

Battle of the Bacteria: Characterizing the Evolutionary Advantage of Stationary Phase Growth

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Cells grown into long-term stationary phase (LTSP – greater than 10 days) are better adapted to the environment, and thus are able to outcompete un-aged cells. This is the growth advantage in stationary phase (GASP) phenomenon. This module is hypothesis driven, and allows the students to alter the culture medium in a manner of their choosing to uncover the variable's impact on the GASP phenotype and its relationship with cell stress and frequency of genome mutation. In doing so, the students are capable of seeing evolution occur within this short module. The below report is an abbreviated and updated summary of work previously published (Kram *et al.*, 2016). For more information, please reference this paper or contact the authors.

Keywords: microbiology, evolution, discovery-driven experiment, long-term stationary phase (LTSP), growth advantage in stationary phase (GASP)

Introduction

While essential to life, student understanding of evolution can be lacking. One common criticism is that species evolution happens on such a large time scale, and that an inability to observe it in real-time makes it somehow less credible. This is clearly not the case with microorganisms though, which reproduce much more rapidly. Bacterial evolution has been demonstrated in many scenarios, one of the most famous being Luria and Delbrück's fluctuation test with bacteriophages (Luria and Delbrück, 1943). This experiment demonstrated that mutations in a population can occur randomly and in the absence of a specific stress, as bacteriophage resistant cells arose whether or not the phages were present in the culture. Selection of these mutations and evolution of the population can then occur in the presence of the stress. This module begins with this premise of mutations occurring naturally and then leads to the ability to characterize the surviving population's phenotype. Does

the GASP population outcompete fresh cells only in the initial selective environment or does this apply to different types of media? Do we observe genetic or physiological changes in a major pathway responsible for survival in LTSP? Does the cell stress or mutation frequency change under various environments and does this impact the degree of the GASP phenotype?

There are four research questions addressed by the different experimental components of the module. It is up to the instructor how many of these to include, and can range from either the first question alone, to that in combination with some or all of the others. The research questions are in Table 1.

The module requires four weeks of the curriculum, including time for students to select a variable and develop a testable hypothesis and perform four different protocols to study various aspects of the phenotype. A rough outline of the experimental schedule, based on our lab course, which meets twice a week, is listed in Table 2.

Table 1. Module Research Questions.

Expt #	Experiment	Research Question(s)
1	GASP assay	<ol style="list-style-type: none"> 1. Does growth into long-term stationary phase select for certain mutations (existing or spontaneous) that result in cells with a growth advantage? 2. Does the addition of Variable X to the LB alter the growth advantage for long-term stationary phase cells? 3. Is the growth advantage observed in LB + Variable X specific for growth in LB + X or evident in regular LB as well?
2	Catalase tests	<ol style="list-style-type: none"> 1. Does RpoS (a transcription factor involved in stationary phase growth) activity change in cultures that are grown to LTSP? 2. If RpoS is being downregulated in LTSP, is this due to a phenotypic or genotypic modification of RpoS? 3. Do the answers to these questions differ for cells grown in LB + Variable X?
3	β -gal stress assay	<ol style="list-style-type: none"> 1. Does the amount of stress experienced by the cells correlate with the intensity of the GASP phenotype? 2. Does the addition of Variable X to the medium alter cell stress?
4	Mutation frequency measurement	<ol style="list-style-type: none"> 1. What is the frequency of mutations (as measured by antibiotic resistance) in the bacteria population? 2. Does this frequency change in LB + Variable X media? 3. Does the frequency of mutation correlate with the results of the GASP experiment?

Table 2. Module Schedule.

Lab Period	Lab Procedure(s) and the experiment # they are tied to
Week 1A	(1) Start <i>E. coli</i> cultures
Week 1B	(2) 1st Catalase test, (4) Rifampicin mutation
Week 2A	(3) β -gal stress assay
Week 2B	N/A
Week 3A	(1) Set up co-culture, (1) 1st dilution plating for co-culture, (2) 2nd Catalase test
Week 3B	(1) 2nd Dilution plating for co-culture, (2) 3rd Catalase Test
Week 4A	(1) 3rd Dilution Plating for co-culture
Week 4B	N/A

Student Outline

Week 1 – Begin Bacteria Evolution Experiment

Evolution of populations occurs in two steps. First, organisms within those populations gain mutations as part of normal biological processes. Second, conditions in the environment allow a few organisms with beneficial mutations to survive longer than their non-mutated counterparts. Those organisms with beneficial mutations are more fit, and will be selected for over time.

Experiments conducted by scientists have shown that mutations arise in a population regardless of the environment. This is because there is a constant mutation rate simply due to mistakes made in DNA replication and repair. One example is the famous fluctuation test done by Luria and Delbruck (1943). The scientists demonstrated that mutations, which confer resistance to bacteriophage attack, occur randomly during growth of bacterial cultures, even when the bacteriophages are not present. This shows that mutations occur during growth with or without the selective environment. If, however, we place the bacteria into selective conditions, we can show that those mutations can be selected out of the population, which leads to evolution. These selective environments generate a number of interesting questions regarding the surviving mutant population. Are the mutations beneficial in other environments, what characteristics of the environment enhance this phenotype, etc.?

In this experiment, we will be growing bacteria in a selective condition, and showing that selection causes the bacteria to be better adapted to their environment after merely a few days of exposure. The selective environment will be long-term stationary phase (LTSP), during which populations of bacteria must survive harsh conditions including low nutrients, basic pH, and high waste product levels.

Bacteria experience five phases of growth during their life cycle: lag phase, exponential phase, stationary phase, death phase, and LTSP (Figure 1).

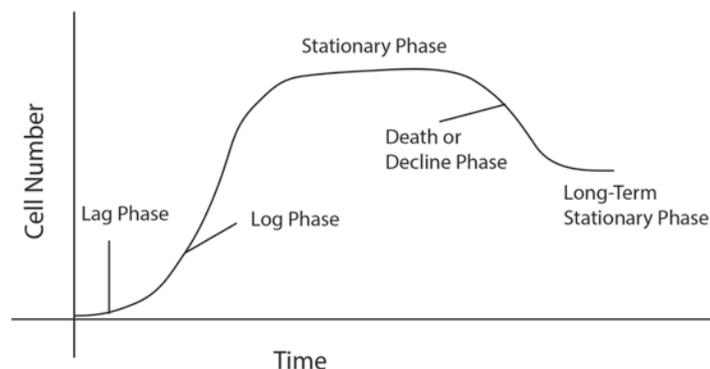


Figure 1. Five growth phases of a bacterial culture.

Most often when we grow bacteria in the lab, we examine them during log phase or early stationary phase growth. However, in the natural environment, and in certain laboratory conditions, bacteria more often experience LTSP. Some strains of *Escherichia coli* can survive in LTSP without the addition of nutrients for years.

We can take bacterial populations that have been grown for over 10 days into LTSP, and compete them with un-aged bacteria in fresh media. The aged bacteria will be better adapted to the environment, and will therefore grow faster than the un-aged cells. We call this phenomenon growth advantage in stationary phase (GASP). Further, we will age our cells in a slightly different environment, which will change the conditions to which it will adapt, and determine whether or not those adaptations also help them in the original environment. Some conditions may select mutations that give the bacteria an advantage in a wide variety of environments, while other conditions may select for mutations that only give an advantage to the bacteria in that specific environment. Using data from this experiment, we can determine what factors influence adaptation.

We will also characterize the GASP phenotype displayed by your cells. Are cells exhibiting the GASP phenotype surviving due to physiological adaptations or genetic mutations? Does increased environmental stress assist or hinder the growth advantage? Do GASP populations also experience a higher mutation frequency? With these experiments, we will illustrate that our aged strain is a genetically different strain from the strain that began the experiment. We will see evolution occurring in real-time! Keep in mind that this is also novel work you will be conducting in collaboration with Dr. Steven Finkel's lab at USC. You have a shot of identifying something that nobody has ever seen!

Below is an outline of the timeline of each experiment, and the specific questions that experiment will be answering. In addition, you will be examining how cells grow in LTSP when the medium is altered. You and your group can choose one of the below variations of LB.

LB + 0.5% NaCl

LB + 50 mM CaCl or MgCl LB + 0.5% tryptone

LB + 0.5% yeast extract

LB + 0.2% glucose or fructose or sucrose

Experiment I - Growth of cells into long-term stationary phase and GASP assay

We need to select for cells that exhibit the GASP phenotype, by first growing a culture of *E. coli* into long-term stationary phase and then adding the survivors to a fresh bacteria culture. We will then compare survival of the aged versus the un-aged cells using different antibiotic resistance markers to distinguish each (Figure 2).

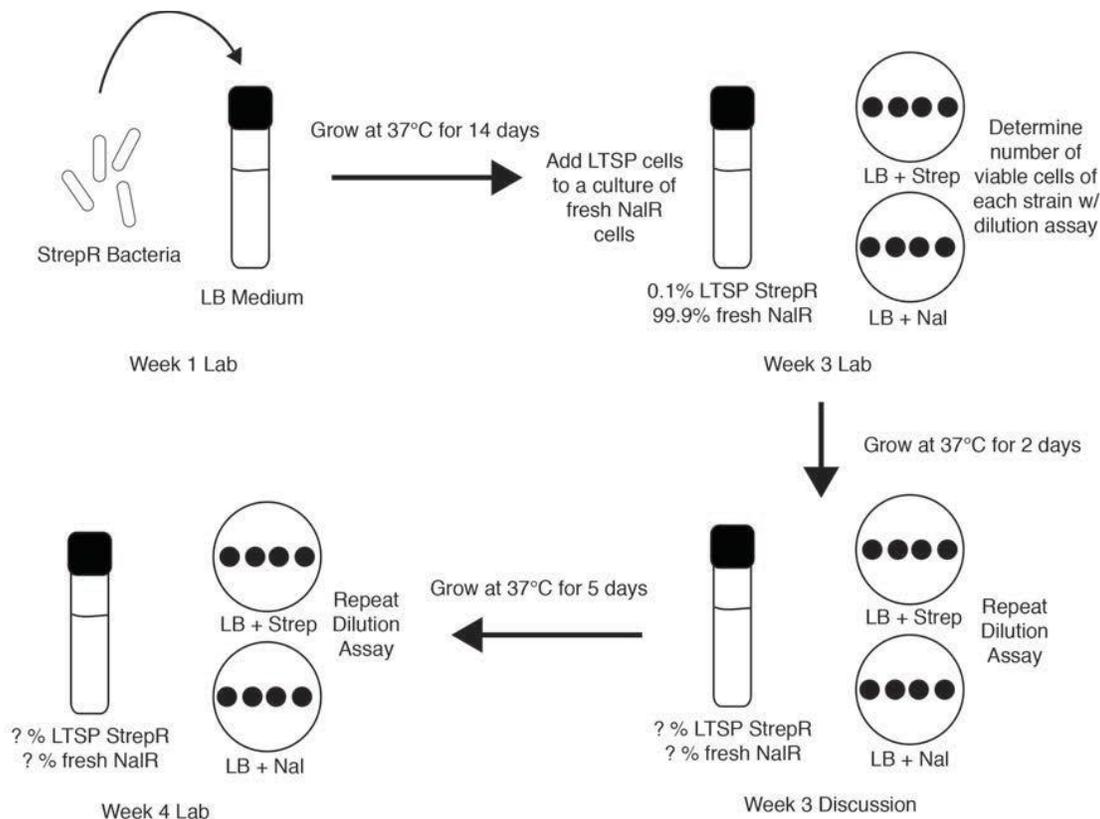


Figure 2. Overview of co-culture experimental design.

In addition, you will be modifying the culture medium in a specific manner (by adding Variable X) to determine how it impacts the GASP phenotype. For this aspect of the experiment, we will need to make slight modifications to the general experimental protocol outlined below.

The experimental questions for experiment I are as follows.

1. Does growth into long-term stationary phase select for certain mutations (existing or spontaneous) producing a culture with a growth advantage?
2. Does the addition of Variable X to the LB alter the growth advantage for long-term stationary phase cells?
3. Is the growth advantage observed in LB + Variable X specific for growth in LB + X or evident in regular LB as well?

Experiment II - Examination of RpoS activity - Genotypic vs. Phenotypic change in extreme conditions

RpoS is a transcription factor that activates genes responsible for fighting cell stress and allowing cells to enter stationary phase. Surprisingly, RpoS activity appears to be detrimental to cell survival in long-term stationary phase, and many cells will downregulate RpoS in LTSP. In this part of the experiment, you will determine whether your LTSP cells have decreased RpoS activity. If so, this may be accomplished through one of two mechanisms, cellular downregulation of RpoS (through

decreased transcription or increased protein degradation, for example) or through mutation, which results in chronically decreased RpoS levels.

One of the targets of RpoS is catalase, and you will measure RpoS activity by examining hydrogen peroxide breakdown by your cells (Figure 3). By conducting this test at various intervals throughout the experiment, you will answer the following questions.

1. Does RpoS activity change in cultures that are grown to LTSP?
2. If RpoS is being downregulated in LTSP, is this due to a phenotypic or genotypic modification of RpoS?
3. Do the answers to these questions differ for cells grown in LB + Variable X?

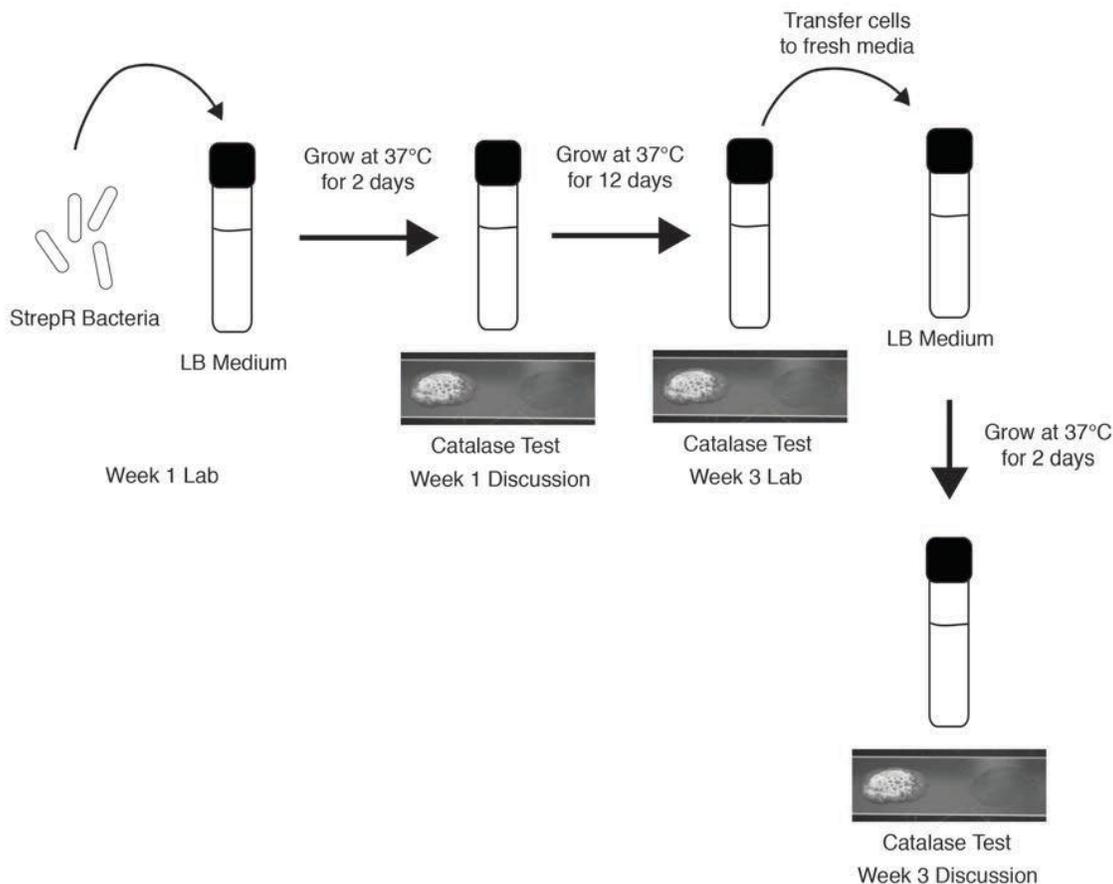


Figure 3. Overview of RpoS activity experimental design.

There are a few potential results you may see.

1. If catalase expression is similar on day 2, 14 and the following day 2, RpoS activity is unaltered by LTSP.
2. If catalase expression decreases on day 14, but is restored when the cells are added back to fresh media, RpoS activity is altered by LTSP conditions. This signifies a physiological, but not genomic change.
3. If catalase expression decreases on day 14 and remains decreased in fresh media, this signifies a genomic change to RpoS.

Experiment III - Cell stress measurement

Environmental stress can be a major factor in driving the evolution of organisms. Bacteria are equipped with cellular mechanisms to handle a variety of stresses, including elevated temperatures, oxidative stress, and low nutrient environments, among others. But in extreme scenarios, it is likely that the stress will overwhelm these pathways and the cells will be unable

to survive. In rare cases, it is also possible that cells contain or acquire beneficial mutations that allow them to survive to a greater extent in these environments compared to the rest of the population.

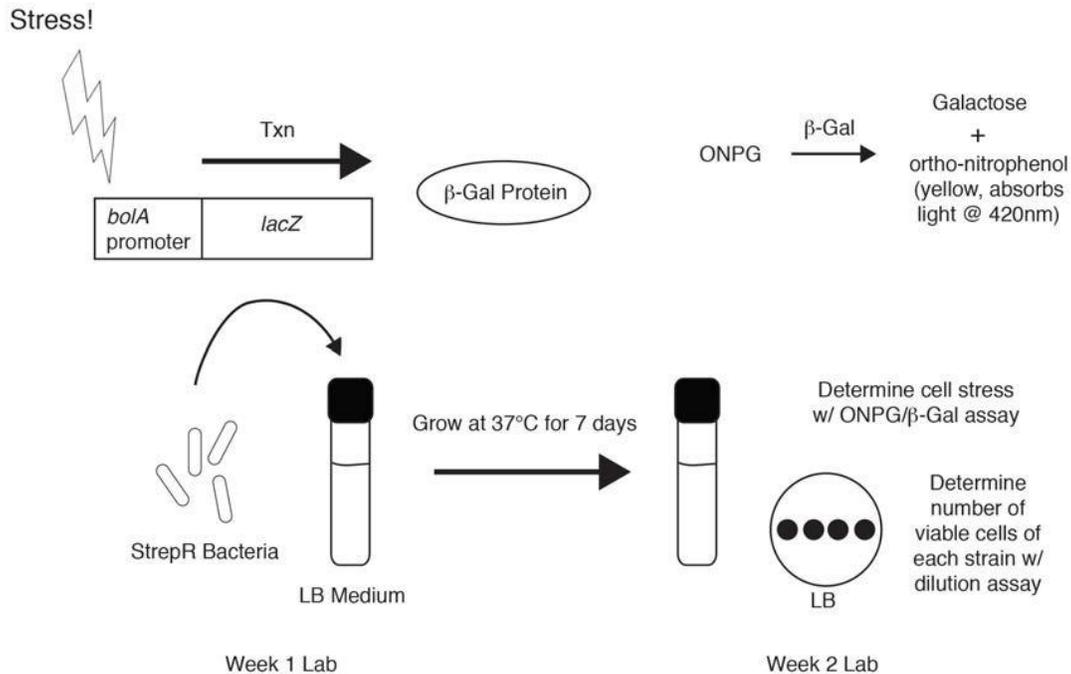


Figure 4. Overview of cell stress experimental design.

We will measure cell stress using a *lacZ* fusion protein, driven by the *bolA* promoter (Figure 4). *bolA* is part of the *E. coli* stress response and is upregulated under carbon starvation, increased osmolarity and oxidative stress. When stressed, the cell will transcribe from the *bolA* promoter, turning on the *lacZ* gene resulting in the production of β -galactosidase protein. We will measure β -galactosidase activity with the addition of its substrate, ortho-nitrophenyl- β -galactoside (ONPG), which is broken down to produce ortho-nitrophenol, a molecule with a yellow color. We can measure the amount of this molecule using the spectrophotometer.

With this reporter protein, we can ask the following questions.

1. Does the amount of stress experienced by the cells correlate with the intensity of the GASP phenotype?
2. Does the addition of Variable X to the media alter cell stress?

Experiment IV - Examination of mutation frequency

Environmental stresses can promote the formation of mutations, for example through increased DNA alterations caused by base damage or decreased activity of DNA repair pathways. While most are likely detrimental or have no effect on the cell, it is also possible a cell will acquire a beneficial mutation. Normally your cells are sensitive to rifampicin, but it is possible for them to become antibiotic resistant. In this experiment, you will be measuring the mutation frequency in your bacterial culture to answer the following questions (Figure 5).

1. What is the frequency of mutations in the bacteria population?
2. Does this frequency change in LB + Variable X media?
3. Does the frequency of mutation correlate with the results of the GASP experiment?

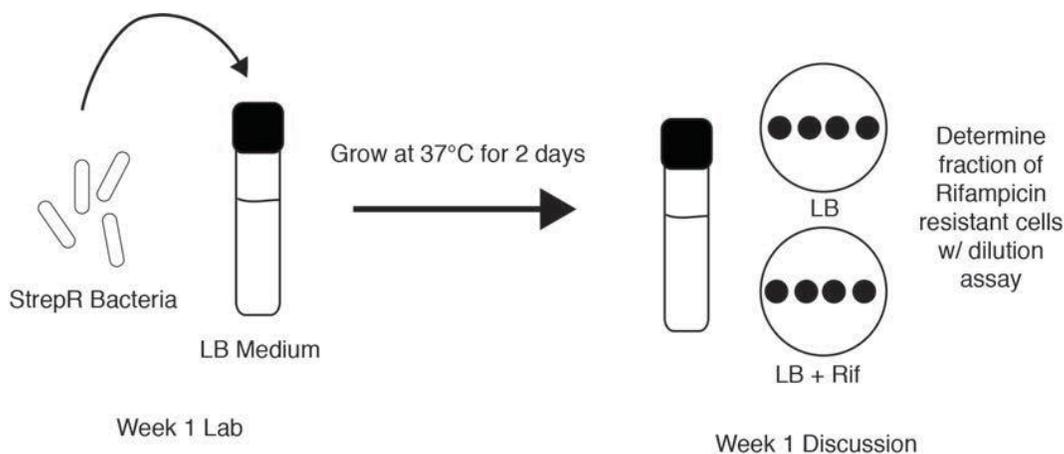


Figure 5. Overview of mutation frequency experimental design.

Week 1 Lab

Experiment I - Growth of Cells Into Long-Term Stationary Phase And GASP Assay

Materials: two test tubes, variable X stock solution, StrepR *E. coli* culture, LB medium.

This week you will start the cultures you will use for the GASP experiment in two weeks. You will be setting up two cultures, one with streptomycin resistant *E. coli* in LB and a second with the same strain in LB + Variable X. When choosing your variable, be sure to think about how it might affect the experiment. Think about the role that the variable plays in the media.

This experiment will be conducted in pairs, although your group (of four) will choose a variable together. That way each pair will perform the same experiment and you can verify each other's results.

The variable you and your group have chosen: _____

1. Set up two tubes. On a piece of tape, label one as LB and one as LB + (your variable). Include your and your partner's initials, the date, and your section day (TUES/WED), time (AM/PM) and room number (226/230). Place the tape on the tube, not the lid.
2. In each tube, add 5 mL of LB and then 5 μ L of the overnight culture (the StrepR *E. coli*).
3. You now want to add your variable X. The stock variable solutions are all 25 X, meaning they are 25 times as concentrated as you need. Calculate how much of the stock you will add to the LB + X culture. In the $C_1V_1 = C_2V_2$ equation, $V_2 = 5$ mL. In addition, to keep the volumes in each tube equal, you will add an equal amount of LB to the LB only tube.

Volume you will add: _____

4. Place each tube in your section's rack for incubation at 37°C.

Week 1 Discussion:

Experiment II - Examination of RpoS activity

Materials: two cultures from lab (LB only, LB + X), microscope slide, H₂O₂

We want to measure catalase activity (and thus RpoS activity) before the cells reach LTSP. This test will be performed similarly to the catalase test performed earlier in the quarter.

1. Agitate each tube if a pellet is at the bottom of the tube.
2. Remove 5 μ L of each of your cultures and add them to a glass slide.
3. Add one drop of H₂O₂ to each aliquot.
4. Note whether bubbles form (large/small, many/few, form immediately/after time, etc.).

Experiment IV - Examination of mutation frequency

Materials: one LB plate, one LB + Rif plate, one empty petri dish, liquid LB

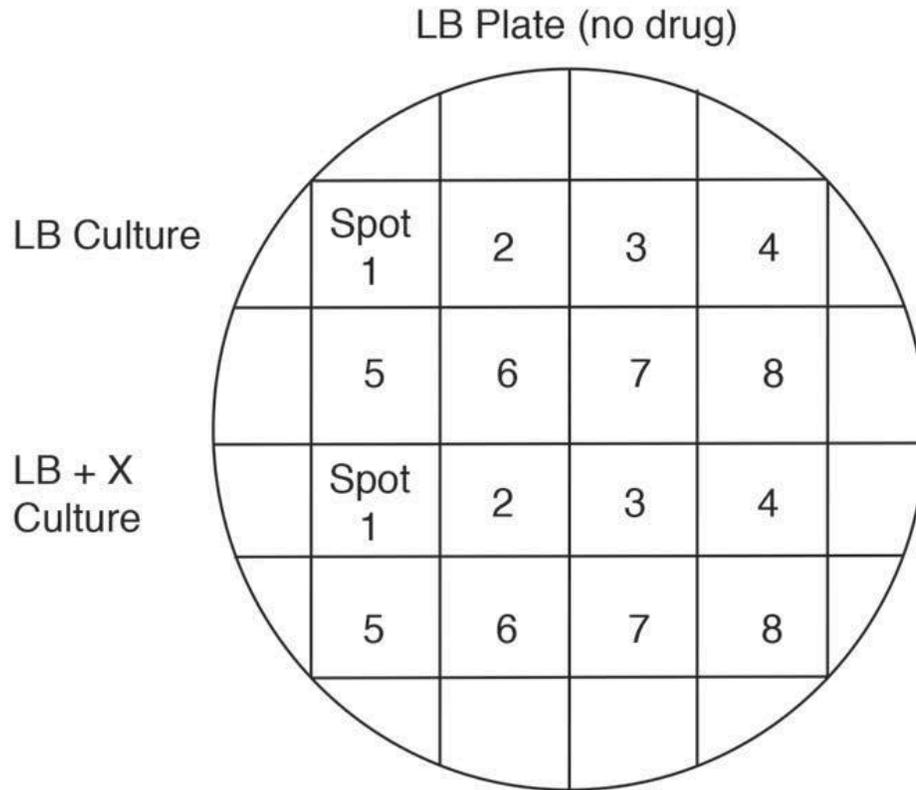
Normally your bacteria are rifampicin sensitive. It is possible that they acquire a mutation that renders them rifampicin resistant. You will examine this by plating your cultures on an LB + Rif plate. In addition, you will plate them on an LB plate to determine the total viable cells in the population to calculate the percentage of the culture that is rifampicin resistant.

For most tests of cell viability, like the LB media plating you will perform today, you will make 1:10 serial dilutions, plating 10 μ L of each dilution on the plate, eight dilutions total.

Each 1:10 dilution will involve adding 90 μ L media and 10 μ L cells. These dilutions will be performed using an empty petri dish (either the lid or the dish), where you add eight spots of 90 μ L LB. You will then transfer 10 μ L of your culture to the first spot, pipette up and down a few times and mix the drop with the pipette tip. The next spot will be made with 10 μ L of the first spot and 90 μ L LB, mixing by pipetting up and down and using the pipette tip. This will be repeated for all eight spots. Your TA will demonstrate this procedure for you.

To determine the frequency of rifampicin resistant cells in the population, you must identify how many cells are rifampicin resistant (by plating on LB + Rif) and comparing this to how many viable cells there are total (by plating on LB alone). We anticipate the frequency of rifampicin resistant cells to be low, so there will be no need to dilute the culture plated on LB + Rif.

1. Take one LB plate and one LB + Rif plate. Label the bottom of these, along with your identifying information.
2. Prepare your LB plate first. In an empty petri dish, you will make eight spots with 90 μ L LB in each. Do not let the drops merge. You should plate one culture in its entirety before moving on to the next.
3. Take 10 μ L of one of your cultures (well-mixed) and add it into the first drop. Pipette up and down and stir the drop with the pipette tip to mix the dilution.
4. Using the same tip, pipette 10 μ L of the first drop onto your LB plate. You will use the grid below to keep track of your spots.
5. Take a new pipette tip and remove 10 μ L of the first drop and pipette it in the second drop. Mix the solution as above and pipette 10 μ L onto the LB plate (onto the #2 on the grid).
6. Repeat for drops three through eight.
7. Repeat steps 1-5 for your second culture.
8. Let the plate dry face up. Then flip upside down and incubate at room temperature. If it does not dry in time, leave face up on the room temperature shelf.
9. Now prepare the LB + Rif plate. Because the number of rifampicin resistant cells will be low, you will not do the above procedure. Instead, plate 100 μ L of your first culture (well- mixed) on an LB + Rif plate.
10. Spread the 100 μ L using the ethanol spreader (dip in ethanol, briefly flame, let cool by placing on a region on the plate where the cells are not).
11. Repeat this with your second culture using another LB + Rif plate.



Next lab period, you will count the number of surviving cells on the LB and LB + Rif plates.

Week 2 – Continue Bacteria Evolution Experiment

This week you will continue incubating your cultures so they can reach long-term stationary phase by the following lab period. There are two questions you will be working to answer: What is the frequency of mutations in the LB and LB + X cultures? And how much stress do the cells experience in LB and LB + X media?

Experiment IV - Examination of mutation frequency

This is a continuation from your work from last discussion where you will analyze the growth observed to determine the frequency of rifampicin resistance.

Your mutation frequency will be based on counting colony forming units (CFU). CFU estimates the number of viable cells by assuming each viable cell is capable of cell division resulting in the formation of a colony. So CFU is determined by counting colony number on a plate.

The equation for the mutation frequency will be:

$$\frac{\text{CFU/ml Rif Res Bacteria (on LB + Rif plate)}}{\text{CFU/ml Total Cells (on LB plate)}}$$

1. To determine the CFU/mL for Rif resistant bacteria you will count the total colonies on the LB + Rif plate. Note that number here: _____ (LB only) _____ (LB + X media). Remember, you plated 100 μL of your original cultures onto the LB + Rif plate. So rather than identifying the CFU/mL, your values above represent the CFU/0.1 mL. This means you need to multiply the numbers above by a factor of _____.

Your CFU/mL values for Rif resistance cells are: _____ (LB) _____ (LB + X)

2. To determine the CFU/mL for all viable cells (plated on the LB media), you will look at your dilution spots. The more concentrated spots are likely completely covered with growth and it is no longer possible to count individual colonies. Instead, select the spot with individual colonies.

Colonies from LB only: _____ Spot # _____ Colonies from LB + X: _____ Spot # _____

Now you need to determine the CFU/mL. Imagine you had two colonies on spot five. The very first spot was created by adding 10 μ L of cells to 90 μ L LB and plating 10 μ L ($1/10^{\text{th}}$) of this dilution. Since you plated $1/10^{\text{th}}$ of that original 10 μ L (from the culture), you actually only added 1 μ L of the original culture. This means the growth in the first spot signifies the CFU/0.001 mL or CFU/ 10^{-3} mL. Spot number two is a 1:10 dilution of the first, so growth in this spot signifies the CFU/ 10^{-4} mL.

This means the two colonies on spot five signify a value of 2 CFU/ 10^{-5} mL or _____ CFU/mL.

Now, based on this little tutorial, calculate your CFU/mL for all viable cells using the values you noted from your LB plates.

LB only CFU/mL: _____ LB + X CFU/mL: _____

And finally, you can calculate your mutation frequency:

$\frac{\text{CFU/mL Rif Resistant Bacteria (on LB + Rif plate)}}{\text{CFU/mL Total Cells (on LB plate)}}$

LB only: _____ LB + X: _____

Experiment III - Cell stress measurement

Materials: Z buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 50 mM BME), phosphate buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4), 0.1% SDS, 4 mg/mL ONPG, 1 M Na_2CO_3 , spectrophotometer

Another goal in our experiment is determine how stressed the cells are in a given medium. This will allow us to see if there is any correlation between cell stress and the GASP phenotype. As mentioned previously, we will measure stress with our *bolA-lacZ* reporter gene. Stress will lead to *bolA* promoter activation, *lacZ* transcription, and β -galactosidase production. β -galactosidase activity can be determined by adding a substrate, ONPG, which is converted to a yellow product. The amount of this yellow product can be measured with a spectrophotometer.

Spectrophotometry allows one to measure the components in a solution. Different molecules are capable of absorption of specific wavelengths of light. This absorption can be measured with a spectrophotometer (Figure 6). A solution is added to a cuvette that is placed into the spectrophotometer. Light of a specific wavelength is directed at the cuvette and may be absorbed by specific molecules. The amount of light, which is not absorbed and passes through the cuvette, is read resulting in the calculation of an absorbance. More of that specific molecule results in more light absorbed and a higher absorbance value, known as the optical density (O.D.).

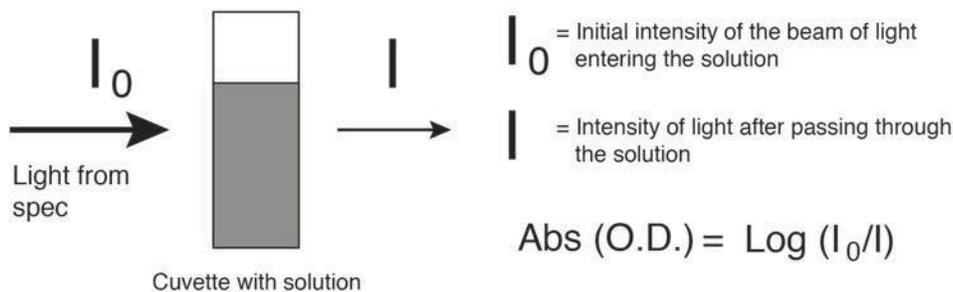


Figure 6. How a spectrophotometer measures optical density of a solution.

For our purposes, there will never be any need to use this equation for absorbance, but it is helpful for understanding how the spectrophotometer is able to measure this value.

Since we want to know the light absorbed by a specific molecule (in this case ortho-nitrophenol), we need to eliminate any light absorbed by the buffer. To eliminate the absorbance by any components in the buffer, we first “blank” the

spectrophotometer with a cuvette containing only buffer. Thus, when we add the cuvette with ONPG and β -galactosidase, the resulting absorbance will be due only to ortho-nitrophenol.

Below is the protocol for the spectrophotometer based β -galactosidase assay. The purpose of the experiment is to lyse the cells to expose the β -galactosidase protein to the ONPG substrate. In addition, you will need to determine the viable cell count in the population similarly to what you did the past week with the rifampicin assay. This is necessary because higher β -galactosidase activity could merely be due to a greater number of viable cells, and not necessarily greater *bolA* activation in each cell.

Before you begin, familiarize yourself with the spectrophotometer (Figure 7).

1. On the screen, confirm that the wavelength is set at 420 nm (not 600 nm as above).
2. Next, add your blank cuvette (Z buffer only) to the spectrophotometer and hit “blank”.
3. After this is complete, you will add your cuvette containing your β -galactosidase assay.
4. Hit the green read sample button. This will generate an absorbance (OD) value.

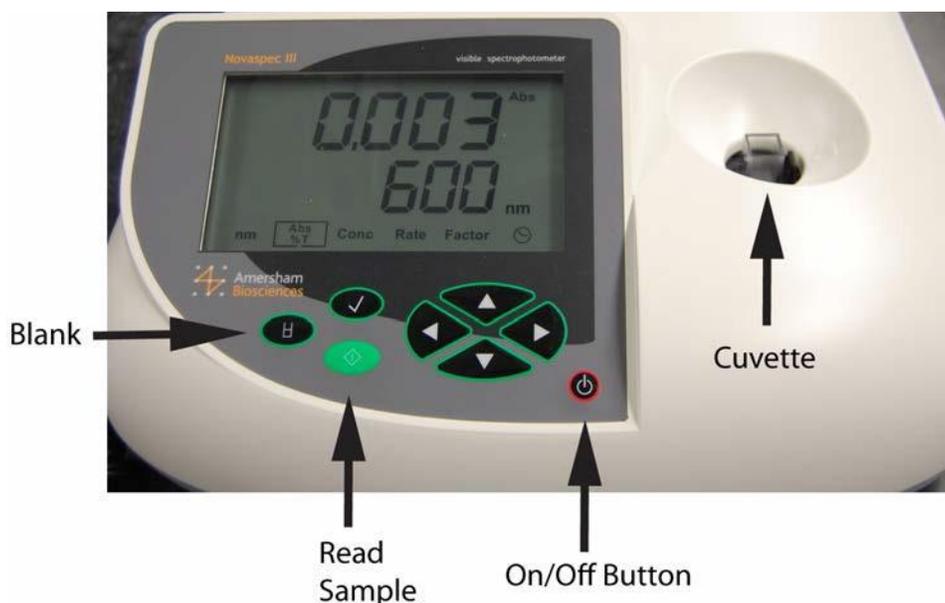


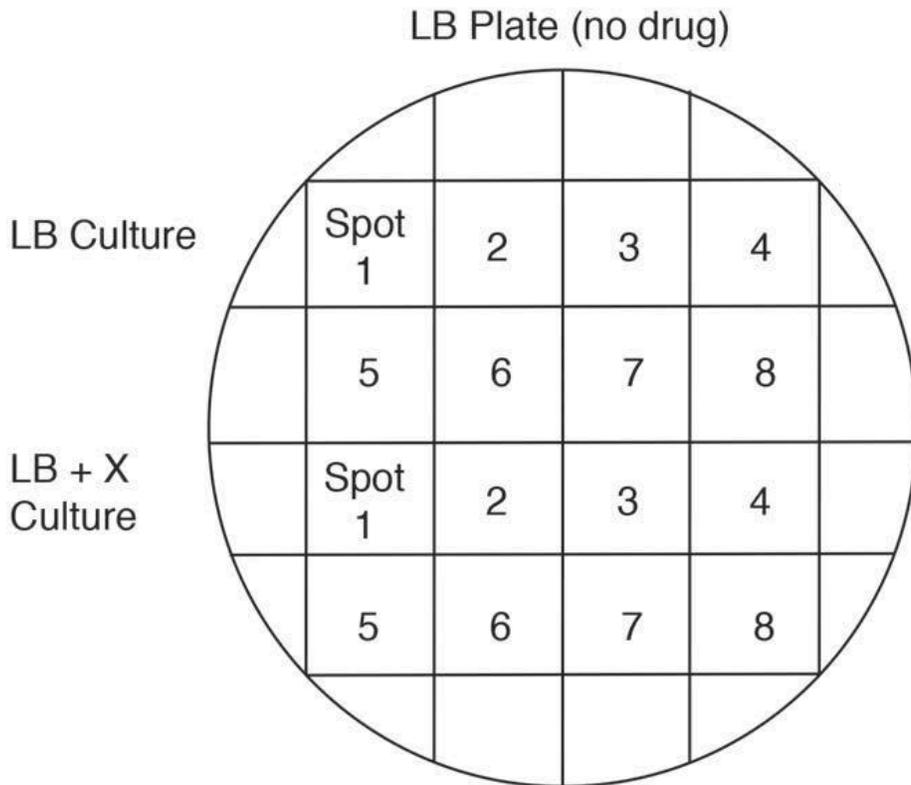
Figure 7. An example of spectrophotometer. The specific machine you use in your lab may vary.

Week 2 Lab

1. Remove 0.5 mL of each culture (one week old LB only, LB + X cultures) to two 1.5 mL Eppendorf tubes. The following steps will be performed for each of the tubes.
2. Centrifuge on full speed for 1 minute to pellet the cells. Pipette off the liquid but do not disturb the pellet. This removes the media, which may contain molecules that impair β -galactosidase activity or light absorbance by the spectrophotometer.
3. Resuspend the pellet in 0.5 mL chilled Z buffer.
4. In a new 2.0 mL tube, add 900 μ L Z buffer and 100 μ L of cells (resuspended in Z buffer).
5. Add 50 μ L SDS and mix by inverting the tube a few times. SDS is a detergent, which will lyse the cells.
6. Let the tubes sit on your bench for 5 minutes at room temperature.
7. Add 200 μ L ONPG to the tube and mix by inverting a few times.
8. Incubate the tube in the 28°C water bath until you see color (usually around 15 minutes).
9. Once you see yellow color in both tubes, stop the reaction by adding 0.5 mL Na₂CO₃ and mix by inverting a few times. The sodium carbonate raises the pH to 11, which inactivates the β -galactosidase protein.
10. Transfer 1 mL of the solution to a new 1.5 mL tube and centrifuge for 5 minutes at max speed.
11. Instructions for using the spectrophotometer are on the following page. Blank the spectrophotometer with 1 mL Z buffer in a cuvette (at OD₄₂₀).
12. Remove the supernatant from the centrifuged tubes to cuvettes.
13. Determine the OD of the tubes. LB only OD₄₂₀: _____ LB + X OD₄₂₀: _____

Determine CFU count for cells

As mentioned above, to accurately measure the β -galactosidase activity, it is necessary to determine the viable cell count in the population. This will be accomplished by performing a dilution assay using LB plates.



1. Prepare your LB plate. In an empty petri dish, you will make eight spots with 90 μ L LB in each. Do not let the drops merge. You should plate one culture in its entirety before moving on to the next.
2. Take 10 μ L of one of your cultures (well mixed) and add it into the first drop. Pipette up and down and stir the drop with the pipette tip to mix the dilution.
3. Using the same tip, pipette 10 μ L of the first drop onto your LB plate. You will use the grid above to keep track of your spots.
4. Take a new pipette tip and remove 10 μ L of the first drop and pipette it in the second drop. Mix the solution as above and pipette 10 μ L onto the LB plate (onto the #2 on the grid).
5. Repeat for drops three through eight.
6. Repeat steps 1-5 for your second culture.
7. Let the plate dry face up. Then flip upside down and incubate at room temperature. If it does not dry in time, leave face up on the room temperature shelf.

Week 2 Discussion:

To determine the CFU/mL for viable cells, you will look at your dilution spots. The more concentrated spots are likely completely covered with growth and it is no longer possible to count individual colonies. Instead, select the spot with individual colonies.

Colonies from LB only: _____ Spot # _____ Colonies from LB + X: _____ Spot # _____

Now you need to determine the CFU/mL using the example from the previous class. Imagine you had two colonies on spot five. The very first spot was created by adding 10 μ L of cells to 90 μ L LB and plating 10 μ L ($1/10^{\text{th}}$) of this dilution. Since you plated $1/10^{\text{th}}$ of that original 10 μ L (from the culture), you actually only added 1 μ L of the original culture. This means the growth in the first spot signifies the CFU/0.001 mL or CFU/ 10^{-3} mL. Spot number two is a 1:10 dilution of the first, so growth in this spot signifies the CFU/ 10^{-4} mL.

This means the two colonies on spot 5 signify a value of $2 \text{ CFU}/10^{-7} \text{ mL}$ or _____ CFU/mL .

Now, based on this little tutorial, calculate your CFU/mL for all viable cells using the values you noted from your LB plates.

LB only CFU/mL : _____ LB + X CFU/mL : _____

And finally, you can calculate your cell stress:

$\frac{\text{OD}_{420}}{\text{CFU}/\text{mL}}$

LB only OD_{420} : _____ LB + X OD_{420} : _____ (values from last class)

LB only: _____ LB + X: _____

Week 3 – Initiate GASP Experiment

This week you will begin your competition experiment to determine whether the LTSP *E. coli* are better able to grow compared to a fresh culture. You will conduct two separate experiments related to this study in lab today.

Experiment I - Growth of cells into long-term stationary phase and GASP assay

Materials: Three test tubes, two aged StrepR *E. coli* cultures (LB, LB + X), fresh NalR *E. coli* cultures, two LB + Strep plates, two LB + Nal plates

Your StrepR *E. coli* have now been growing for two weeks, well into long-term stationary phase. You will now compete them against another strain of *E. coli*, which is resistant to nalidixic acid, but is otherwise identical to the strains you aged. It is because each strain possesses a different resistance marker that we can tell them apart. The competition experiment will start in favor of the new NalR *E. coli*, as they will be found at roughly 1,000 times higher concentration than the aged StrepR strain. To see whether the LTSP culture possesses the GASP phenotype, you will survey the mixed culture today, in discussion and next week in lab. What results would you expect to see if the StrepR *E. coli* are capable of GASP?

In addition, keep in mind that you grew two cultures, one with LB only and one with LB + Variable X. To determine whether growing in Variable X generated a phenotype specific for Variable X or a more general one (that is evident in LB only as well), you will examine three different conditions.

1. LTSP StrepR *E. coli* grown in LB → Add cells to fresh NalR bacteria in LB
2. LTSP StrepR *E. coli* grown in LB + X → Add cells to fresh NalR bacteria in LB
3. LTSP StrepR *E. coli* grown in LB + X → Add cells to fresh NalR bacteria in LB + X

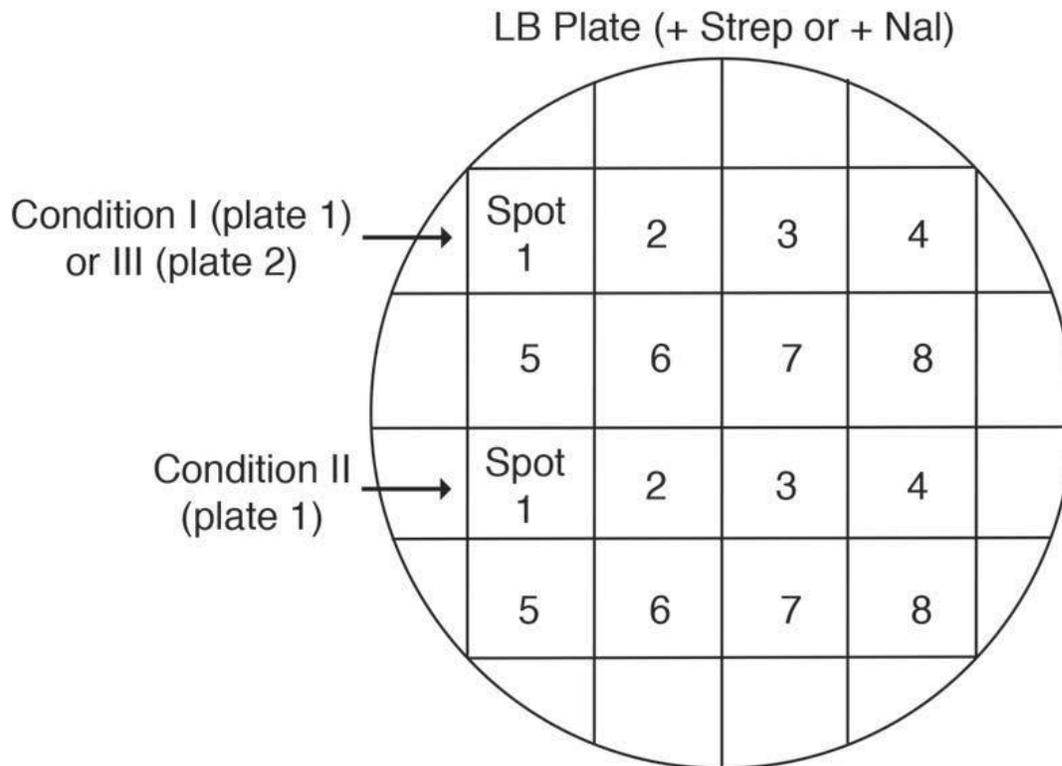
Below is the protocol for today's part of the experiment. This will be similar to the set-up that you used to start the StrepR culture in Week 1.

1. Set up three tubes. On a piece of tape, label one as Condition I (which will consist of LB media/StrepR *E. coli* grown in LB), one as Condition II (which will consist of LB media/StrepR *E. coli* grown in LB + X), and one as Condition III (which will consist of LB + X media/StrepR *E. coli* grown in LB + X). Include your and your partner's initials, the date, and your section day (TUES/WED), time (AM/PM) and room number (226/230).
2. In each, add 5 mL of the NalR overnight culture. This is the NalR bacteria grown in LB.
3. You now want to add your variable X to condition III. Add the same amount of the stock solution to tube III as you did in Week 1. In addition, to keep the volumes in each tube equal, you will add an equal amount of LB to tubes I and II.
4. Shake each tube to mix.
5. Now you need to add the aged StrepR bacteria. Pay careful attention that you add the correct bacteria to the correct tubes! For each, you will add only 5 μL of the LTSP culture. When done, keep the LTSP cultures for the next experiment!
 - To tube I – add 5 μL of the LB only LTSP culture
 - To tube II – add 5 μL of the LB + X LTSP culture
 - To tube III – add 5 μL of the LB + X LTSP culture
6. At the end of the class period, place each tube in your section's rack for incubation at 37°C. But in the meantime there are a few additional things you need to do.

Remember, the goal is to monitor how the LTSP StrepR bacteria grow compared to the fresh NalR bacteria. This will be determined by looking at growth on an LB + streptomycin plate (where only the StrepR bacteria will survive) versus growth on an LB + nalidixic acid plate.

The plating will occur similarly to the rifampicin plating, although that only had 16 spots (two cultures), whereas this will have 24 spots (three cultures). This means you will need to use two of each plate rather than one. Plate 1 will contain conditions I and II. Plate 2 will be for Condition III only. Details are below.

1. Mix the tubes thoroughly before removing any liquid. You should plate one strain in its entirety before moving on to the next.
2. In an empty petri dish, you will make eight spots with 90 μ L each. Do not let the drops merge. Eventually you will have 24 spots, which may require you to use both the lid and bottom of the empty petri dish.
3. Take 10 μ L of one of your cultures (start with condition I) and add it into the first drop. Pipette up and down and stir the drop with the pipette tip to mix the dilution.
4. Using the same tip, pipette 10 μ L of the first drop onto your LB + Strep plate. You will use the grid below to keep track of your spots. Then pipette 10 μ L of the same drop onto your LB + Nal plate. Use the grid from both your and your partner's manuals so that each will be used for one of the plates.
5. Take a new pipette tip and remove 10 μ L of the first drop and pipette into the second drop. Mix and pipette 10 μ L onto both plates.
6. Repeat for drops three through eight.
7. Repeat steps 2-6 for your second culture.
8. Repeat steps 2-6 for your third culture on plate two.
9. Let the plate dry face up. Then flip upside down and incubate at room temperature. If it does not dry in time, leave face up on the room temperature shelf.



Experiment II - Examination of RpoS activity

Materials: two cultures (LB only, LB + X), microscope slide, H₂O₂

We want to measure catalase activity (and thus RpoS activity) now that the cells have reached LTSP.

1. Shake each tube if a pellet is at the bottom of the tube.
2. Remove 5 μ L of each of your cultures and add them to a glass slide.

3. Add one drop of H_2O_2 to each aliquot.
4. Note whether bubbles form (large/small, many/few, form immediately/after time, etc.).

We next want to see whether the catalase activity observed in LTSP is altered when the cells are returned to fresh media. To accomplish this we need to set up new cultures in fresh media. We cannot use the StrepR/NalR mixed cultures you just set up because we are unable to distinguish between the catalase activity of the LTSP StrepR cells and the NalR cells.

1. Take two tubes. On a piece of tape, label one as LB and the other as LB + X. Include your and your partner's initials, the date, and your section day (TUES/WED), time (AM/PM) and room number (226/230).
2. Add 5 mL fresh LB to each. Add the appropriate amount of Variable X to one tube and the same amount of LB to the other.
3. Add 5 μ L of the LTSP culture to each. The LTSP culture in LB will be added to the fresh LB while the LTSP culture in LB + X will be added to the fresh LB + X tube.
4. Stick both tubes in your section's rack to be incubated at 37°C.

Week 3 Discussion:

Experiment I - Growth of cells into long-term stationary phase and GASP assay

It has now been two days since starting your mixed culture. Today, you will be analyzing the plates you inoculated in lab (at day 0) and setting up a new set to see the ratio of StrepR and NalR bacteria today.

First, examine the plates from lab. You want to determine the ratio of StrepR CFU/NalR CFU.

As you did with the rifampicin experiment, find the highest dilution where single colonies are easily distinguished.

LB + Strep

Colonies from Cond I: _____ Spot # _____
 Colonies from Cond II: _____ Spot # _____
 Colonies from Cond III: _____ Spot # _____

LB + Nal

Colonies from Cond I: _____ Spot # _____
 Colonies from Cond II: _____ Spot # _____
 Colonies from Cond III: _____ Spot # _____

Now we need to convert this to CFU/mL for each condition/plate as you did in Week 1 and 2 labs. Be sure to set up your new LB + Strep/Nal plates for today before performing the below calculations. Instructions for this are below.

Calculate the CFU/mL for all viable cells using the values you noted from your LB plates.

LB + Strep

Condition I CFU/mL: _____
 Condition II CFU/mL: _____
 Condition III CFU/mL: _____

LB + Nal

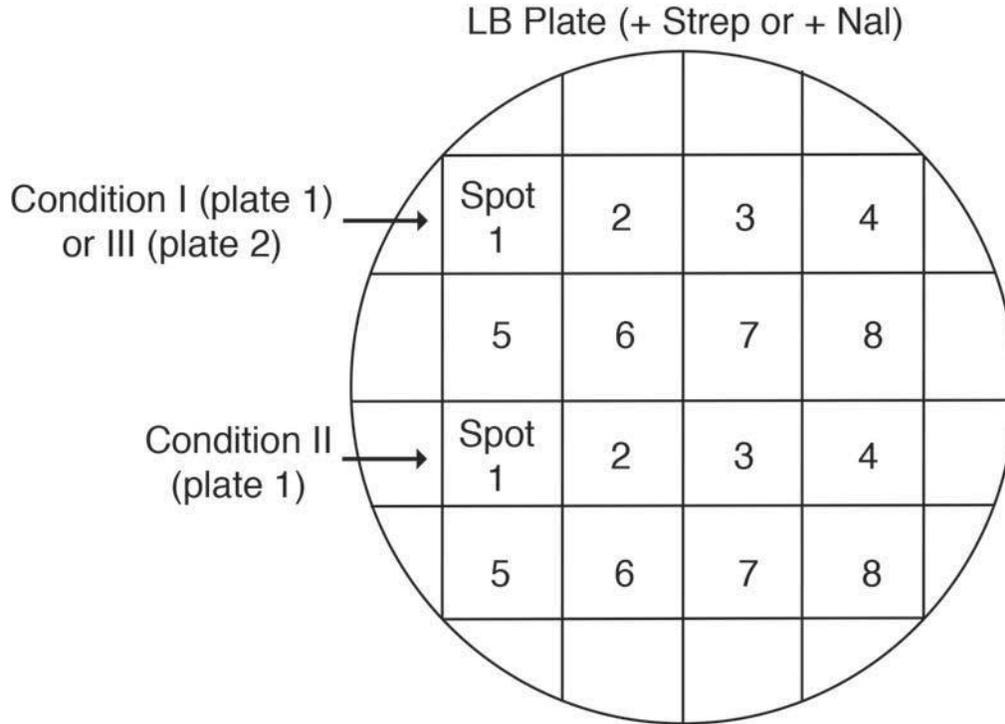
Condition I CFU/mL: _____
 Condition II CFU/mL: _____
 Condition III CFU/mL: _____

And finally, we will now see compare levels of StrepR and NalR cells in the culture. Since these were taken immediately after creating the cultures, we expect them to be in the starting ratio of 1:1,000.

StrepR CFU/NalR CFU: Condition I: _____ Condition II: _____ Condition III: _____

LB + Strep, LB + Nal plating

Materials: two LB + Strep plates, two LB + Nal plates, three cultures (Condition I, II, III)



1. Mix the tubes thoroughly before removing any liquid. You should plate one strain in its entirety before moving on to the next.
2. In an empty petri dish, you will make eight spots with 90 μ L each. Do not let the drops merge. Eventually you will have 24 spots, which may require you to use both the lid and bottom of the empty petri dish.
3. Take 10 μ L of one of your cultures (start with condition I) and add it into the first drop. Pipette up and down and stir the drop with the pipette tip to mix the dilution.
4. Using the same tip, pipette 10 μ L of the first drop onto your LB + Strep plate. You will use the grid above to keep track of your spots. Then pipette 10 μ L of the same drop onto your LB + Nal plate. Use the grid from both your and your partner's manuals so that each will be used for one of the plates.
5. Take a new pipette tip and remove 10 μ L of the first drop and pipette into the second drop. Mix and pipette 10 μ L onto both plates.
6. Repeat for drops three through eight.
7. Repeat steps 2-6 for your second culture.
8. Repeat steps 2-6 for your third culture on plate 2.
9. Let the plate dry face up. Then flip upside down and incubate at room temperature. If it does not dry in time, leave face up on the room temperature shelf.

Experiment II - Examination of RpoS activity

Materials: two StrepR cultures (LB only, LB + X), microscope slide, H₂O₂

We want to measure catalase activity (and thus RpoS activity) now that the cells have reached LTSP.

1. Shake each tube (the tubes with only StrepR *E. coli*) if a pellet is at the bottom of the tube.
2. Remove 5 μ L of each of your cultures and add them to a glass slide.
3. Add one drop of H₂O₂ to each aliquot.
4. Note whether bubbles form (large/small, many/few, form immediately/after time, etc.).

Compare the data from Week 3 Discussion (day 2), Week 4 lab (day 14) and today (following day 2) to see whether you are observing changes in RpoS downregulation that have a genetic or physiological basis.

1. If catalase expression is similar on day 2, 14 and the following day 2, RpoS activity is unaltered by LTSP.
2. If catalase expression decreases on day 14, but is restored when the cells are added back to fresh media, RpoS activity is altered by LTSP conditions. This signifies a physiological, but not genomic change.
3. If catalase expression decreases on day 14 and remains decreased in fresh media, this signifies a genomic change to RpoS.

Week 4 – Continue GASP Experiment

This week you will determine whether your LTSP *E. coli* were able to outcompete the fresh NalR *E. coli*. You will be given a worksheet that must be filled out and submitted to the EEE dropbox within 24 hours after your discussion period. Failure to do so on time will result in a loss of points. As in discussion last week, you will be analyzing the results from the previous class (examining growth 2 days into the GASP experiment) and setting up the 7 day growth plates.

Week 4 Lab

Experiment I - Bacteria Growth After Stationary Phase (GASP) Assay

First, examine the plates from discussion. You want to determine the ratio of StrepR CFU/NalR CFU. As you did previously, find the highest dilution where single colonies are easily distinguished.

LB + Strep

Condition I CFU/mL: _____
 Condition II CFU/mL: _____
 Condition III CFU/mL: _____

LB + Nal

Condition I CFU/mL: _____
 Condition II CFU/mL: _____
 Condition III CFU/mL: _____

Now we need to convert this to CFU/mL for each condition/plate.

LB + Strep

Condition I CFU/mL: _____
 Condition II CFU/mL: _____
 Condition III CFU/mL: _____

LB + Nal

Condition I CFU/mL: _____
 Condition II CFU/mL: _____
 Condition III CFU/mL: _____

And finally, we will now compare levels of StrepR and NalR cells in the culture.

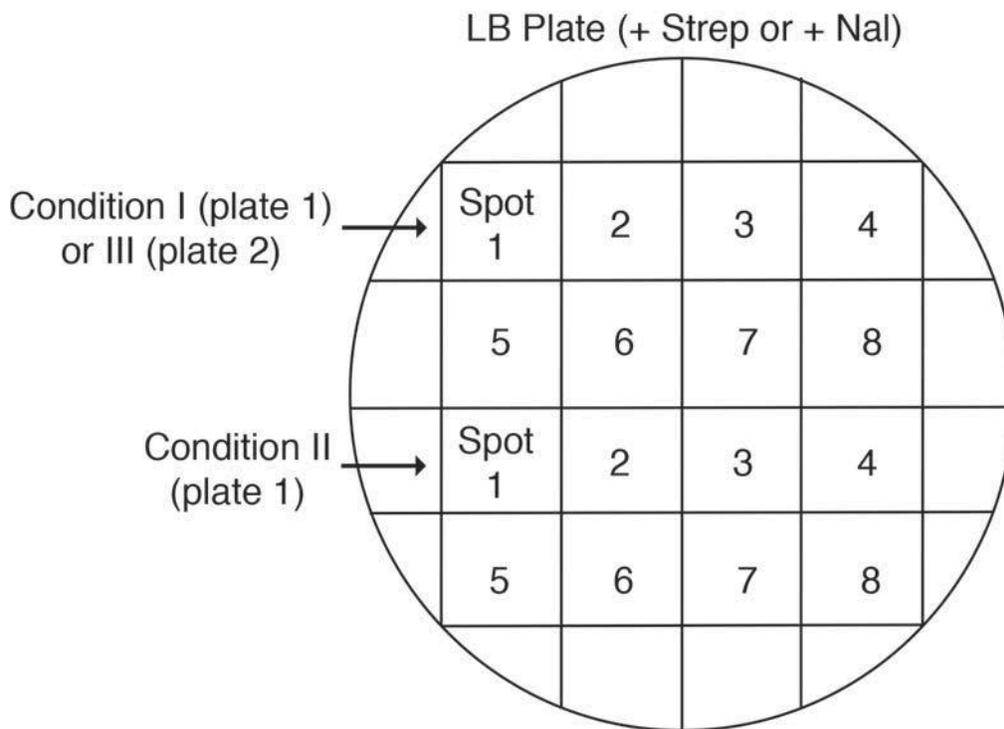
StrepR CFU/NalR CFU: Condition I: _____ Condition II: _____ Condition III: _____

You also need to set up your final LB + Strep and LB + Nal plates to examine the StrepR/NalR ratio at day two of the experiment.

Materials: two LB + Strep plates, two LB + Nal plates, three cultures (Condition I, II, III)

1. Mix the tubes thoroughly before removing any liquid. You should plate one strain in its entirety before moving on to the next.

2. In an empty petri dish, you will make eight spots with 90 μL each. Do not let the drops merge. Eventually you will have 24 spots, which may require you to use both the lid and bottom of the empty petri dish.
3. Take 10 μL of one of your cultures (start with condition I) and add it into the first drop. Pipette up and down and stir the drop with the pipette tip to mix the dilution.
4. Using the same tip, pipette 10 μL of the first drop onto your LB + Strep plate. You will use the grid below to keep track of your spots. Then pipette 10 μL of the same drop onto your LB + Nal plate. Use the grid from both your and your partner's manuals so that each will be used for one of the plates.
5. Take a new pipette tip and remove 10 μL of the first drop and pipette into the second drop. Mix and pipette 10 μL onto both plates.
6. Repeat for drops three through eight.
7. Repeat steps 2-6 for your second culture.
8. Repeat steps 2-6 for your third culture on plate 2.
9. Let the plate dry face up. Then flip upside down and incubate at room temperature. If it does not dry in time, leave face up on the room temperature shelf.



Week 4 Discussion:

As you did previously, find the highest dilution where single colonies are easily distinguished.

LB + Strep

Condition I CFU/mL: _____
 Condition II CFU/mL: _____
 Condition III CFU/mL: _____

LB + Nal

Condition I CFU/mL: _____
 Condition II CFU/mL: _____
 Condition III CFU/mL: _____

Now we need to convert this to CFU/mL for each condition/plate.

LB + Strep

Condition I CFU/mL: _____

Condition II CFU/mL: _____

Condition III CFU/mL: _____

LB + Nal

Condition I CFU/mL: _____

Condition II CFU/mL: _____

Condition III CFU/mL: _____

And finally, we will now compare levels of StrepR and NalR cells in the culture.

StrepR CFU/NalR CFU: Condition I: _____ Condition II: _____ Condition III: _____

Materials

- Micropipettes (P20, P200, P1000) – one set per group of four
- Bench top centrifuge – one per section of 20 students
- Glass tubes (20 mm x 150 mm)
- Spectrophotometer

- E. coli* – both strains express the *bolA-lacZ* fusion protein
- Naladixic acid resistant – SF2054 (W3110 *deltalacU169 tna2 Nalr lambda-bolA::lacZ-Kan-r*)
 - Streptomycin resistant – SF2055 (W3110 *deltalacU169 tna2 Strr lambda-bolA::lacZ-Kan-r*)
 - More information can be found in Farrell and Finkel, 2003.

Lysogeny broth (LB) – for bacteria growth

LB + Drug plates

- Rifampicin – 100 µg/mL (100 mg/ml Rifampicin stock in DMSO)
- Naladixic acid – 20 µg/mL (20mg/mL Naladixic acid stock in water, add NaOH to get in solution)
- Streptomycin – 25 µg/mL (25 mg/mL Streptomycin stock in water)

Reagents per experiment (per group of 4)

Experiment is performed in pairs, although two pairs (a group of four) will perform the identical experiment for repeatability purposes.

25 X Variable solutions (others can be used as well) – make in water, sterile filter before use

- 12.5% NaCl, 1.25M CaCl₂, 12.5% tryptone, 12.5% yeast extract, 5% glucose, 5% fructose

Starting cultures to generate LTSP conditions

- Four glass tubes, 25 mL LB

GASP Assay

- Variable stock solutions, 10 glass tubes, 25 mL LB
- Eight LB + Nal plates, eight LB + Strep plates, and eight empty petri dishes (four for day 0, four for day 2)
- 5 mL sterile water

rpoS downregulation (catalase test)

- a few drops of 3% H₂O₂, microscope slides

Mutation frequency (Rifampicin resistance)

- Two LB plates, four LB + Rif plates, two empty plastic petri dishes, 5 mL sterile water

Cell Stress (β-galactosidase Assay)

- Z buffer (must be made within a few days of use) – 8 mL
- To make 150 mL - 2.4 g Na₂HPO₄•7H₂O, 0.84 g NaH₂PO₄•H₂O, 1.5 mL 1M KCl, 0.15 mL 1 M MgSO₄, 0.405 mL β-mercaptoethanol. Adjust pH to 7.0, then bring to 150 mL with water, keep at 4°C (12 tubes per class, on ice)
- ONPG solution (must be made within a few days of use) – 4 mg/mL in water
- 0.1% SDS solution – 300 µL
- 1 M Na₂CO₃ – 2.5 mL
- Two LB plates, two empty plastic petri dishes
- 5 mL sterile water

Notes for the Instructor

The goal for this module is to collect novel data regarding how environmental changes impact the GASP phenotype. To this end, we plan to establish an online data collection instrument to gather information from other institutions utilizing this experiment. If you are interested in participating, please contact the authors.

As mentioned previously, the specific experiments incorporated from this module can vary based on the available time, reagents, and class schedule. The GASP experiment (experiment I) is essential, but the remaining three experiments do not need to be incorporated. In cases where we have less time available, we have chosen to only include the stress assay (experiment III).

For labs that do not meet twice a week, it is also possible to make modifications to the experimental schedule. For example, the dilution assays associated with the co-culture experiment can be performed on day 7 alone or day 7 and 14 (instead of 2 and 7) for labs that meet weekly. Another option is to have students in different lab groups meet

There may be also some chemicals used in the modules that require specialized collection, depending on your campus' safety regulations. These could include ONPG, β-mercaptoethanol, and SDS, as well as the liquid bacterial cultures and agar plates.

For any additional questions, please do not hesitate to contact the authors.

Literature Cited

- Farrell, M. J., and S. E. Finkel. 2003. The growth advantage in stationary-phase phenotype conferred by *rpos* mutations is dependent on the pH and nutrient environment. *Journal of Bacteriology*, 185: 7044–7052.
- Kram, K. E., K. M. Yim, A. B. Coleman, and B. K. Sato. 2016. Battle of the Bacteria: Characterizing the Evolutionary Advantage of Stationary Phase

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