respectively). For each dilution, tubes initially have 9 ml of liquid in them so that the final volume will be 10 ml after the addition of 1 ml of culture or diluted culture.

Test Yourself

- ? If you want to make a 1:10 dilution in a tube that holds a maximum volume of 5 ml, how much sample should you transfer into how much liquid? (Hint: remember that you always want the amount of sample added to be one tenth of the final volume for a 1:10 dilution.)
- ? If you want to make a 1:100 dilution in a single step (instead of using two serial 1:10 dilutions), how could you do this? (Hint: here you want the amount of sample added to be 1/100th of the final volume.) *Interestingly, doing a 1:100 dilution in a single step tends to be less accurate than doing two serial 1:10 dilutions.*

Preparation for Week One's Experiment

The Parent Culture: Testing for Streptomycin Sensitivity and Viability

Serratia marcescens, strain D1 was purchased from Carolina Biological Supply in 2009 and maintained in phosphate buffered saline at 3 degrees Celsius. Cells were plated on no-antibiotic agar plates and a single red-pigmented *Serratia* colony (a collection of genetically identical cells) was added to nutrient broth and shaken overnight at 30°C.

- (a) Samples of this culture were plated onto agar plates with nutrients to test whether the cells are viable (do they grow?). If you do not test for viability and the cells do not grow after you expose them to antibiotics (treatment plate), you will not know whether the lack of colonies is the result of the antibiotic or the cells were already dead before you plated them.
- (b) Another sample will be plated onto plates containing streptomycin to test if the parent culture was sensitive to streptomycin. If the parent culture is sensitive (as hoped) to streptomycin, we expect no growth on these plates, but if the parent culture is already resistant to streptomycin, you will not be able to use that culture for your experiments.

The Parent Culture: Estimating Cell Density

Cells suspended in liquid scatter and absorb light. The more cells, the lower the percentage of transmitted light and the higher the measured absorbance, or **optical density (OD)**. For *Serratia*, optical density measured at 620 nm (OD_{620}) varies directly with cell concentration for values between 0.06 and 0.99 such that 1.0 OD unit $\approx 5.80 \times 10^8$ cells per ml (Haddix et al., 2000). The parent culture's cell density was estimated using a spectrophotometer to measure the OD of a 1:10 dilution. This cell density estimate was then used to determine the dilution necessary to have only 50 to 500 cells in 0.1 ml. This dilution was then used to inoculate the nutrient broth to start ten replicate cultures (Fig. 8). You will be given the OD of the parent culture and asked to calculate the concentration of cells in 1:10 and other dilutions of this parent culture as part of your Mutation and Selection Worksheet.

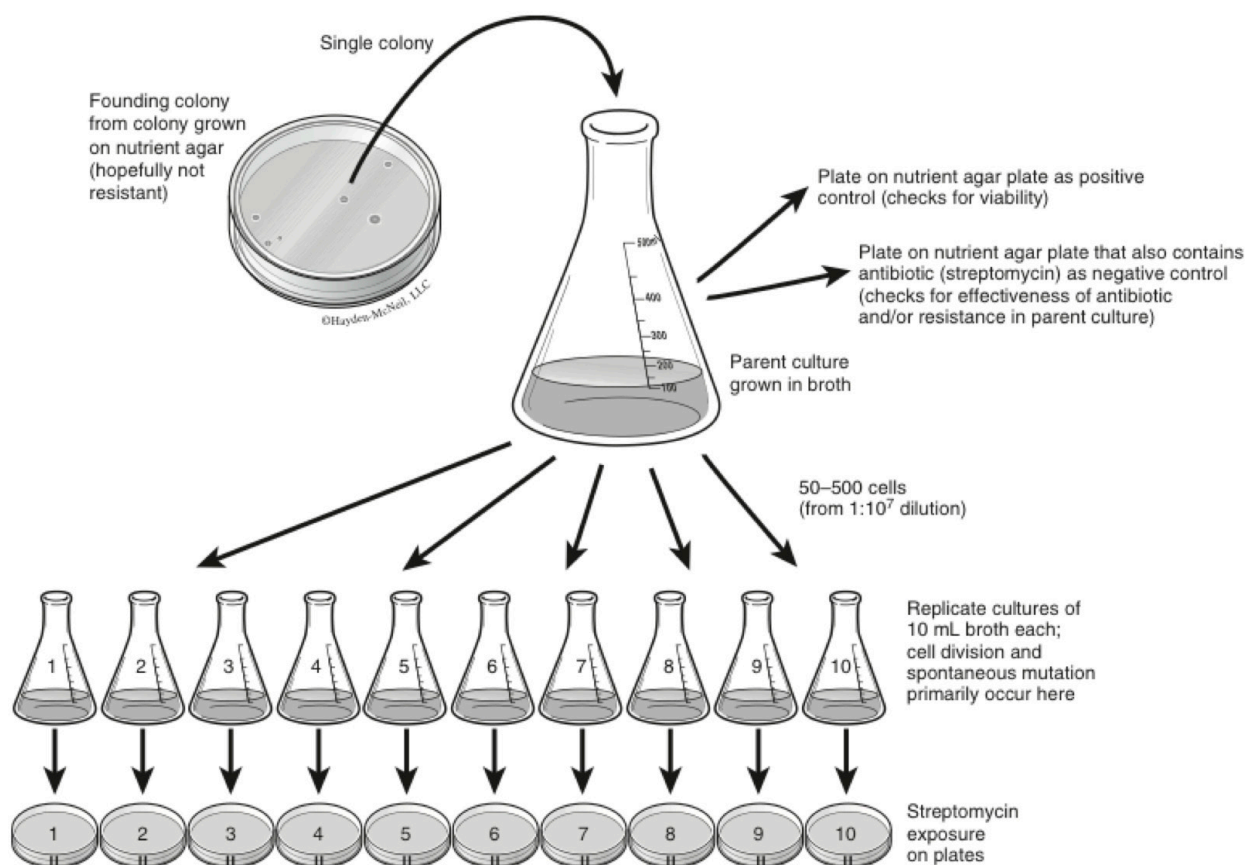


Figure 8. Preparation for week one's class wide experiment started with growth of a parent culture inoculated with a single *Serratia* colony. The cell density of the parent culture was estimated by optical density and a culture sample was diluted sufficiently to permit the transfer of only 50 - 500 cells into each of ten replicate cultures. These bacteria went through many generations of growth before replicate cultures were diluted 1:10 (not shown) in preparation for plating. Cells are grown in nutrient broth and are not exposed to streptomycin until they are plated on streptomycin-containing plates by you in lab (the presence of streptomycin in the plate is indicated by the marking on the side of the plates).

Replicate Cultures

Following inoculation, the ten replicate cultures were grown in a shaking water bath at 30°C. After about 22 hours, OD_{620} was measured for a few of these cultures (so we can estimate final cell concentration). A sample of each replicate culture was plated on streptomycin plates and the rest of each replicate culture was diluted 1:10 in cold phosphate buffered saline (PBS) to halt growth while preserving live cells. You will be plating the 1:10 dilutions of the replicate cultures in your week one lab. Note that by knowing the approximate number of cells that started each culture and the final cell concentration, we can use our understanding of bacterial growth to estimate the number of cell divisions if the cultures are assayed before reaching stationary phase.

Using Luria-Delbrück's Fluctuation Test to Estimate Mutation Rate

You learned earlier that the observed frequency of resistant mutants is not the same as the mutation rate (the probability of a new mutation occurring per cell division). You can relatively easily calculate the frequency of mutants – just divide the number of mutants detected by the total estimated number of cells plated. However, this frequency includes the offspring of mutant cells from earlier generations as well as cells with de novo mutations in the generation plated. Luria and Delbrück (1943) devised a clever way to estimate the mutation rate despite the difficulty of distinguishing number of mutants from number of mutations. They knew that the occurrence of mutation events should follow the Poisson distribution (Fig. 9). The Poisson distribution describes the probability of a certain number of events occurring for a defined sample of time or space and is particularly useful for describing the probability of very rare events (Rosner, 1995). Although this distribution does not describe the distribution of mutants in the final generation, it does describe the distribution of the number of new mutations (a very rare event) that occur over the time period/number of generations represented by each replicate culture (our sample). For this scenario, we can use the following mathematical description of the Poisson distribution:

$$P_x = e^{-m}m^x/x! \quad (\text{Equation 2})$$

P = the probability of a mutation occurring exactly x times in a sample (a replicate culture)

e = the base of the natural logarithm (\ln) and is approximately equal to 2.71828

m = the average number of mutational events during growth of a replicate culture

$!$ = the symbol for factorial, the product of the specified number and each positive integer less than itself down to and including 1 (so that $5! = 5*4*3*2*1$). By definition $0!$ is equal to 1.

We, like Luria and Delbrück (1943), want to find m , but it is impossible for us to tell exactly which replicate cultures had one, two, or three mutational events since the end number of mutants includes mutants that inherited their mutation from an earlier generation. However, it is possible to determine how many replicate cultures have zero mutational events if we plate out all (or at least most of) each replicate. These replicate cultures will have no mutants on any of the plates. By noting the proportion of replicate cultures with no mutants (our estimate of P_0) we can solve this equation for the case where $x = 0$. Fortunately for us, this greatly simplifies the math. Since $m^0 = 1$ and $0! = 1$, $P_0 = e^{-m}m^0/0!$ simplifies to:

$$P_x = e^{-m} \quad (\text{Equation 3})$$

To solve for m , we just take the natural log of each side: $\ln(P_0) = \ln(e^{-m}) = -m$, or

$$m = -\ln P_0 \quad (\text{Equation 4})$$

This last equation (Equation 4) is the version you need to know and use for this course, although you should understand from the previous discussion that it is derived from the Poisson distribution.

Once we have used our observed proportion of replicate cultures with zero mutants to estimate m (the average number of mutational events per replicate culture), we can estimate the **mutation rate per cell division** merely by dividing m by the estimated number of cell divisions. Conveniently, the number of cells in the replicate cultures following exponential growth provides a good estimate of the number of cell divisions that occurred during the growth of the culture. While you will estimate the mutant frequency from your own lab section's data, we will use course-wide data to estimate both m and the mutation rate per division.

Note that we will only be able to use this method to estimate the average number of mutational events per replicate culture if we have at least one replicate culture without any observed mutants (resistant cells) across all class sections. If all replicate cultures have at least some observed resistant cells, we cannot estimate P_0 . (There actually is a way to still make an estimate, but the mathematics are much more complicated and are beyond the scope of our course - for additional information see Pope et al., 2008)

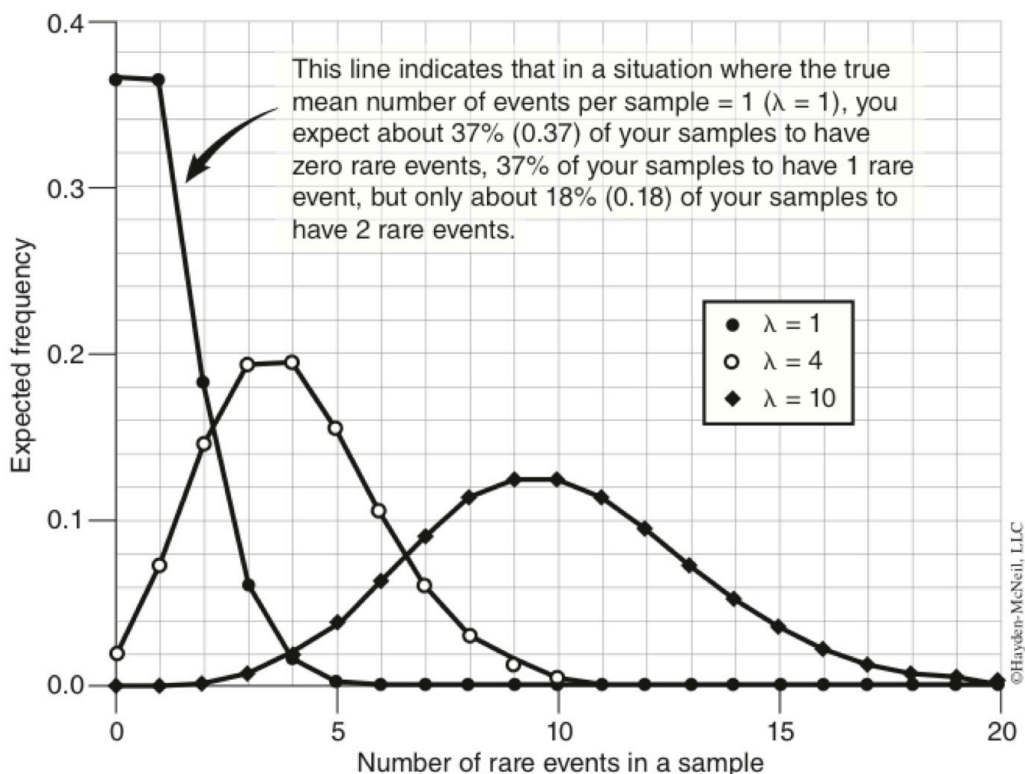


Figure 9. The Poisson distribution is shown for cases in which the true mean number of events per sample (λ) is 1, 4, or 10. When the mean number of events per sample is large (e.g. $\lambda = 10$), the curve is similar to the normal distribution. The rare event of interest in our case is the new mutation; the Poisson distribution describes the expected distribution for the number of spontaneous mutational events for a given trait across replicate cultures (i.e. one replicate culture might have one mutational event whereas another replicate culture might have two mutational events or no mutational events). In contrast, the distribution of resistant cells in the final generation of a culture should have significantly more variation than shown in the distribution above since, according to the spontaneous mutation model, many colony-forming cells inherit their resistance (not a rare event) rather than experiencing a new mutation themselves.

Procedures - Week One

NOTE: Throughout this lab, minimize both your exposure to Serratia and the possibility of contamination by (1) washing your hands before and after the activity, (2) cleaning your work area with alcohol before and after working with Serratia and (3) keeping all bacterial samples covered as much as possible. Gloves are available if you wish to use them.

Work in groups of two or three.

Estimating Cell Concentration Using Optical Density

1. Label one cuvette “B” for blank, label another “1:10” for your first dilution and the next “1:100” for your second dilution. Use the 5 ml volumetric pipette to dispense 4 ml of broth into the “B” tube and 3.6 ml of broth into the two dilution tubes.
2. Set your micropipettor to 200 μ l and pipette 400 μ l (2 x 200 μ l) of the sample *Serratia* culture into your “1:10” cuvette containing 3.6 ml of nutrient broth. This will bring the total volume up to 4.0 ml and constitutes a 1:10 dilution of the culture (since 0.4 ml is one tenth of the final volume of 4.0 ml). Cover the top with Parafilm® and invert to mix. The *Serratia* used here is just an example culture since the ten replicate cultures were diluted 1:10 after about 22 hours of growth (before reaching stationary phase).

3. Follow the directions describing how to use the Spectrophotometer to measure the OD_{625} of your “1:10” dilution. Note: the prep staff was able to measure OD_{620} , but some lab room spectrophotometers have bulbs that only transmit light down to 625 nm, so you will use this wavelength for your estimates (results for these two wavelengths are very similar). Use plain nutrient broth as your blank solution.

$$\%T = \text{Absorbance (OD)} =$$

4. Now put a new tip on your micropipettor and transfer 400 μl of your 1:10 dilution into the 3.6 ml of broth in your tube labeled “1:100”. Measure the OD of this dilution, again using plain nutrient broth as your blank solution.

$$\%T = \text{Absorbance (OD)} =$$

5. Use the information given below to estimate the cell density of your 1:10 and 1:100 dilutions.

$$\frac{1.0 \text{ OD}}{5.80 \times 10^8 \text{ cells per ml}} = \frac{\text{our OD reading}}{\text{your cells per ml}}$$

$$\text{so your cells per ml} = \text{your OD reading} * \frac{(5.80 \times 10^8 \text{ cells per ml})}{1.0 \text{ OD}} \quad (\text{Equation 5})$$

- ? Estimated cell density (cells/ml) of 1:10 dilution _____
- ? Of 1:100 dilution _____
- ? How many cells per ml were present in the original culture you used for your dilution?
- ? Class average of cell density for the original culture _____

You should now understand how optical density measurements are used to estimate cell concentrations and are ready to move on to our experiment. You will use OD_{620} data collected by the course staff on the parent culture and on the ten replicate cultures to estimate the final cell concentration and cell number in each of these cultures as part of your Mutation and Selection Worksheet. In our experiment we dilute 1:10 before measuring OD because our undiluted cultures have too many cells to accurately measure using this method (almost all light scattered or absorbed by undiluted culture).

Testing the Parent Cultures Before the Experimental Set-Up

1. Observe the parent culture sample's growth on the agar plates (no antibiotics) and on the streptomycin plates. The agar plates were plated with the same number of cells used to start a single replicate culture (50 – 500 cells). The streptomycin plates were plated with 100 X the number of cells used to start a single culture.
 - ? Was the parent culture viable? How do you know?
 - ? Was the parent culture sensitive to streptomycin? How do you know?

Plating Serratia Using Sterile Technique

1. Obtain your plates and label them with your group initials and the replicate number (1 - 10). Most groups will be plating just on streptomycin plates (green stripe), but each section will have at least one group that also plates a sample of each replicate culture on an agar plate for culture viability.
2. Clear your work area and wipe it with a paper towel dampened with alcohol.
3. Check that your micropipettor is still set to deliver 200 μl . You will be plating 200 μl of the 1:10 dilution of each replicate culture on the plate numbered for that replicate. When plating, be sure to change pipet tips before you take a sample from a new replicate culture dilution.

An assembly-line approach should be used to set up the plates, with different group members responsible for adding the cell suspensions and spreading the suspensions on the agar surface. Note: as cell suspension is added to each plate it must be spread immediately so teamwork is essential. Sterile technique is required in setting up these plates.

In the following procedure, there is a real danger of fire. To minimize this danger arrange your equipment on the work area so that the alcohol dish is well removed from the alcohol burner and flaming spreading rod, as shown in Fig. 10. Be sure to immediately replace the lid on the alcohol dish each time it is used.

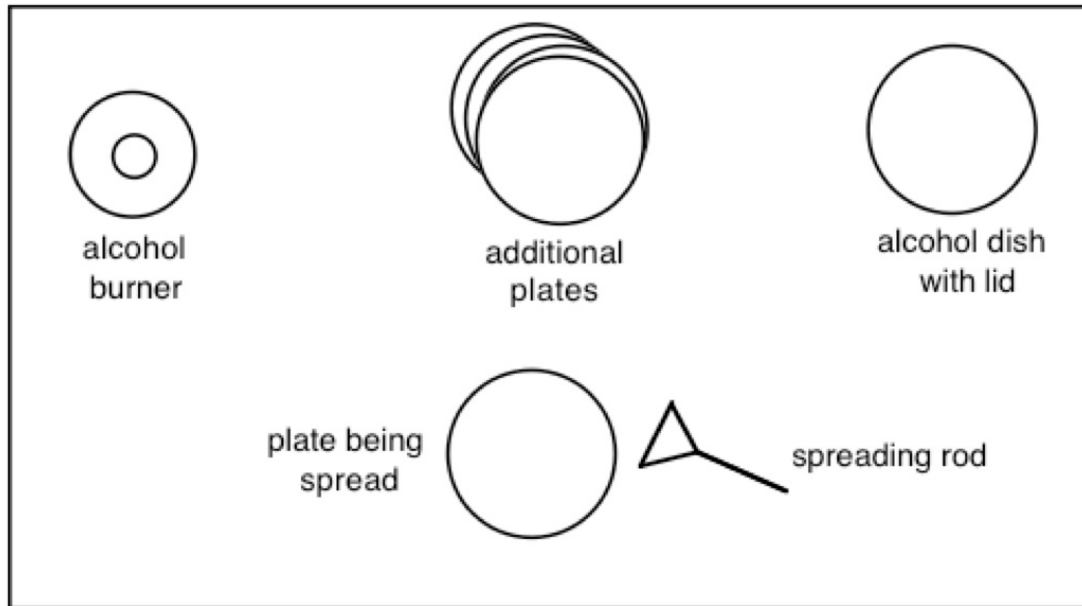


Figure 10. Suggested spatial arrangement of major materials used for plate spreading.

1. One person will dispense 200 μl of the replicate culture dilution into the middle of a plate and the second person will immediately spread the liquid as follows.
2. Dip the spreading rod into a petri dish with alcohol. Note: **immediately** replace the lid on the petri dish containing the alcohol.
3. Pass the rod through the flame of your alcohol lamp to ignite the alcohol.
4. Remove the rod from the alcohol flame and allow the alcohol to burn off. Note: hold the spreader at a downward angle to prevent flaming alcohol from running on to your hand.
5. Lift the lid of a petri dish only enough to allow insertion of the spreading rod. Cool the rod by touching it to the inside lid of the plate.
6. When the rod is cool, spread the cells by moving the spreading rod back and forth across the agar surface several times.
7. Rotate the plate 1/4 turn and repeat the spreading motion. Rotate the plate 1/4 turn again and spread once more so that the *Serratia* are spread over the entire plate.
8. Repeat this transferring and spreading procedure for each replicate culture, being sure to insert a clean tip each time until all replicate culture dilutions have been plated.
9. When you are done, cap your burner and tape your group's plates in stacks of five, labeling each stack with your lab day and time. Place all of your group's plates in the porcelain pan or tray labeled for your lab section.
10. Put away all supplies, clean your workspace with alcohol and wash your hands.

Designing your Group Experiment for Next Week:

When you are designing your experiment, your control should be different from the treatment by only one variable. Below you will find 5 different experimental groups that focus on 5 different variables. You have a wide variety of experiments you can design within each group:

1. Determination of the minimum inhibitory concentration (MIC)

The variable in this experiment is antibiotic concentration. The **minimum inhibitory concentration** is the lowest concentration of antibiotic that will be effective against a bacterium. By varying the concentration of the antibiotic a dose response curve can be constructed to determine the MIC. A standard dose response curve is shown in Fig. 11. The minimum inhibitory concentration would be the lowest concentration that results in inhibition of bacterial growth and can be found by locating the linear range on a dose response curve. For the Streptomycin dose curve use a Streptomycin resistant colony from the class project, for Kanamycin and Ampicillin dose curves use the parental culture of the red strain. Determine the dose response curve and test whether there is a correlation between *Serratia* susceptibility and the concentration of the antibiotic. You can use the disc diffusion method for this experiment (see below).

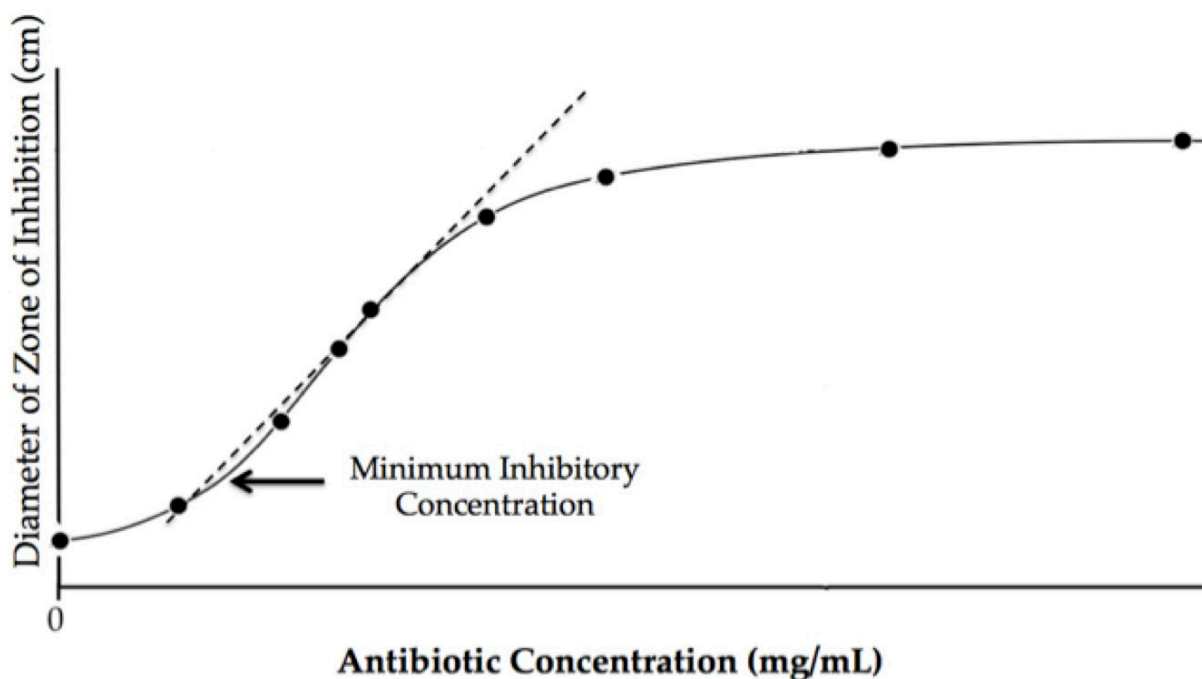


Figure 11. A typical dose response curve. Note the threshold region at low concentrations indicating a certain level of tolerance that must be exceeded before inhibition of growth occurs. Saturation effects are also noticeable on a dose response curve as greater concentrations fail to produce greater effects.

2. Comparison of low-pigmented (WF) and red *Serratia* strains

The variable in this experiment is the *Serratia* strain. Different colored strain of *Serratia* differ in their ability to produce the prodigiosin pigment. In this experiment you will be able to test whether production of this pigment is related to antibiotic resistance by comparing the resistance of the two strains on the same concentration of the same antibiotic. Plate the red strain and the WF strain of *Serratia* on 6.25 $\mu\text{g/ml}$ Streptomycin or 300 $\mu\text{g/ml}$ Ampicillin, or use the disc diffusion method with a constant concentration of streptomycin, kanamycin or ampicillin.

3. Comparison of resistance levels of different colonies

The variable in this experiment is the Streptomycin resistant colony. In this experiment you will test whether different colony types show different patterns of resistance to antibiotics. Use the disc diffusion or the colony count method to compare two Streptomycin resistant colonies from the class project (from the same or different replicate cultures) on a constant concentration of Streptomycin.

4. Cross-resistance of a Streptomycin resistant cell

The variable in this experiment is Streptomycin resistance. To test whether a selected Streptomycin resistant *Serratia* colony is more resistant than the original replicate culture to Ampicillin, use either the disc diffusion or the colony count method. For Kanamycin use the disc diffusion method only.

5. Antibiotic comparison

The variable in this experiment is the antibiotic type. Use the disc diffusion method to compare *Serratia* susceptibility to the same concentration of different antibiotics. You can compare Streptomycin with Kanamycin, Streptomycin with Ampicillin or Ampicillin with Kanamycin, as long as all antibiotics are diluted to the same concentration.

Collecting Background Information

After deciding on a general topic in consultation with your lab instructor, your group should look at the folders of papers on antibiotic resistance. You do not need to understand every detail of these articles, but each article should have at least one potentially relevant piece of information. You should also search for additional papers by accessing library databases.

Methods and Materials

After brainstorming for a few minutes, settle on one hypothesis and check it with your lab instructor. After your lab instructor verbally agrees with your design, submit your detailed methods and materials so your lab instructor can give this information to the course preparatory staff. You will receive all the materials needed for your study next week. You have a great level of freedom designing your experiment. You may use a red *Serratia* strain or a low-pigmented (WF) *Serratia* strain. You can also use colonies that survived exposure to Streptomycin from the class wide project. You may choose Streptomycin, Kanamycin or Ampicillin as your antibiotic. All groups will have plating supplies and a maximum of 20 plates per group.

Your experiment will use either the disc diffusion or the colony count methods.

Disc Diffusion

When a filter paper disc saturated with an antibiotic is placed on a plain agar plate the antibiotic will diffuse from the disc into the agar as shown in Fig. 12. This diffusion will result in a chemical gradient around the disc with higher concentrations occurring closer to the disc and decreasing with distance. If an organism is plated on the agar it will not grow in the area around the disc if it is susceptible to the chemical. This area of no growth around the disc is known as a **zone of inhibition**. The size of the zone of inhibition is related the degree of susceptibility to the antibiotic as more resistant bacteria will be able to tolerate higher concentrations of antibiotic.

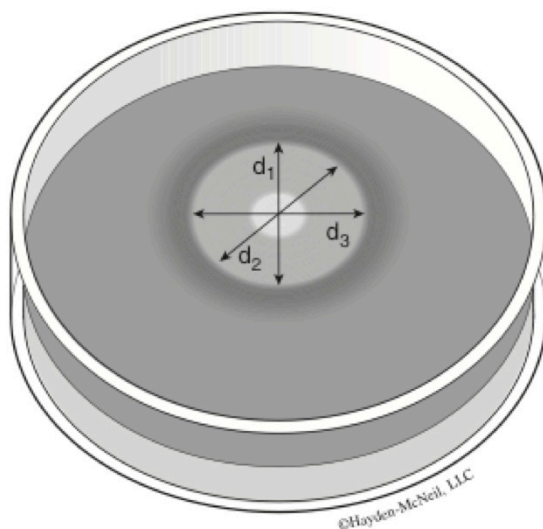


Figure 12. Example results from a disc diffusion experiment, showing a clear zone of inhibition. The zone of inhibition is often irregularly shaped and d_1 , d_2 and d_3 are not necessarily equal. To correct for any asymmetry, the diameter of the zone of inhibition should be measured at multiple locations.

Colony Counting

In this method, antibiotics are incorporated into the agar itself, creating a consistent antibiotic concentration across the entire plate. Spreading *Serratia* on these plates will select for the cells resistant to the antibiotic at that concentration. Much like the class experiment, each resistant cell that survives will grow into a single colony. This method describes the incidence of antibiotic resistance in the *Serratia* culture.

Mutation and Selection Experiment Plans

1. Start your background search by looking at the primary literature articles in the appropriate folder.
2. Start your biological investigation by establishing your Research Hypothesis:
3. What prediction(s) of your research hypothesis will you test?
4. Experiment Design/Methods: Treatment group? Control group? Variable? Replicates?
5. Other (statistical test you plan to use, sources of error, etc.)?
6. Obtain approval of your lab instructor.

Procedures - Week Two

Class Data Collection

NOTE: Once again, minimize both your exposure to Serratia and the possibility of contamination by (1) washing your hands before and after the activity, (2) cleaning your work area with alcohol before and after working with Serratia and (3) keeping all bacterial samples covered as much as possible. Gloves are available if you wish to use them.

1. Observe your plates from last week and count all colonies, entering data in Table 1. Also note bacterial growth on your section's control plates.

Table 1. Number of colonies observed on streptomycin plates from the ten replicate cultures.

1	2	3	4	5	6	7	8	9	10

? What did bacterial growth look like on the control plates?

? Do you have any other observations about bacterial growth observed on the plates?

2. Enter your group data on the class data chart. Use your class data to estimate the frequency of mutants for each replicate culture (the number of mutants detected divided by the total estimated number of cells plated). Enter these frequencies in Table 2.

Table 2. Frequency of mutants for each of the ten replicate cultures.

1	2	3	4	5	6	7	8	9	10

? What is the average mutant frequency over all 10 replicates? _____

Data from all sections will be combined in our attempt to estimate the mutation rate for streptomycin resistance in *Serratia*. Your TA can email you a copy of your section's excel file with all of your section's data.

Group Experiments

NOTE: Once again, be very careful with fire. Please review ALL details of the plating procedure before starting to plate so that you can stay safe and have good sterile technique.

Creating a culture of *Serratia* from a plate.

1. Flame-sterilize the wire loop by holding the loop end in the ethanol lamp until it glows orange. DO NOT dip flame loop in ethanol!
2. Use the loop to scrape a colony off the surface of a plate, making sure not to take pieces of the agar. When making a culture from a lawn, a loop-full, similar in size to a colony is sufficient.
3. Transfer the bacteria from the plate to a fresh tube of Luria broth.
4. Cap the tube and shake well.
5. Use the Spec20 to measure the optical density of the culture. Additional dilutions may be needed based on your experimental design.

Disc Diffusion Protocol

If you choose the disc diffusion protocol, you will receive agar plates without antibiotics. You will need to plate *Serratia* cells on these plates. These cells will originate either from liquid cultures or from the colonies you plated earlier in the class wide project. Antibiotics will be applied on paper discs, and you will be able to quantify the effect of the antibiotic by measuring the diameter of the halo around the disc (Fig. 12)

1. Obtain your agar plates. Label the plates on the bottom using a Sharpie pen for all your treatments.
2. First you will plate the *Serratia* on the agar plates. Obtain your liquid cultures or your colonies from the class wide experiment. If you are using resistant colonies, you will need to use an inoculation loop. DO NOT DIP THE LOOP INTO ALCOHOL! This loop is sterilized by holding it in the flame for 2 seconds. It is then used to scrape up part of a colony or lawn. The colony is then transferred to a tube with Luria broth and has to be shaken to make a homogenous mix. You will not need to dilute your cultures when using the disc diffusion method.
3. Set your micropipettor to 200 μ l and spread this amount of the *Serratia* dilution on all plates. Set plates aside to dry.
4. Now you will make the proper antibiotic dilution. Obtain several 5 ml tubes (one for each antibiotic dilution). Label each tube for the dilutions you wish to make. Refer to Table 3 below for making dilutions. In general, $C_1V_1 = C_2V_2$ where C_1 = the concentration of the initial solution, V_1 is the volume you use of it, C_2 = the concentration you want and V_2 is the total volume you want. 0.2 ml of each dilution is plenty for dipping discs in. You only have 2 ml of your initial antibiotic stock solution. Table 3 was developed to help you set up the serial dilutions for the different antibiotic concentrations. It should be used as a guideline for diluting any antibiotic solutions for your group experiment.
5. Now that you have your dilutions, you are ready to dip your discs and put them on your plates:
 - Use the forceps to separate discs.
 - Dip each disc in the appropriate dilution and drag disc across top of tube to remove excess liquid.
 - Place disc in the center of the prepared and labeled plate.
 - Rinse and dry the forceps before picking up the next disc.
6. Tape your plates in stacks of 5 again, but **leave your plates right side up to be sure the disc does not fall off.**

After you set up your group experiment, your plates will be incubated at room temperature (check lab thermostat \sim 21-22 $^{\circ}$ C) so *Serratia* colonies can grow. After 3 days, when the colonies become visible, the plates will be moved to 3 $^{\circ}$ C to stop further growth.

Table 3. You will need to use the glass pipets for measuring the volume you add. Use the micropipettor for all volumes of 200 μ l or less. The volumes included in this table are reported for SERIAL dilution.

Ampicillin/Streptomycin					
(C ₁)	(C ₂)	(V ₁)		(V ₂)	(V ₂ : V ₁)
Starting Antibiotic Concentration (mg/ml)	Final Antibiotic Concentration (mg/ml)	Volume of antibiotic (ml)	Volume of Water (ml)	Final volume(ml)	Ratio of dilution
100	90	1.8	0.2	2	1 : 1.11
90	80	1.78	0.22	2	1 : 1.24
80	70	1.75	0.25	2	1 : 1.14
70	60	1.71	0.29	2	1 : 1.17
60	50	1.67	0.33	2	1 : 1.2
50	40	1.6	0.4	2	1 : 1.25
40	30	1.5	0.5	2	1 : 1.33
30	20	1.33	0.77	2	1 : 1.5
20	10	1	1	2	1 : 1
10	5	1	1	2	1 : 1
0	0	0	2	2	1 : 0

Kanamycin

25	10	0.8	1.2	2	1 : 2.5
10	5	1	1	2	1 : 2
5	1	0.4	1.6	2	1 : 5
1	0.5	1	1	2	1 : 2
0.5	0.1	0.4	1.6	2	1 : 5
0.1	0.05	1	1	2	1 : 2
0.05	0.01	0.4	1.6	2	1 : 5
0.01	0.005	1	1	2	1 : 2
0	0	0	2	2	1 : 0

Colony Count Protocol

If you chose the colony count protocol, your treatment plates will have antibiotics added to the agar already. You need to obtain the *Serratia* liquid culture or colony, prepare the required dilution and similarly to the class wide project, plate the cells on the treatment and control plates.

1. Obtain the plates and the appropriate *Serratia* cells. On the side of the plate you can identify the antibiotic treatment:

Single purple line: 6.25 μ g/ml Streptomycin

Double green line: 25 μ g/ml Streptomycin,

Single black line: 6.25 μ g/ml Kanamycin

Single red line : 300 μ g/ml Ampicillin

2. Before plating, gather your supplies and label all plates on the bottom (because the top can swivel around and change its position).

3. Dilute your bacterial cultures to the appropriate level. The suggested dilutions of the *Serratia* plated on the antibiotics are as follows:

Streptomycin plates: *Serratia* colonies should be diluted to 1:125,000

Ampicillin plates: *Serratia* colonies should be diluted to 1:50

Kanamycin plates: No dilution of the *Serratia* colonies are necessary

4. Set your micropipettor to 200 μ l and spread this amount of the dilution on all plates. In addition to plating on plates containing the new antibiotic, you probably should plate on control plates. Plating on no-antibiotic plates will test whether the cells are still viable, if you are using resistant colonies, plating on Streptomycin plates will test whether the cells are still resistant to Streptomycin.
5. Start by plating your resistant colonies, so if you run short of the mix, you have enough replicates of the treatment plates.
6. Tape your plates in stacks of 5 again, but **leave your plates right side up to be sure the disc does not fall off.**

After you set up your group experiment, your plates will be incubated at room temperature (check lab thermostat \sim 21-22 $^{\circ}$ C) so *Serratia* colonies can grow. After 3 days, when the colonies become visible, the plates will be moved to 3 $^{\circ}$ C to stop further growth.

Procedures - Week Three

Group Experiment Data Collection

Disc Diffusion

1. Record the diameter of the zone of inhibition in 3 directions for all plates (see Fig. 12)
2. Calculate the mean diameter of zone of inhibition for each plate

Colony Counting

1. Record the number of colonies on each plate
2. If there are too many colonies to count on a plate, divide the plate into quarters and count the number of cells in one quarter of the plate. Multiply this value by 4.

Materials

This section lists the supplies and equipment required for the class wide experiment, during which students screen for streptomycin resistant *S. marcescens*. The essence of this laboratory module is to provide a variety of experiments for the student groups, therefore the materials and methods vary from one lab section to another, and from one semester to

another. For the possible experimental combinations please see the examples listed in the notes for the instructors, and consult Appendix C and Fig. 13.

Lab Room Materials

Tables 4 and 5 are materials lists for 18 students, forming six lab groups in each lab section.

Table 4. Materials used during the class project (Week One)

Equipment/ Material	Quantity; Remarks
Spectrophotometer (Spec 20)	6
Shaking water bath at 30 C.	1
Metal tube rack	6
Cuvette rack	6
10 ml plastic test tubes	18
Cuvettes	18
Kimwipes	3 boxes
Adjustable pipettor (20-200 μ l)	6
20-200 μ l pipettor tips	6 boxes
Ethanol (95%)	1 liter
Glass petri dishes for ethanol	6
Disposable gloves	3 boxes
Red food coloring	6 tubes, for pipetting demonstration
5 ml pipettes	8
Pipette fillers for 5 ml pipettes	8
Luria broth	3 x 5 ml tubes
Parafilm	1 roll
Bacteria spreaders	6
Marker pens	6
Scissors	3
Matches	6 boxes
Alcohol lamps	6
Masking tape	6 rolls
Agar plates with 25 μ g/ml streptomycin	60, marked with single purple stripe
<i>S. marcescens</i> D1 strain replicate cultures 1-10	2 ml each
<i>S. marcescens</i> in water bath	300 ml, for serial dilution and to measure optical density
Agar plates with without antibiotics	10; these will be used for control

Table 5. Materials used during the group project (Week Two)

Equipment/ Material	Quantity; Remarks
Sterile tubes	As many as requested. Only used if dilution of resistant colonies is needed
Metal tube rack	6
Ethanol (95%)	1 liter
Glass petri dishes for ethanol	6
Vortex mixer	3
Masking tape	6 rolls
Agar plates with and without antibiotics	As requested. See Appendix C and Fig. 13 for clarification.
Agar plates with streptomycin resistant <i>S. marcescens</i> colonies	100 plates. Results of set up in week one.
<i>S. marcescens</i> cultures	2 ml tubes of D1, WF strains. Amounts depend on how many groups use these strains
Adjustable pipettor (20-200 μ l)	6
20-200 μ l pipettor tips	6 boxes
Forceps	6
Disposable gloves	3 boxes
5 ml pipettes	8
Fillers for 5 ml pipettes	8
Luria broth	5 ml tubes. As many as requested for colony dilutions
Parafilm	1 roll
Bacteria spreaders	6
Marker pens	6
Scissors	3
Matches	6 boxes
Alcohol lamps	6
Paper discs	As requested. Depends on how many groups use disc diffusion experiments
Inoculating loops	6

Serratia marcescens Strains

The *S. marcescens* strain D1 was purchased from Carolina Biological Supply in 2009 and maintained in phosphate buffered saline (PBS) at 3 degrees Celsius. Cells were plated on no-antibiotic agar plates and a single red-pigmented *Serratia* colony (a collection of genetically identical cells) was added to nutrient broth and shaken overnight at 30°C (Fig. 8). Ten replicate cultures were started and after 22 hours each replicate culture was diluted 1:10 in cold PBS to halt growth while preserving live cells. Students receive these cultures for their week one class project, to screen for Streptomycin resistant bacteria. Students can use these replicate cultures for their week two group projects, if they choose.

Notes for the Instructor

The following pages include handouts for Laboratory Instructors. These are currently being used at Cornell University and may be modified by individual instructors, depending on

how they wish to adapt this lab module.

Preparatory Notes for Week One

In this lab module’s first week, students will learn how optical density measurements can be used to estimate cell density of bacteria growing in broth and set up a course-wide experiment to look at mutations for streptomycin resistance in *Serratia*. We have chosen to use an antibiotic as our selective agent in this artificial selection activity to capture student interest, but much of this activity is more about the basics of mutation and selection than about antibiotic resistance per se. At the end of this week’s lab, students will look at selected primary sources on related topics and design experiments for next week’s lab. Students must submit their methods and material requests before leaving lab. You must sign their INDIVIDUAL experimental sheets. Last semester many students complained that they did not have the experimental design because their group mate was the one who took notes.

TA Name: Leo Day: Tues Room: 1120 Time: PM * RETURN ON THE CART *

Check the types of *Serratia* and/or antibiotic stock solutions (for Disc Diffusion) that each group needs below. Maximum of 6 groups per lab.

<i>Serratia</i> Source	Streptomycin lowest effective dose	Kanamycin lowest effective dose	Ampicillin lowest effective dose	Low Pigment		Colony Comparison		Antibiotic Comparison		Cross Resistance		Totals
				Disks	Colony Count	Disks	Colony Count	gp 1	gp 2	Disks	Colony Count	
5 ml (5000µl) of 1:10 PBS dilution of red strain	NA	NA	NA			NA	NA		✓	NA	NA	1 ✓
5 ml (5000µl) of 1:10 PBS dilution of low pigment (WF) strain	NA	NA	NA			NA	NA			NA	NA	0 ✓
Resistant colonies from the Class experiment	✓	NA	NA	NA	NA			NA	NA	✓		2
Control plates from the Class experiment		✓	✓	NA	NA	NA	NA	✓		✓		4
Disks	✓	✓	✓		NA		NA	✓	✓	✓	NA	6 ✓
Antibiotic Solutions:												
2ml of 100 mg/ml streptomycin	✓	NA	NA		NA		NA		✓		NA	2 ✓
2ml of 25 mg/ml kanamycin	NA	✓	NA		NA	NA	NA	✓	✓		NA	3 ✓
2ml of 100 mg/ml ampicillin	NA	NA	✓		NA	NA	NA	✓		✓	NA	3 ✓

Please enter the number of each type of plate each group needs below. Each group is limited to 20 plates total (all types combined!)

Type of plates	Streptomycin lowest effective dose	Kanamycin lowest effective dose	Ampicillin lowest effective dose	Low pigment		Colony Comparison		Antibiotic Comparison		Cross Resistance		Totals
				Disks	Colony Count	Disks	Colony Count	gp 1	gp 2	Disks	Colony Count	
(<i>Serratia</i> media) no antibiotics	20	20	20					20	20	20		120 ✓
6.25µg/ml streptomycin	NA	NA	NA	NA		NA		NA	NA	NA	NA	-
300µg/ml ampicillin	NA	NA	NA	NA		NA	NA	NA	NA	NA		-
	1	1	1					1		2	20	6 ✓

Figure 13. Materials request sheet summary for 6 lab groups (18 students total). After students complete the individual request sheets (see Appendix C), the laboratory instructors summarize the requested materials, so the preparatory staff can deliver everything by the next lab.

Schedule of Activities

Pipetting instructions	10 min.
Serial Dilutions/ Optical density	30 min.
Intro. to class mutation study	5 min.
Reference plates of parent culture	5 min.
Plating Demonstration	5 min.
Plating replicate cultures for class mutation experiment	40 min.
Clean-up	5 min.

Answers to Prelab Questions:

1. For a typical bacterial population, what are the four phases of growth?

Lag phase, Exponential phase, Stationary phase and Death phase

2. If you have a starting population of 50 cells, how many cells will you have after 10 generations of exponential growth? After 15?

After 10 generations: $N = N_0 2^n = (50)(2^{10}) = (50)(1024) = 51,200$ cells

After 15 generations: $N = (50)(2^{15}) = (50)(32,768) = 1,638,400$ cells

3. If you have a culture with 10^6 cells/ml, how could you use serial dilutions to obtain a solution with 103 cells/ml?

This problem requires a total overall dilution of 1:1000. You could take 1 ml of your starting culture and add it to 9 ml of buffer (a 1:10 dilution) to get 10^5 cells/ml. You could then repeat that process two more times to get a solution with 103 cells/ml. You could also do one 1:10 dilution and one 1:100 dilution [adding 100 μl which equals 0.1 ml into 9.9 ml of buffer].

4. For Luria and Delbrück's experiment, what type of results would have supported the induced mutation model?

A low variation among the ten replicate cultures would have supported the induced mutation model.

5. Where do streptomycin and penicillin originally come from?

Both antibiotics were initially isolated from naturally occurring organisms – streptomycin from a soil bacteria and penicillin from fungi.

6. How do aminoglycoside and beta-lactam antibiotics differ in their modes of action?

Aminoglycoside antibiotics inhibit translation

(by binding to the 30S ribosomal unit and preventing it from attaching to the 50S unit during the initiation of translation). Beta-lactam antibiotics affect transpeptidase, an enzyme essential in bacterial cell wall synthesis.

Group Considerations

For this lab module's activities, students should work in groups of 3 with no more than 6 groups per lab.

Safety Considerations

We have chosen to work with *Serratia marcescens* for this activity because it normally is not pathogenic. Nonetheless, it has been known to be pathogenic in cases where it gets into the bloodstream (i.e. contaminated IV syringes) or into internal organs or cavities (i.e. during surgery). While we are not requiring gloves of all students, gloves are available and you and students should wear them if you wish. Anyone with a cut on their hand should wear gloves, and those who are immune-compromised might also want to wear gloves. Everyone should wash his or her hands before and after working with *Serratia*. This week please enforce the no food or drink in lab policy.

Students will also be working with fire while plating. Risks are minimal if the lab manual directions are followed. It is your job to emphasize proper technique and *immediately* correct any groups with open ethanol dishes or other problematic procedures. Please tell students that they will lose subjective points if they are careless with the plating procedure. You will need to be walking around the lab while burners are in use both this week and next week. Should anything happen, just cap the ethanol dish immediately, use the fire blanket or, if necessary, the fire extinguisher.

Pipetting technique

Proper micropipettor technique is an essential skill. The students will use the micropipettors throughout the semester so mastering the technique is important to their success in the course. Go over how to set the volume of the pipettor and make sure the students know not to exceed the volume range listed on the pipettor. Have the students hold the pipettors and feel the first and second stops. Demonstrating proper pipetting technique is helpful to the students.

Serial Dilutions

Students should know what volumes of sample and solvent add to reach a desired volume of a particular dilution. The students should also understand the affect of dilutions on the cell concentration. Start by assigning a random cell concentration of an undiluted culture. Each step on the table should represent a 1:10 dilution (see the provided example). Students should also be able to calculate the number of cells in a given sample by multiplying by the volume. Emphasize the units of cell density (cells/ml) and volume (ml) and how

they cancel during this calculation. Student can use this calculation to arrive at the number of cells they are spreading on a plate. Remind students that one colony on a plate represents one-cell surviving in a culture. Students can work backwards from the number of cells on a plate to determining how many cells were in a culture. This method also works for determining how far to dilute a culture to achieve a countable number of cells.

Table 6. An example of demonstrating the relationship between cell density and dilution.

Colony type	Colony Comparison
Streptomycin Resistance	Cross resistance

Estimating Cell Concentration Using Optical Density

The goal of this exercise is to help students understand how we can use absorbance/optical density measurements to estimate the number of cells per ml in a growing culture. Students will have 3 cuvettes to work with. One of them is a blank. Since the students are diluting the cultures with Luria broth, they should also blank the spec with Luria. Have the students make a 1:10 dilution from the culture in the shaking water bath and measure the optical density. From there the students should make a 1:10 dilution of the solution they just examined (1:100 total dilution) and measure the optical density. You might want to ask students how they could verify the accuracy of the equation we are using (answer: plate out dilutions of your optical density measures and count cells ... but you have to dilute quite a bit to get a countable number per plate).

Parent Culture Streptomycin Sensitivity

Briefly go over our class-wide experiment. Hold up the example cultures of the parental cultures and pass them around for students to observe. This lets students see the nice red color of mature *Serratia* cultures. Since none of our negative controls grew any colonies, it is highly unlikely that there were any resistant cells among those used to start the replicate cultures.

Ask the following questions:

Why should we plate more cells on the streptomycin plate?

–Because only a small proportion of the cells survive on the antibiotics (the probability of antibiotic resistant mutation is small).

Why look at control plate growth?

–Growth on the control plate tells us whether the cells are alive (viable).

Why look at Streptomycin plate growth?

–To check if there are any resistant cells in the parent culture.

Was the parent culture viable?

– Yes, because the control plate has a lawn.

Was the parent culture sensitive to streptomycin?

– Yes, because we do not see colonies growing on this plate.

Plating Replicate Cultures for Class Mutation Experiment

Before the students start plating, you should demo proper sterile technique by plating on of the controls. Shake each culture to mix before passing out to students. Make sure all students understand that they will be plating 200 μ l of each replicate culture on only one of their plates (1:10 dilution of replicate one plate on plate one; 1:10 dilution of replicate two plated on plate two, etc.). They will then need to pass the stock tube of each replicate culture on to the next group. Whichever group finishes first should go ahead and plate the control plates. Students should tape plates together in groups of 5 and place them upside down to prevent condensation from dripping. All students should wash their hands and wipe down their work area with alcohol.

Method Design for Next Week's Group Experiments.

Students need to fill out the study proposal form, following these steps:

- 1. Choosing a variable.** The experiments they design should vary only in that ONE variable.

Table 7. Possible variable choices for the five experiments.

Variable	Experiment
Antibiotic Concentration	Dose response Curve
Pigmentation	Comparing Red and Low Pigmentation
Antibiotic type	Comparing Effectiveness of 2 antibiotics

- 2. Finding appropriate primary literature.**

- 3. Developing a hypothesis.** Before following through with a complete experiment the students need to write down a hypothesis. This will help them frame the question and stay on track while designing the experiment. The hypotheses in these experiments are essentially fixed but you should be able to guide students to the correct hypothesis.

- 4. Picking a Method.** After picking a variable the students will pick an appropriate method. There are 2 methods used for the experiments in our class: disc diffusion or colony counting

- 5. Choosing Controls.** The students will need to identify the proper controls for their experiments. The proper controls are listed below but try to talk the students

through their experiments so that they reach the design on their own.

6. Choosing number of replicates/statistical method.

Students can receive a total of 20 plates to run and replicate their experiment. Make sure they are allotting plates for their controls. Remember a Spearman rank correlation needs a minimum of 4 different treatment levels. The best design is to have 5 data points replicated 4 times or 6 data points replicated three times. For the rank sum and t test students should try as many replicates as possible. The treatment groups should have the same number of replicates if possible. Students should test their data for normality before choosing the rank sum test or the t test.

7. Make predictions.

Description of Experiments:

Dose Response: Students will use disc diffusion to construct a dose response curve for the antibiotic they are interested in. For the streptomycin dose curves, the students will use a streptomycin resistant colony from the class experiment. For the kanamycin and ampicillin dose responses the students will use the parent culture of the red strain. Control: Students should include a 0 mg/ml antibiotic solution (DI water only). Statistic: Spearman rank correlation test.

Comparison of low and red *Serratia* strains: The variable in this experiment is the pigmentation of the *Serratia*. Students will expose the two strains to identical treatments to look for a difference in their response to antibiotics. If the students use disc diffusion, they should use 1 concentration of the antibiotic for both strains. The students can also use the colony count method. This will mean they plate the red and low pigment strains on plates containing 6.25 µg/ml streptomycin, 6.25 µg/ml kanamycin or 300 µg/ml ampicillin. Control: Both of the strains of *Serratia* need to be plated on plain agar plates to confirm that they have similar growth under normal conditions. Statistic: T-test/ Rank Sum test.

Colony Comparison: The students will pick two resistant colonies and expose them to identical antibiotic treatments. The colonies should be morphologically different in size or color. Colony counting or disc diffusion can be used in this experiment. Control: Each of the *Serratia* cultures from the different colonies needs to be plated on a plain agar plate to confirm that there is no difference in normal growth. This way the student can be sure any differences between the colonies are due to the antibiotic. Statistic: T test or rank sum test.

Antibiotic Effectiveness: Disc Diffusion can be used to compare two different antibiotics. Any combination of antibiotics can be used as long as antibiotics are equal in concentration. The students will use the parent culture of the red strain of *Serratia*. Colony counting **cannot** be used for this method. Control: Since this is a disk diffusion study, the students will include a control with the disk soaked in DI

water. This confirms that the disc itself does not influence the growth and the differences observed are due to antibiotic type. Statistic: T test or rank sum test.

Cross-Resistance: Students will take Streptomycin resistant *Serratia* from the class experiment, and treat them with other antibiotics to look for cross-resistance. If the students want to examine cross-resistance to ampicillin they may use the colony count or the disc diffusion method. Due to kanamycin's effectiveness against *Serratia*, cross-resistance to kanamycin should be done using disc diffusion only. Control: Students should use *Serratia* from control plates of the group experiment and plate them on the antibiotic. Be sure that the students are using the control plate from the same culture they picked the resistant colony from. This way the students can test for differences between the resistant colonies and the non-resistant parent culture and the differences will be related to the resistance variable. Statistic: T test or rank sum test

Preparatory Notes for Week Two

Students start with the Antibiotic Treatment Simulation followed by observing the results of the course-wide experiment from last week, looking at mutations for streptomycin resistance in *Serratia*. This week's lecture will touch on natural selection and how mutations propagate through a population. The challenge of this week's lab will be to get through the initial activity and calculations efficiently and then help guide the groups setting up their experiments. Before the groups start the experimental set-up, we would like you to talk personally to each group and ask them to **briefly** describe their design and help them organize their materials and finalize their experiments. By discussing this at the beginning, the possible errors with experimental design can still be corrected. Have the students write down a to do list for the group experimental set-up. Groups that finish early may start writing their methods section.

Schedule of Activities

Pre-lab quiz	10 min.
Data Collection & Frequency	
Calculation	45 min.
Set-up Group Experiments	45 min.
Clean-up	5 min.
(do not dump anything in the sink)	

Answers to Prelab Questions:

1. Why does the Poisson distribution describe the distribution of the number of new mutation that occurs within each replicate culture, but not the distribution of mutants in the final generation?

The Poisson distribution describes the probability of very rare events. New mutations are rare events, but once the mutation has occurred, the

inheritance of the new mutation from a dividing mutant cell is not a rare event (it is also not an independent event since all subsequent daughter cells would normally share the same mutation).

2. In the equation $m = -\ln P_0$, m is the average number of mutational events over the time period/number of generations examined (i.e. the time the bacteria were growing in a replicate culture). What do you need to do to calculate a mutation rate once you know m ?

Divide m by the estimated number of cell divisions (the # of cells in the replicate cultures).

3. What is the difference between mutation rate and frequency of mutants?

The frequency of mutation is the frequency of resistant cells at the end of many generations of growth. Mutation rate is the probability of a new mutation occurring per cell division.

4. If you look at 10 replicate *Serratia* cultures plated on antibiotic plates, how do you know whether it was induced or spontaneous mutation?

If the mutation was induced, then it should have happened at the same time on all the plates, so the number of cells on each plate should be approximately the same, and the variance among plates should be really small. If the mutations occurred spontaneously, at different times, then some plates will have many colonies, while some will have very few.

5. What is the relationship between sensitivity to an antibiotic and the diameter of the zone of inhibition in a disc diffusion experiment?

The more sensitive the culture is to the antibiotic the larger the zone of inhibition will be. Directly correlated.

6. On a dose response curve, what causes the flat regions at low concentrations and high concentration?

There is a threshold concentration that must be reached in order for cells to start dying which is why there is a flat portion at the start of a dose response curve. At very high concentrations there is a saturation effect, which occurs when additional levels of antibiotic don't produce any greater effect on the bacteria.

Data Collection and Frequency Calculation

Students should retrieve their plates and record the number of colonies (if any) on each streptomycin plate in the top row of Table 1. Each group should then come up to the front com-

puter and enter their data in the class excel file. Also ask students to take a look at your section's control plates (laid out on side bench). All should have a nice lawn on them. When all data have been entered, go through the frequency calculation. Discuss the difference between the mutant frequency and the mutation rate using examples provided. We will not be calculating mutation rate this year so the examples given will be sufficient and the students should be able to repeat this sort of calculation on the prelim.

Setting Up Group Experiments

Before the students start to set up their group experiments, ask each group about their experimental design. You should ask for their hypothesis, and experimental design. Make sure to ask for the treatment and control. This is a great last minute check to make sure that each group has enough replicates and control plates. Additionally help the students understand how they will use all the materials you have provided them. In particular groups who are doing dilutions of antibiotic solutions will need some extra attention to sort everything out. Tell the students to write out a set of step-by-step instructions for themselves before starting the experiment. This will also be useful in writing their methods section. Ask which statistical methods they will use. If they didn't have enough replicates to run proper statistical analysis, then help them adjust their experimental design.

Dose Response: This group will need to make a culture from one of the resistant colonies (streptomycin) or a loop full of cells from the control plates (kanamycin and ampicillin) from the class experiment. These groups will have a tube of antibiotic. They will need to use the glass pipettes provided to do serial dilutions with the antibiotic solution to reach different concentrations. The table in the lab manual tells them exactly what volumes to add to reach the different concentrations. Emphasize that this is a serial dilution and they have to dilute to one concentration before further diluting to the next. The control in this experiment is a disc with no antibiotic (DI water). The students only need .2 ml to soak the disks. Have them do ALL the dilutions first before placing any discs. While or two students are diluting, the others can plate the cultures.

Pigmentation: These students will be given a culture of the red pigmented and white pigmented *Serratia* strains. The disc diffusion group will need to pick what concentration of antibiotic to use/. Both strains should receive the exact same antibiotic treatment. Controls in this study should be a set of each culture plated without antibiotic treatment to ensure that the cultures grow equally well under normal conditions. This doesn't require more than 1 plate each.

*Note** - Kanamycin should only be done with disc diffusion not with colony count. Otherwise the students won't have any data to work with.

Colony Comparison: These students will make two cultures, one from each of the colonies they pick. When picking colonies the students should try to pick colonies that show

distinct morphological differences. If all the colonies look exactly the same then make sure the two colonies are coming from different replicate cultures. The controls are the same as the pigmentation. The students need to plate both cultures on plates without antibiotic to ensure there is no significant difference in how the bacteria grow under normal conditions.

Cross-resistance: These students will make 2 cultures; One from a resistant colony and another from a control plate. These cultures should come from the exact same replicate culture. The culture from the control plate serves as a control in this experiment. The students then treat these cultures with the antibiotic they are interested in. Antibiotic concentration should be exactly the same.

Antibiotic comparison: This study is disc diffusion only. The students will be comparing two different antibiotics. In order for this to be a precise comparison the students need to dilute the antibiotic solutions so that both antibiotics are at the same concentration.

Preparatory Notes for Week Three

Data Collection

This third week of the Mutation and Selection lab module students observe the results of their group experiments from last week. If they have a very large number of colonies, they can draw a cross on the back of the plate and only count the colonies in one quadrant. If there are too many colonies to count, they may still be able to do some sort of qualitative scoring if there are differences in the density of growth. Encourage students to also notice any other differences in their colonies such as color or size variation.

Disc diffusion groups should measure the distance between the edge of the disc and the edge of colony growth. If this distance seems variable around the disc they can measure this distance in two or three places and calculate the mean for each disc.

Colony count measures resistance, as all surviving cells (each colony started from one cell) are resistant. Disk diffusion measures susceptibility, as cells may be less sensitive to lower concentrations, etc. However resistant colonies may grow between the disk and the edge of the halo. These isolated colonies are resistant colonies, which were not killed by the antibiotics. These colonies should be noted, but not included in the zone of inhibition measurement.

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APPENDIX A

Student Group Experiment Plans

Names of group members:

Start your background search by looking at the primary literature articles in the appropriate folder. Use the space below to take notes about these articles. Use additional pages if necessary:

Start your biological investigation

Establish your Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Experiment Design/Methods

Treatment group:

Control group:

Variable:

Replicates:

Other (statistical test you plan to use, sources of error, etc.)

Approval of your lab instructor: _____

Appendix B

Table B-1. Percent Transmittance-Absorbance Conversion Table

	T(%) ABSORBANCE					T(%) ABSORBANCE			
	1*	2*	3*	4*		1*	2*	3*	4*
	(0.0)	(.25)	(.50)	(.75)		(0.0)	(.25)	(.50)	(.75)
1	2.000	1.903	1.824	1.757	51	.2924	.2903	.2882	.2861
2	1.699	1.648	1.602	1.561	52	.2840	.2819	.2798	.2777
3	1.523	1.488	1.456	1.426	53	.2756	.2736	.2716	.2696
4	1.398	1.372	1.347	1.323	54	.2676	.2656	.2636	.2616
5	1.301	1.280	1.260	1.240	55	.2596	.2577	.2557	.2537
6	1.222	1.204	1.187	1.171	56	.2518	.2499	.2480	.2460
7	1.155	1.140	1.126	1.112	57	.2441	.2422	.2403	.2384
8	1.097	1.083	1.071	1.059	58	.2366	.2347	.2328	.2310
9	1.046	1.034	1.022	1.011	59	.2291	.2273	.2255	.2236
10	1.000	.989	.979	.969	60	.2218	.2200	.2182	.2164
11	.959	.949	.939	.930	61	.2147	.2129	.2111	.2093
12	.921	.912	.903	.894	62	.2076	.2059	.2041	.2024
13	.886	.878	.870	.862	63	.2007	.1990	.1973	.1956
14	.854	.846	.838	.831	64	.1939	.1922	.1905	.1888
15	.824	.817	.810	.803	65	.1871	.1855	.1838	.1821
16	.796	.789	.782	.776	66	.1805	.1788	.1772	.1756
17	.770	.763	.757	.751	67	.1739	.1723	.1707	.1691
18	.745	.739	.733	.727	68	.1675	.1659	.1643	.1627
19	.721	.716	.710	.704	69	.1612	.1596	.1580	.1565
20	.699	.694	.688	.683	70	.1549	.1534	.1518	.1503
21	.678	.673	.668	.663	71	.1487	.1472	.1457	.1442
22	.658	.653	.648	.643	72	.1427	.1412	.1397	.1382
23	.638	.634	.629	.624	73	.1367	.1352	.1337	.1322
24	.620	.615	.611	.606	74	.1308	.1293	.1278	.1264
25	.602	.598	.594	.589	75	.1249	.1235	.1221	.1206
26	.585	.581	.577	.573	76	.1192	.1177	.1163	.1149
27	.569	.565	.561	.557	77	.1135	.1121	.1107	.1093
28	.553	.549	.545	.542	78	.1079	.1065	.1051	.1037
29	.538	.534	.530	.527	79	.1024	.1010	.0996	.0982
30	.523	.520	.516	.512	80	.0969	.0955	.0942	.0928
31	.509	.505	.502	.498	81	.0915	.0901	.0888	.0875
32	.495	.491	.488	.485	82	.0862	.0848	.0835	.0822
33	.482	.478	.475	.472	83	.0809	.0796	.0783	.0770
34	.469	.465	.462	.459	84	.0757	.0744	.0731	.0718
35	.456	.453	.450	.447	85	.0706	.0693	.0680	.0667
36	.444	.441	.438	.435	86	.0655	.0642	.0630	.0617

37	.432	.429	.426	.423	87	.0605	.0593	.0580	.0568
38	.420	.417	.414	.412	88	.0555	.0543	.0531	.0518
39	.409	.406	.403	.401	89	.0505	.0494	.0482	.0470
40	.398	.395	.392	.390	90	.0458	.0446	.0434	.0422
41	.387	.385	.382	.380	91	.0410	.0398	.0386	.0374
42	.377	.374	.372	.369	92	.0362	.0351	.0339	.0327
43	.367	.364	.362	.359	93	.0315	.0304	.0292	.0281
44	.357	.354	.352	.349	94	.0269	.0257	.0246	.0235
45	.347	.344	.342	.340	95	.0223	.0212	.0200	.0188
46	.337	.335	.332	.330	96	.0177	.0166	.0155	.0144
47	.328	.325	.323	.321	97	.0132	.0121	.0110	.0099
48	.319	.317	.314	.312	98	.0088	.0077	.0066	.0055
49	.310	.308	.305	.303	99	.0044	.0033	.0022	.0011
50	.301	.299	.297	.295	100	.0000	.0000	.0000	.0000

*Columns 1, 2, 3, and 4 give absorbance values corresponding to the whole % value of T given in the left hand column plus the fraction of a % indicated below the column number (e.g., 1.50 %T corresponds to an absorbance of 1.824)

Appendix C

Material Request Sheets for Each Group Project

Topic: Streptomycin lowest effective dose Group Members: _____

Background facts about topic (use back if necessary):

Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Variable(s):

Control(s):

Experimental Design/Methods (think about controls, replicates, sources of error, use back if necessary). We recommend you draw a picture of your experimental set-up.

Use tables below to note specific supplies needed.

<i>Bacterial Source?</i>	<i>Check if needed</i>
Resistant colonies from Class experiment	
Control colonies from Class experiment	
<i>Antibiotic source?</i>	
2 mL of 100 mg/ml streptomycin	

<i>Plates (20 max) and Discs</i>	<i>Indicate # needed</i>
Agar plate with no antibiotics	
Discs	

Topic: Kanamycin lowest effective dose **Group Members:** _____

Background facts about topic (use back if necessary):

Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Variable(s):

Control(s):

Experimental Design/Methods (think about controls, replicates, sources of error, use back if necessary). We recommend you draw a picture of your experimental set-up.

Use tables below to note specific supplies needed.

<i>Bacterial Source?</i>	<i>Check if needed</i>
Resistant colonies from Class experiment	
Control colonies from Class experiment	
<i>Antibiotic source?</i>	
2 mL of 25 mg/ml kanamycin	

<i>Plates (20 max) and Discs</i>	<i>Indicate # needed</i>
Agar plate with no antibiotics	
Discs	

Topic: Ampicillin lowest effective dose **Group Members:** _____

Background facts about topic (use back if necessary):

Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Variable(s):

Control(s):

Experimental Design/Methods (think about controls, replicates, sources of error, use back if necessary). We recommend you draw a picture of your experimental set-up.

Use tables below to note specific supplies needed.

<i>Bacterial Source?</i>	<i>Check if needed</i>
Resistant colonies from Class experiment	
Control colonies from Class experiment	
<i>Antibiotic source?</i>	
2 mL of 100 mg / ml ampicillin	

<i>Plates (20 max) and Discs</i>	<i>Indicate # needed</i>
Agar plate with no antibiotics	
Discs	

Topic: Low-pigmented *Serratia* Colony Count Group Members: _____

Background facts about topic (use back if necessary):

Antibiotic used in this experiment:

Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Variable(s):

Control(s):

Experimental Design/Methods (think about controls, replicates, sources of error, use back if necessary). We recommend you draw a picture of your experimental set-up.

Use tables below to note specific supplies needed.

Bacterial Source?	Check if needed
5 ml (5000 μ l) of 1:10 PBS dilution of red strain	
5 ml (5000 μ l) of 1:10 PBS dilution of low pigment (WF) strain	

<i>Plates (20 max) and Discs</i>	<i>Indicate # needed</i>
Agar plate with no antibiotics	
6.25 μ g/ml streptomycin	
6.25 μ g/ml kanamycin	
300 μ g/ml ampicillin	

Topic: Low-pigmented *Serratia* Disc Diff. Group Members: _____

Background facts about topic (use back if necessary):

Antibiotic used in this experiment:

Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Variable(s):

Control(s):

Experimental Design/Methods (think about controls, replicates, sources of error, use back if necessary). We recommend you draw a picture of your experimental set-up.

Use tables below to note specific supplies needed.

Bacterial Source?	Check if needed
5 ml (5000µl) of 1:10 PBS dilution of red strain	
5 ml (5000µl) of 1:10 PBS dilution of low pigment (WF) strain	
<i>Antibiotic source?</i>	
2 mL of 100 mg/ml streptomycin	
2 mL of 25 mg/ml kanamycin	
2 mL of 100 mg/ml ampicillin	

<i>Plates (20 max) and Discs</i>	<i>Indicate # needed</i>
Agar plate with no antibiotics	
Discs	

Topic: Antibiotic comparison Group 1. **Group Members:** _____

Background facts about topic (use back if necessary):

Two antibiotics compared in this experiment: _____ **vs.** _____

Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Variable(s):

Control(s):

Experimental Design/Methods (think about controls, replicates, sources of error, use back if necessary). We recommend you draw a picture of your experimental set-up.

Use tables below to note specific supplies needed.

<i>Bacterial Source?</i>	<i>Check if needed</i>
Control colonies from Class experiment	
5 ml (5000 μ l) of 1:10 PBS dilution of red strain	
5 ml (5000 μ l) of 1:10 PBS dilution of low pigment (WF) strain	
<i>Antibiotic source?</i>	
2 mL of 100 mg/ml streptomycin	
2 mL of 25 mg/ml kanamycin	
2 mL of 100 mg/ml ampicillin	

<i>Plates (20 max) and Discs</i>	<i>Indicate # needed</i>
Agar plate with no antibiotics	
Discs	

Topic: Antibiotic comparison Group 2. **Group Members:** _____

Background facts about topic (use back if necessary):

Two antibiotics compared in this experiment: _____ **vs.** _____

Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Variable(s):

Control(s):

Experimental Design/Methods (think about controls, replicates, sources of error, use back if necessary). We recommend you draw a picture of your experimental set-up.

Use tables below to note specific supplies needed.

<i>Bacterial Source?</i>	<i>Check if needed</i>
Control colonies from Class experiment	
5 ml (5000 μ l) of 1:10 PBS dilution of red strain	
5 ml (5000 μ l) of 1:10 PBS dilution of low pigment (WF) strain	
<i>Antibiotic source?</i>	
2 mL of 100 mg/ml streptomycin	
2 mL of 25 mg/ml kanamycin	
2 mL of 100 mg/ml ampicillin	

<i>Plates (20 max) and Discs</i>	<i>Indicate # needed</i>
Agar plate with no antibiotics	
Discs	

Topic: Cross Resistance Disc Diff. Group Members: _____

Background facts about topic (use back if necessary):

Antibiotic used in this experiment:

Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Variable(s):

Control(s):

Experimental Design/Methods (think about controls, replicates, sources of error, use back if necessary). We recommend you draw a picture of your experimental set-up.

Use tables below to note specific supplies needed.

Bacterial Source?	Check if needed
5 ml (5000 μ l) of 1:10 PBS dilution of red strain	
5 ml (5000 μ l) of 1:10 PBS dilution of low pigment (WF) strain	
<i>Antibiotic source?</i>	
2 mL of 100 mg/ml streptomycin	
2 mL of 25 mg/ml kanamycin	
2 mL of 100 mg/ml ampicillin	

<i>Plates (20 max) and Discs</i>	<i>Indicate # needed</i>
Agar plate with no antibiotics	
Discs	

Topic: Colony comparison Colony Count **Group Members:** _____

Background facts about topic (use back if necessary):

Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Variable(s):

Control(s):

Experimental Design/Methods (think about controls, replicates, sources of error, use back if necessary). We recommend you draw a picture of your experimental set-up.

Use tables below to note specific supplies needed.

<i>Bacterial Source?</i>	<i>Check if needed</i>
Resistant colonies from Class experiment	
Control colonies from Class experiment	

<i>Plates (20 max) and Discs</i>	<i>Indicate # needed</i>
Agar plate with no antibiotics	
6.25 µg/ml streptomycin	

Topic: Colony comparison Disc Diffusion **Group Members:** _____

Background facts about topic (use back if necessary):

Research Hypothesis:

What prediction(s) of your research hypothesis will you test?

Variable(s):

Control(s):

Experimental Design/Methods (think about controls, replicates, sources of error, use back if necessary). We recommend you draw a picture of your experimental set-up.

Use tables below to note specific supplies needed.

<i>Bacterial Source?</i>	<i>Check if needed</i>
Resistant colonies from Class experiment	
Control colonies from Class experiment	
<i>Antibiotic source?</i>	
2 mL of 100 mg/ml streptomycin	

<i>Plates (20 max) and Discs</i>	<i>Indicate # needed</i>
Agar plate with no antibiotics	
Discs	

Appendix D

Example of Student Worksheet Questions and Answers

Question 1. This question refers to the parent culture, its dilution, and inoculation of the 10 replicate cultures.

The optical density (OD) of a 1:10 dilution of the parent culture was 0.52. (*You may wish to use actual measured parent culture OD here*).

- 1a. According to Haddix et al. (2000), there is a direct linear relationship between cell concentration and optical density for OD readings between 0.06 and 0.99 such that 1.00 OD unit = 5.8×10^8 cells/ml. Use this relationship to estimate the cells/ml in the 1:10 dilution. Show your calculation.

$$0.52 (5.8 \times 10^8 \text{ cells/ml}) = 3.016 \times 10^8 \text{ cells/ml in 1:10 dilution}$$

- 1b. The 1:10 dilution was diluted 1:100 to get a 1:1000 dilution of the parent culture. How much further would this 1:1000 dilution need to be diluted to obtain a 1:10⁵ dilution of the original parent culture? *100x*

Approximately how many cells/ml would be in this 1:10⁵ dilution?

$$3.016 \times 10^8 \text{ cells/ml divided by } 100 \times 100: 3.016 \times 10^4 \text{ cells/ml (may be written as 30,160 cells/ml)}$$

- 1c. The 1:10⁵ dilution was diluted yet again to obtain a 1:10⁷ dilution of the original parent culture. If each replicate culture was inoculated with 200 μ l of this 1:10⁷ dilution of the parent culture, about how many cells were added to each of the 10 cultures? Show your calculation.

$$\text{Since the } 1:10^5 \text{ dilution had ca. } 30200 \text{ cells/ml, the concentration in the } 1:10^7 \text{ dilution will be around } 302 \text{ cells/ml. } 200 \mu\text{l} = 0.200 \text{ ml, therefore } 0.200 \text{ ml} \times 302 \text{ cells/ml} = 60.4 \text{ (~60) cells added to each of the 10 cultures}$$

Question 2. This question refers to the 10 replicate cultures after 22 hours of growth.

When bacteria grow in culture, there is an initial lag phase before bacteria start dividing. Once bacteria start dividing, they grow in an exponential fashion until they saturate the culture and reach a stationary phase (for *Serratia marcescens* at a density of about 5.8×10^9 cells/ml). During the exponential phase, the number of cells can be estimated using the equation: $N = N_0 2^n$, where N_0 is the number of cells in the starting population and n is the number of generations.

- 2a. If the 10 replicate cultures had an initial time lag of 2 hours and 30 minutes before starting to divide and a generation time, or doubling time, of 45 minutes, how many new generations (i.e. how many doublings) would there be in 22 hours? Show your calculation.

$$22 - 2.5 = 19.5 \text{ hours} \times 60 \text{ minutes/hour} = 1170 \text{ minutes of exponential growth } 1170 \text{ minutes} / 45 \text{ minutes/generation} = 26 \text{ new generations (or doublings) in 22 hours}$$

- 2b. If replicate cultures were started with exactly 50 cells and grew exponentially for the number of generations you calculated above, how many total cells would you expect there to be in the culture at the end of 22 hours? Show your calculation.

$$50 \times 2^{26} = 3.35 \times 10^9 \text{ cells}$$

- 2c. The mean transmittance reading for 1:10 dilutions of the 10 replicate cultures after 22 hours of growth was 28%. Each culture contained 10 ml of broth. Use this information to calculate the following:

$$\text{Mean Optical Density of the 1:10 dilutions of the 10 cultures: } 0.553$$

$$\text{Approximate cells/ml in undiluted replicate culture: } 3.20 \times 10^9 \text{ cells/ml}$$

$$\text{Since ... } 0.553 \times (5.8 \times 10^8 \text{ cells/ml}) \times 10 = 3.20 \times 10^9 \text{ cells/ml}$$

$$\text{Approximate total number of cells in one replicate culture: } 3.20 \times 10^{10} \text{ cells}$$

2d. You assume that *Serratia* grows as described above, but when you measure the optical density on a particular strain after 22 hours of growth, you get a higher value than expected. Give two possible explanations besides measurement error for the higher value.

Correct explanations include:

- 1) *the actual lag time was shorter than 3 hours,*
- 2) *the actual generation time was shorter than 45 minutes,*

2e. In lab, you plated out 200 μ l of the 1:10 dilution for each replicate culture. About how many cells would you expect in the 200 μ l that you spread on each plate? Show your calculation.

$$0.2 \text{ ml} \times (3.20 \times 10^8 \text{ cells/ml}) = \text{about } 6.4 \times 10^7 \text{ cells}$$

2f. You realize that you won't be able to count this number of colonies growing on a plate, so you decide to further dilute a 1:10 dilution of one of the replicate cultures to get a countable number of colonies on a control plate (assume that you don't want to count more than 500 cells).

How much of the 1:10 dilution would you transfer into broth to make a total volume of 10 ml to get a 1:10,000 dilution of the original (undiluted) replicate culture? (Assume that you have the right amount of broth to start with so that when you add your amount of 1:10 dilution you end up with exactly 10 ml.) _____ 10 μ l (0.01 ml) _____

Would you plate from this dilution? ___ *No, it is too concentrated* _____

If yes, what volume would you plate and why?

If no, what dilution would you use for plating and about how many colonies would you expect to see?

In order to easily count cells, you need less than 200 cells per plate. A dilution of 1:10000 still has about 3.2×10^5 cells/ml, but a dilution of 1:10⁶ would have about 3.2×10^3 cells/ml and you could plate 100 μ l to get about 320 cells/plate. A 1:10⁷ dilution could also work (32 cells in 100 μ l and 64 cells in 200 μ l). A 1:10⁵ dilution should only get partial credit since it is difficult to effectively plate only 10 μ l and more than 320 cells/plate will start getting hard to count.

Haddix, P. L., E. T. Paulsen, and T. F. Werner. (2000) Measurement of Mutation to Antibiotic Resistance: Ampicillin Resistance in *Serratia marcescens*. *Bioscience*, 26(1), 17–21.