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BACTERIAL RESPONSES TO ANTIBIOTIC-INDUCED STRESSES

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ABSTRACT

Understanding how bacteria respond to a stressful environment can be beneficial when studying antibiotic resistance. The knowledge gained can suggest methods to reduce the amount of resistance that arises in the future. This thesis focuses on two approaches for exploring how bacteria respond to an antibiotic-induced stressful environment. The first studies the importance of the Rcs phosphorelay, a cell envelope stress response pathway in *E. coli*, and how it contributes to resistance. We monitored how the deletion of various components of the pathway affects contribution of the response to resistance. The second is an ecological approach, where factors related to antibiotic treatment were manipulated and the outcome of the response was monitored. The first method was accomplished by selectively deleting components of the Rcs phosphorelay and observing the difference in the number of colonies that grew when added to ampicillin. Upon deletion of each component, partial dependence on the pathway was observed, as there was a decrease but not a complete reduction in the number of colonies. The most dependence was on the RcsF component when plated on solid medium and the RcsB component in liquid medium. The second method involved manipulation of three ecological factors: timing, intensity, and frequency. We varied the amount of antibiotic added, when the antibiotic was added with respect to the growth curve, and the number of times the antibiotic was added. It was it was determined that timing is most the important factor. The results showed that the addition of amp at the beginning of the growth curve is more effective compared to the middle or end. Additionally, the data reflected that higher intensities of 15 and 20 μg/ml amp given in split amounts across the growth curve was equivalent to smaller intensities of 5 and 10 μg/ml given all at once. This suggests a possible correlation between intensity and frequency.

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Introduction

A major health problem in today's society is the increase in antibiotic resistance in infectious bacteria (**3, 26, 29**). Unfortunately, there is a transition occurring where commercial drugs that have been in use year after year are no longer effective against the most infectious strains of bacteria. Understanding how bacteria become resistant is crucial for developing new drug therapies, but understanding how they respond to antibiotic-induced stress can shed light on how to avoid a problem like this in the future. The knowledge gained from this research could prove useful when dealing with a strain that has acquired resistance to one or a multitude of antibiotics; because it allows us to better understand how bacteria can respond differently to antibiotic treatment.

There are many different types of microbes, for example, bacteria, viruses, fungi, and parasites. While the relationship between most microbes and humans tends to be more commensal, there are some cases where microbes, called pathogens, can lead to infectious diseases. All microbes, except viruses, have the ability to develop resistance to antibiotics (**18, 27**). It is not necessarily a problem when just a few microbes have acquired their resistance, but when this resistance spreads to many different strains, the effect is drastic as these resistant microbes become harder and harder to effectively treat.

This project focuses on two experimental approaches, both pertaining to the question, how do bacteria respond to a stressful environment? The first is looking at cell envelope stress response pathways in bacteria and how altering or inhibiting these pathways affects the initial response to an antibiotic. The second approach is from an ecological perspective and how the

response to antibiotic treatment is influenced through the manipulation of factors related to antibiotic induced stress.

Antibiotic Targeting and Resistance

In recent years, our society has seen an increase in the number of strains that have become resistant to many common antibiotics. This increase in antibiotic resistance has rendered some of our once effective antibiotics completely ineffective (**3, 22, 29**). Unfortunately, there is a common misconception in the public as to what antibiotic resistance really is and what it means for us in the near future. Most people who are uneducated about the topic of antibiotic resistance are under the assumption that humans have acquired the resistance to antibiotics. That, however, is not that case; it is the bacteria inside us that are becoming resistant (**27**).

There are many ways that bacteria can become resistant to antibiotics; specifically, intrinsic resistance and acquired resistance (**6, 9, 11**). Intrinsic resistance occurs when the bacteria has an innate ability to resist an antimicrobial agent. These can be structural or functional, but they are typically inherited, not acquired. For example, many strains of bacteria are naturally resistant through certain structural components, like efflux pumps. Efflux pumps are used to pump the antibiotic out of the cell before it is able to do any real damage (**6**). Acquired, as it suggests, means the bacteria have gained their resistance by changing in a way that allows for their protection. Typically, acquired resistance is the result of genetic changes in which a gene of resistance forms. For example, a mutation in the DNA sequence that codes for a particular protein could alter the shape or function of that protein. An antimicrobial agent that normally uses that protein to access the bacteria will no longer be able to recognize the target

protein, and therefore the bacteria will be unaffected. Another way bacteria can acquire their resistance is through horizontal gene transfer, in which one bacterium is able to copy and transfer their genetic material to different bacteria (**25**). This is especially dangerous because several genetic transfers can lead to multi-drug resistance, making it even harder to treat.

The antibiotic of interest for the work done in this thesis is ampicillin (amp), which belongs to a class known as β-lactam antibiotics (**1, 15**). Each β-lactam antibiotic targets a set of enzymes, called penicillin-binding proteins (PBPs), which are involved in the synthesis of the peptidoglycan layer in *E. coli* (**5, 28**, **30**). The peptidoglycan layer is a key component that makes up the cell wall found in bacteria and is important for forming the structure of the bacterium and is a necessary protection for their survival. PBPs help catalyze the final crosslinking of the peptidoglycan layer. Without them, the formation of the layer is not complete and cell wall synthesis is compromised. β-lactam antibiotics work by targeting these PBPs. The structure of β-lactam antibiotics is analogous to the terminal amino acid of the NAM/NAG-peptide subunits that make up the peptidoglycan layer (**7**). Because of this structural similarity, the β-lactam antibiotics interact with the active site of PBPs in place of the NAM/NAG subunits, their natural substrate. Once bound to PBPs, the β-lactam antibiotic initiates an irreversible inhibition of the PBPs, thereby preventing the final crosslinking of the peptidoglycan layer (**7**).

Unfortunately, strains have acquired resistance to β-lactam antibiotics through the production of an enzyme called β-lactamase (**9**). This enzyme specifically targets β-lactam antibiotics by altering the antibiotic's structure through a hydrolysis reaction. The enzyme cleaves the β-lactam ring structure, deactivating the molecule's antibacterial properties and thereby resulting in a decreased activity of the β-lactam antibiotic.

Significance of Establishing Dosing Regimens

One of the main reasons for the recent increase in antibiotic resistance is the misuse of antibiotics (**10, 20**, **23**). For years, we have been prescribing without realizing the problems that can arise because of constantly using antibiotics. Antibiotic resistance can result from taking antibiotics the wrong way or when they are not needed (**20**). Bacteria change or adapt over time; this is especially true of bacteria that are exposed to an antibiotic but not killed (**10**). When bacteria survive a first course of antibiotics, a second course must be used. If the bacteria become resistant to the second course, yet a third course is required. The problem can continue until a particular bacterium cannot be treated at all, a phenomenon called multi-drug resistance (**10**). Used correctly, antibiotics treat infections caused by bacteria. When prescribed for bacterial infections, antibiotics are taken a certain number of hours apart for a specified number of days. Antibiotics must be taken as long as prescribed, even if the patient may feel better before finishing their prescription. The timing allows the concentration of the antibiotic in the bloodstream to be maintained at an effective level.

Besides doctor prescriptions, another event that has led to antibiotic resistance is overuse in agriculture and farming (**8, 21**). In recent years, considerable controversy over the use of antibiotics to promote growth and reduce mortality in animals raised for food has arisen. The idea was introduced in the 1940s after chickens that were fed by-products of tetracycline fermentation grew much faster and healthier than those without (**29**). Since that discovery, growth promotion in livestock grew and the longstanding effects were not taken into consideration. Recently, many scientists have suggested a strong correlation between antibiotic use in animals and resistance in humans (**14**). The transfer of resistance comes from considering the epidemiology of zoonoses, a disease that animals can transmit to humans (**14**). Animals

carrying or infected by a resistant organism are hazardous, not only to those that work with them, but to the public as well. The infectious bacteria survive and proliferate in the gut and the transfer can occur through many different possibilities. Farmers who work frequently with animals that have been exposed can indirectly obtain the resistant bacteria and spread them to others when handling food. Crops and soils can also become contaminated through excretes by these infected animals due to grazing or use as a fertilizer, and thereby vegetables that we eat become contaminated as well.

The bottom line is that misusing an antibiotic, whether for personal health or in agriculture, can be a problem because if a specific antibiotic is needed to treat infectious bacteria, it may no longer be effective (**4, 23)**.

Cell Envelope Stress Response Pathways in Response to Antibiotics

The outer membrane of *E. coli* selectively allows for entry of various nutrients and limits the entry of foreign molecules. It is the first, major line of defense that bacteria use against threats introduced to them, either through natural competence or uptake of their surroundings, and is a target for numerous antibiotics (**30**). Therefore, monitoring and maintaining the cell envelope in the presence of stressful agents is crucial for survival.

Gram-negative bacteria possess an array of stress responses that maintain the integrity of the cell envelope. A cell envelope stress response is one type of response generated by bacteria when under a stressful situation (**2, 13, 24**). This kind of response differs from a cytoplasmic response based on where the stress is occurring. A cytoplasmic response is when the stressor is in the cytoplasm but not the cell envelope. General stressors can activate both responses, while a

stress in the cell envelope, for example a chemical or an antibiotic, typically activates cell envelope stress responses (**2**, **12**). There are many cell envelope stress response pathways that have currently been identified in *E. coli*; including σ^E, Cpx, Rcs, phage-shock protein (Psp), and Bae responses (**15**). Except for the Bae response pathway, all pathways identified are important for combating envelope stress in other organisms besides *E*. coli (**16**).

Knowing that antimicrobials specifically target and overcome barriers provided by the cell envelope, it is not surprising that changes made to affect the function of these barriers can have an influence on antimicrobial susceptibility. Understanding resistance and how best to avoid it in the future is linked to observing the impact of stress and how the strain responds to that stress.

The Rcs Phosphorelay

Previous research showed that the Rcs phosphorelay becomes active when *E. coli* are treated with β-lactam antibiotics, which is the key reason why amp was chosen for the experiments (**16**). The Rcs phosphorelay consists of a group of proteins that work through a signaling cascade to activate the response, initiated by the environmental stress signal (Figure 1). The components are RcsF, RcsC, RscD, RcsB, and RcsA. The RcsF component faces the periplasmic space and senses the stress, which is thereby responsible for the stimulation of the pathway. Upon activation by the stress, RcsF triggers the next component RcsC to auto phosphorylate. RcsC is the component found on the inner membrane of the bacteria and is one of the core components for the Rcs system. RcsC then phosphorylates the next component, RcsD – a phosphotransferase protein for the pathway. Next, RcsD phosphorylates RcsB, the response

regulator. RcsB can then homodimerize to form $RcsB_2$ or it can heterodimerize with RcsA to form RcsAB; both of which are transcription factors that regulate gene expression when activated. One useful aspect of the pathway is the ability of RcsD and RcsC to dephosphorylate RcsB, causing the pathway to reset.

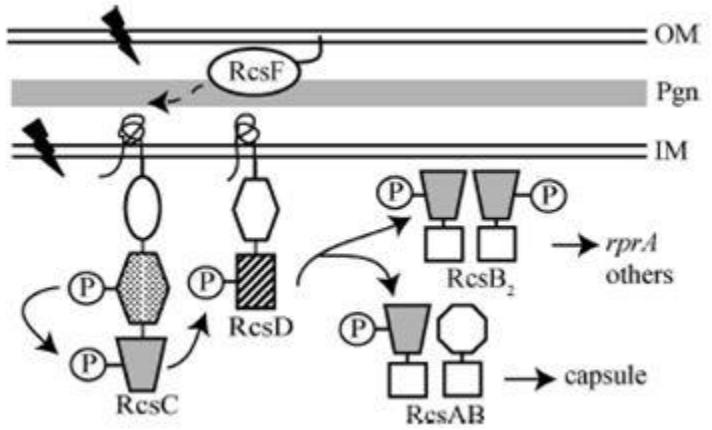

Figure 1: The Rcs Phosphorelay (16)
This is a visual representation of the Rcs phosphorelay and all of its components. Upon external stimulation along the outer membrane (OM), there is transfer of the RcsF component to the inner membrane (IM), where the phosphorylation cascade takes place.

We investigated the importance of the Rcs phosphorelay by placing *E.coli* under an antibiotic-induced stressful environment and monitored growth to see if deletion of the pathway would affect the response. By understanding how the response is affected, we can determine if there is dependence on the pathway and if that dependence is contributing to the resistance that has occurred.

Ecological Factors and Their Influence on Antibiotic-Induced Stress Responses

The effects of disturbance regimes on species diversity have always been of central interest for many ecologists. It is a popular topic for ecologists to study because disturbance

regimes in various locations tend to change rapidly and the changes can have profound effects. Each disturbance event that affects a particular system or location has aspects that are measurable; specifically, intensity, timing, duration, extent, and disturbance-interval (**17, 19**). The best way to understand these factors is to consider the event of a flood. A flood consists of many different factors: a maximum depth represented by *intensity*, a date of onset signified by *timing*, an inundation period shown by *duration*, an area of inundation characterized by *extent*, and a time since the last occurrence indicated by the *disturbance interval*. Utilizing the aspects measured, a sample distribution is constructed. In this manner, frequency and predictability can be determined.

With bacteria as the population of interest, analogous to the flood scenario, we designed an experiment to test certain disturbance events and how each factor affects the bacteria's ability to grow when treated with an antibiotic, individually. The factors of interest included timing, intensity, and frequency. The timing is the point at which the drug is administered, the intensity is the amount that is being administered, and the frequency is how often the drug is administered. By selectively manipulating each factor, we can determine how timing, intensity, and frequency affect the outcome of the antibiotic-induced stress response both individually and in combination with one another. We can use this relationship to determine which ecological factors – intensity, timing, and frequency – affect survival in the presence of amp. This is important as it allows us to determine a more efficient way for treating infectious bacteria.

Materials and Methods

Isolation of Bacterial Strains

An initial MIC test (see Liquid MIC Determination below) using a wild-type MG1655 Δ*lac E. coli* strain (WT) yielded an MIC of 10 μg/ml amp. Overnight cultures of WT were grown in lysogeny broth (LB) at 37^{o}C for 16 hours. The liquid cultures were spread onto solid LB medium containing three varying concentrations of ampicillin (LB+AMP), 10 μg/ml, 15 μg/ml, and 20 μg/ml. The plates were placed into 37^{o}C overnight. Following incubation, colonies were counted and arbitrarily selected. These selected colonies that grew were called resistant mutant strains. Each selected resistant mutant was streaked onto new LB and LB+AMP plates at the initial concentration they were isolated on to access the efficacy of survival upon restreaking. Mutant colonies that survived restreaks were chosen and liquid cultures of each mutant were prepared by inoculating each in LB at 37^{o}C for 16 hours. Frozen stocks of each mutant were prepared by mixing 1 ml of overnight liquid cultures with 0.7 mls of 50% glycerol and frozen at -80^{o}C. In total, 10 mutant strains were isolated and characterized.

Liquid Minimum Inhibitory Concentration (MIC) Determination

Each resistant mutant strain and corresponding transductant was subjected to MIC determination and the level of resistance was determined compared to the WT strain. Stock preparations of ampicillin (100 μg/ml) were serially diluted (using 8 tubes) 2-fold in LB starting at 40 μg/ml and ending at 0.31 μg/ml. 2 more additional tubes were prepared. A ninth tube containing LB only and a tenth tube containing LB+AMP with one more 2-fold dilution past the

eighth tube, ending at 0.16 μg/ml amp. An overnight liquid culture of each mutant strain was prepared by inoculation in LB at 37^{o}C for 16 hours. Overnights of the wild-type and mutant strains were diluted 100-fold to a final concentration of roughly 10^{6} cell/ml of LB. 100 μl of the diluted overnights were transferred into tubes 1-9, but not 10, as a negative control. Each tube was placed into 37^{o}C for 20 hours overnight. The last tube containing no growth was defined as the MIC.

Spot Titer Determination

To determine the amount of colony forming units per ml of cell culture (CFU/ml), spot titer assays were performed for each strain – WT, mutants, and transductants. An overnight culture of each strain was prepared individually by inoculation in LB at 37^{o}C for 16 hours. The strains were serially diluted 10-fold to 10^{-7}. From each 10-fold dilution, 10 μl were plated onto plain LB plates as well as LB+AMP at varying concentrations of 5 μg/ml, 10 μg/ml, 15 μg/ml, and 20 μg/ml. The plates were incubated overnight at 37^{o}C, and then analyzed.

P1 Lysate and Transduction

P1 transduction was used to replace the *rcsF*, *rcsA*, and *rcsB* components of the Rcs phosphorelay, individually, with an antibiotic resistance marker, kanamycin or chloramphenicol, depending on which lysate the cells were being subjected to. For lysates used to replace the *rcsB* and *rcsA* components, a kanamycin resistance marker was used. Lysate used to replace the *rcsF* used a chloramphenicol resistance marker instead. The result was a corresponding transductant

signified as having a lack of the specified Rcs gene – for example, Δ*rcsB* is a strain that is lacking *rcsB* of the pathway.

For making each lysate, first a donor strain was inoculated into LB at 37^{o}C for 16 hours. Each lysate was made from a different donor strain, depending on which Rcs component was going to be targeted for in the Mutant strains (for example, the lysate for deletion of *rcsB* was SEA3060 Δ*rcsB*). The overnight was diluted 1:100 in LB, and from the dilution, 150 µl was added to two separate flasks containing 15 ml LB; one to serve as a control and the second to be infected with P1 phage. Both flasks were incubated at 37^{o}C until reaching an OD600 of 0.2 Once the proper OD600 was reached, 150 µl of 1M $CaCl_2$ was added. The flasks were incubated at 37^{o}C for an additional 4 hours. After 4 hours, 500 µl of chloroform was added to and transferred to a 15 ml conical tube. The mixture was vortexed and centrifuged for 10 minutes are 4.5K rpm. The supernatant was collected at transferred to a new tube containing 500 µl of chloroform.

With the lysate generated, the transduction could then be performed. Overnight cultures of the wt and each mutant strain were prepared by inoculation in LB at 37^{o}C for 16 hours. From the overnight cultures, 1 ml was centrifuged for 2 minutes at 13.2K rpm. The supernatants were discarded and the cells were resuspended in 500 µl of 10 mM $MgSO_4$, 5 mM $CaCl_2$. Next, 3 separate samples were prepared; the first contained 100 µl of resuspended cells only, the second contained 100 µl of resuspended cells and 50 µl of lysate, and the last contained 100 µl of lysate only. Each tube was incubated for 30 minutes in a 37^{o}C water bath. After the 30 minutes, 1 ml of LB was added and the tubes were incubated in the 37^{o}C water bath for 1.5 hours. Following the second incubation, the samples were centrifuged for 2 minutes at 13.2 rpm. The supernatants were removed, except for 100 µl used to resuspend the pellets. Cells that were subjected to replacement of *rcsB* and *rcsA* were plated onto LB+KAN plates and cells that were subjected to

replacement of *rcsF* were plated onto LB+CAM plates, all at concentration of 25 μg/ml. The plates were incubated overnight in 37°C. Lack of growth with the first and last samples (cells only and lysate only, respectively) indicated a successful transduction. Of the colonies that grew from the second sample (cells + lysate), 2 colonies were restreaked onto 15 μg/ml LB+KAN or 15 μg/ml LB+CAM, as well as plain LB to ensure survival during the transfer. The restreaked plates were incubated overnight at 37°C. The next day, 1 colony from each restreak was selected and an overnight liquid culture was prepared in LB at 37°C for 16 hours. Frozen cultures were created by taking 1 ml of each overnight and 0.7 ml of 50% glycerol. Each frozen culture was stored at -80°C.

Growth Curve Establishment

Before beginning the manipulation of the three ecological factors, initial growth curves of the strains were created to figure out the time points to add ampicillin based on the growth curve of the bacteria used. The wild-type MG1655 Δ*lac E. coli* from the Rcs phosphorelay experiments was used for the second set of experiments as well; however, a second *E. coli* strain (MG1655 Δ*tolC*). Overnight cultures of each strain were prepared by inoculation into LB at 37°C for 16 hours. Each culture was diluted 1:100 in LB. From the dilutions, mixed ratios of WT and Δ*tolC* (dT) were created; specifically 1:1 WT:dT, 1:10 WT:dT, and 10:1 WT:dT. In a 96 well microtiter plate, 150 μl of each sample (WT, dT, 1:1, 1:10, and 10:1) was aliquoted in designated rows, as well as 150 μl of plain LB as a control. The 96 well microtiter plate was placed into 37°C shaker at 250 rpm for a total of 8 hours. Each half hour, OD600 readings were recorded. At

the end of the 8 hours, a final OD600 reading was obtained. Additionally, spot titer determinations were performed using LB+XGAL plates at a concentration of 50 μg/ml Xgal.

Manipulation of Timing and Intensity

Overnight cultures of WT and dT were prepared by inoculation in LB at 37^{o}C for 16 hours. Both overnights were diluted 1:100 in LB. From the diluted WT overnight, 150 μl was aliquoted into designated wells of a 96-well microtiter plate, while 150 μl of diluted dT was aliquoted into separate designated wells. An initial OD600 reading of each well was recorded. The microtiter plate was incubated in a shaker at 37^{o}C and 250 rpm. An established dose of 5 μg/ml amp was added at varying intensities of 1x to 4x the dosing size at specified time points, beginning, middle, and end of the growth curve to each designated well with WT or dT present. Every half hour, OD600 readings were taken. Cells were grown for a total of 4 hours. Following the final OD600 reading, the CFUs/ml were determined via spot titer determination on LB+XGAL plates at a concentration of 50 μg/ml Xgal.

Manipulation of Frequency

Overnight cultures of WT and dT were prepared by inoculation in LB at 37^{o}C for 16 hours. Both overnights were diluted 1:100 in LB. From the diluted WT overnight, 150 μl was aliquoted into designated wells of a 96-well microtiter plate, while 150 μl of diluted Δ*tolC* was aliquoted into separate designated wells. An initial OD600 reading of each well was recorded. The microtiter plate was incubated in a shaker at 37^{o}C and 250 rpm. An established dose of 5 μg/ml amp was selected. A portion of the established dose, either 1/2 or 1/3 – depending on the

specified frequency – was added to its designated wells at one indicated time point of beginning, middle, or end of the growth curve, while the remaining was added to the same well at the next indicated time point of beginning, middle, or end. Every half hour, OD600 readings were taken. Cells were grown for a total of 4 hours. Following the final OD600 reading, the CFUs/ml were determined via spot titer determination on LB+XGAL plates at a concentration of 50 μg/ml Xgal.

Results

Deletion of the Rcs Phosphorelay

The Analysis and Classification of Isolated Strains through MIC Determinations

An initial MIC determination yielding 10 μg/ml amp was determined for the WT. In order to acquire mutant strains, liquid cultures of WT were spread onto plates with higher concentrations of amp and any growth that appeared on the higher concentrations were believed to be mutants. WT cultures were plated onto 15 μg/ml, 20 μg/ml, and 25 μg/ml. Fewer colonies were observed as the concentration of amp increased. No colonies were observed on the 25 μg/ml plates. A total of 10 mutant strains were isolated for further analysis, most of which were taken from the 15 μg/ml plate and only a few taken from the 20 μg/ml. The level of resistance in MIC assays for each mutant was determined relative to the WT, as shown in Figure 2. Of the 10, there were 5 that showed the most resistance to ampicillin at 20 μg/ml, 3 that showed less resistance at 15 μg/ml, and 2 that were not much more resistant compared to the WT, at 10 μg/ml.

To determine if the Rcs phosphorelay contributed to increased resistance of the mutants, the *rcsB* gene was deleted and the MIC was measured for each Δ*rcsB* derivative. The *rcsB* gene is a key transcription factor for the pathway and deletion of the gene disrupts the ability of both $RcsB_2$ and RcsAB responses (Figure 1). Two other components of the pathway were also deleted individually, *rcsA* and *rcsF*, to determine if those components played any significant role in the pathway's ability contribute to the resistance seen by the mutants. Deletion of *rcsA* disrupts the RcsAB response and deletion of *rcsF* prevents activation of *rcsC* and all other components

following in the phosphorylation cascade, if the signal is sensed by RcsF (Figure 1). The most significant change in MIC overall is upon deletion of *rcsB* (Figure 3), in which there are significant decreases in all but three of the strains, corresponding to mutants 1501, 1521, and 1522. With the deletion of *rcsA*, only two of the strains, corresponding to mutants 1518 and 1523 show a decrease in MIC, and even then, the decrease is only a slight one (Figure 3). The Δ*rcsA* strain corresponding to mutant 1520 actually showed an increase in MIC, but that could be due to human error since it does not follow the general trend and an increase in MIC would not make sense here. Therefore the less significant change observed in liquid MIC determination, was upon deletion of *rcsA*. The intermediate between *rcsB* and *rcsA* was *rcsF*, upon deletion of *rcsF* (Figure 3), where two mutants showed a slight decrease in MIC; Mutants 1501 and 1518. Interestingly, deletion of *rcsF* was the only case where the MICs of the most resistant strains, 1501, 1521, and 1522, decreased compared to deletion of the other components.

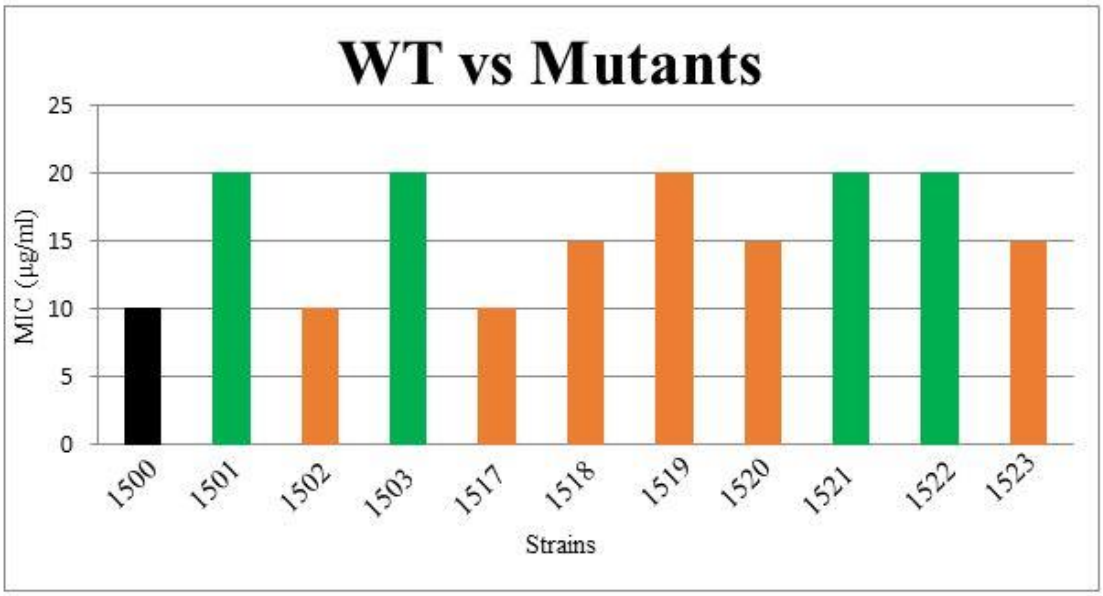

Figure 2: WT and Mutant MIC Determinations
Figure 2 shows the MICs for the WT compared to each isolated mutant. Mutants in green were taken from 20 µg/ml amp and mutants in yellow were taken from 15 µg/ml amp.

Table 1 Strain Classifications

Strain	Classification
SEA1500 (MG1655 Δ*lac*)	**Wildtype**
SEA1516 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (Wildtype)
SEA1510 (MG1655 Δ*lac* Δ*rcsA*)	Δ*rcsA* (Wildtype)
SEA1513 (MG1655 Δ*lac* Δ*rcsF*)	Δ*rcsF* (Wildtype)
SEA1501 (MG1655 Δ*lac*)	**Mutant 1**
SEA1507 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (mutant 1)
SEA1511 (MG1655 Δ*lac* Δ*rcsA*)	Δ*rcsA* (mutant 1)
SEA1514 (MG1655 Δ*lac* Δ*rcsF*)	Δ*rcsF* (mutant 1)
SEA1502 (MG1655 Δ*lac*)	**Mutant 2**
SEA1508 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (mutant 2)
SEA1503 (MG1655 Δ*lac*)	**Mutant 3**
SEA1509 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (mutant 3)
SEA1512 (MG1655 Δ*lac* Δ*rcsA*)	Δ*rcsA* (mutant 3)
SEA1515 (MG1655 Δ*lac* Δ*rcsF*)	Δ*rcsF* (mutant 3)
SEA1517 (MG1655 Δ*lac*)	**Mutant 4**
SEA1524 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (mutant 4)
SEA1531 (MG1655 Δ*lac* Δ*rcsA*)	Δ*rcsA* (mutant 4)
SEA1538 (MG1655 Δ*lac* Δ*rcsF*)	Δ*rcsF* (mutant 4)
SEA1518 (MG1655 Δ*lac*)	**Mutant 5**
SEA1525 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (mutant 5)
SEA1532 (MG1655 Δ*lac* Δ*rcsA*)	Δ*rcsA* (mutant 5)
SEA1539 (MG1655 Δ*lac* Δ*rcsF*)	Δ*rcsF* (mutant 5)
SEA1519 (MG1655 Δ*lac*)	**Mutant 6**
SEA1526 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (mutant 6)
SEA1533 (MG1655 Δ*lac* Δ*rcsA*)	Δ*rcsA* (mutant 6)
SEA1540 (MG1655 Δ*lac* Δ*rcsF*)	Δ*rcsF* (mutant 6)
SEA1520 (MG1655 Δ*lac*)	**Mutant 7**
SEA1527 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (mutant 7)
SEA1534 (MG1655 Δ*lac* Δ*rcsA*)	Δ*rcsA* (mutant 7)
SEA1541 (MG1655 Δ*lac* Δ*rcsF*)	Δ*rcsF* (mutant 7)
SEA1521 (MG1655 Δ*lac*)	**Mutant 8**
SEA1528 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (mutant 8)
SEA1535 (MG1655 Δ*lac* Δ*rcsA*)	Δ*rcsA* (mutant 8)
SEA1542 (MG1655 Δ*lac* Δ*rcsF*)	Δ*rcsF* (mutant 8)
SEA1522 (MG1655 Δ*lac*)	**Mutant 9**
SEA1529 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (mutant 9)
SEA1536 (MG1655 Δ*lac* Δ*rcsA*)	Δ*rcsA* (mutant 9)
SEA1543 (MG1655 Δ*lac* Δ*rcsF*)	Δ*rcsF* (mutant 9)
SEA1523 (MG1655 Δ*lac*)	**Mutant 10**
SEA1530 (MG1655 Δ*lac* Δ*rcsB*)	Δ*rcsB* (mutant 10)
SEA1537 (MG1655 Δ*lac* Δ*rcsA*)	Δ*rcsA* (mutant 10)
SEA1544 (MG1655 Δ*lac* Δ*rcsF*)	Δ*rcsF* (mutant 10)

Table 1 shows the strains utilized for the first half of this thesis (Rcs phosphorelay dependence). After deletion of *rcsB*, it was decided that Mutant 1502 not be continued with further testing since it had relatively no difference from the WT.

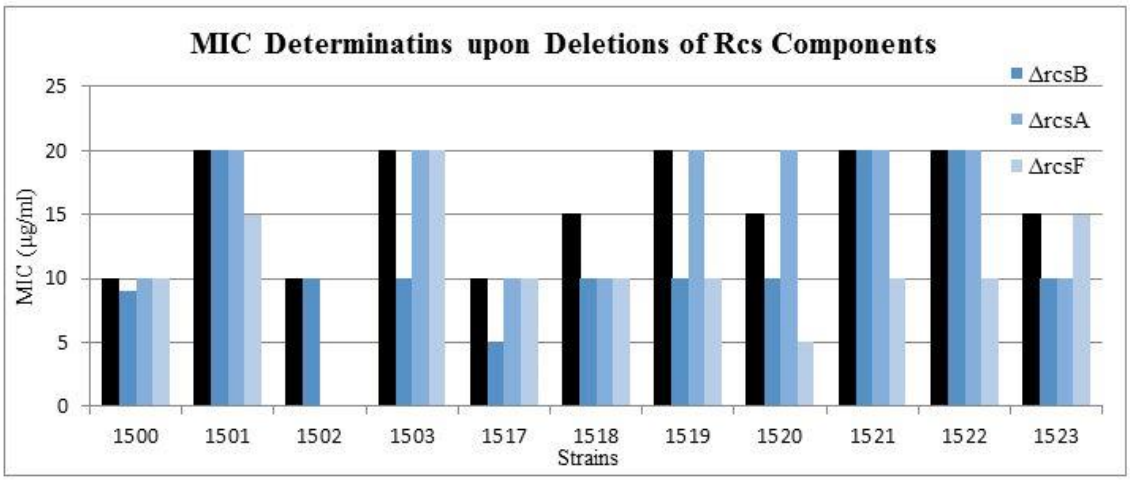

Figure 3: MICs for Each Corresponding Transductant

Figure 3 shows the MICs for WT and Mutants, as well as each corresponding transductant with removal of the indicated Rcs component. The dark blue is each deletion of *rcsB*. The light blue is each deletion of *rcsA*. The lightest blue/gray is each deletion of *rcsF*. After deletion of *rcsB*, it was decided that Mutant 1502 not be continued with further testing since it had relatively no difference from the WT, so there is no rcsA or rcsF data for Mutant 1502.

Spot Titer Determinations for Deletion of *rscB*

The second method for measuring resistance the mutant strains compared to each corresponding transductant was through spot titer assays carried out on solid media containing amp. Among the many differences between using MIC or spot titer determinations to measure resistance, the main difference is that MIC is a measure of every cell present in the culture – completely dead, fully functioning, and anything in between. Spot titer determinations were performed for data analysis in addition to MIC determinations because spot titers are a more direct measurement of the viable cells that survive when treated with amp. It is important to do both assays because some cells may have a different degree of resistance in liquid media compared to on solid media. Additionally, the bacterial cell physiology appears different on plates. Moreover, with spot titer determinations, the growth is monitored in the presence of a different concentrations of amp, which may include other concentrations besides the minimum

concentration needed to prevent growth. For the spot titer results, we saw a change in the dependence on each individual component. In liquid media, *rcsB* appeared to be the most important component. Whereas on solid media, that is no longer the case. The cfu/ml results from spot tier determinations for each mutant are shown in Figures 7, 8, 9, and 10. The plates for two mutants, 1501 and 1503, are shown in Figures 4, 5, and 6 to explain the cfu/ml results in more detail.

Upon deletion of the *rcsB* component, the results while looking at that component alone showed partial dependence on that component of the pathway for all mutants except Mutants 1503 and Mutant 1519. As shown in Figure 4a, Mutant 1501 still grows effectively on LB+AMP at a concentration of 5 μg/ml, even when *rcsB* has been deleted, while Mutant 1503 loses its ability to grow after deletion. This suggests that Mutant 1503 is completely dependent on *rcsB*, while Mutant 1501 is only partially dependent, and therefore there must be some other means for the acquired resistance seen in Mutant 1501. However, upon an increase from 5 μg/ml to 10 μg/ml (Figure 4b), there is a significant decrease in Mutant 1501's ability to grow after deletion of *rcsB*, indicating that *rcsB* is important for resistance when there is a higher concentration of amp.

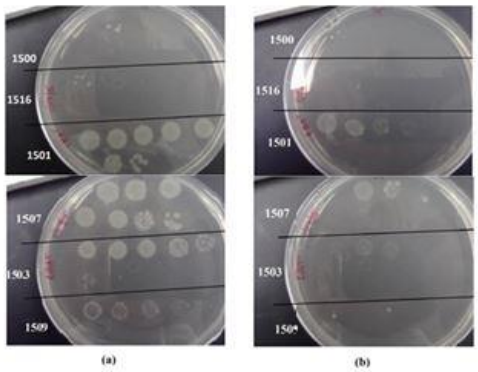

Figure 4: Spot Titer Results upon Deletion of *rcsB*
(a) Plating of wt (1500), mutant 1501, and mutant 1503 strains with corresponding Δ*rcsB* transductants (1516, 1507, and 1509) on LB+AMP at a concentration of 5 μg/ml. (b) Plating of wt, mutant 1501, and mutant 1503 strains with corresponding Δ*rcsB* transductants on LB+AMP at a concentration of 10 μg/ml. Each spot was a taken from a serial dilution of an overnight culture for the specified strains.

Spot Titer Determinations for Deletion of *rcsA*

As was the case with deletion of *rcsB*, the deletion of *rcsA* also suggests an overall partial dependence on the Rcs Pathway. Interestingly, there was not much of a change in MIC upon deletion of *rcsA*. However, analysis with spot titer determinations shows there was a significant change in the number of CFUs for each mutant after *rcsA* was removed. Spot titer determinations for Mutants 1501 and 1503 are shown below as an example for strains that were partially dependent and completely dependent on the Rcs phosphorelay, respectively (Figure 5). The same general trend that was seen with deletion of *rcsB* was present in the data show upon deletion of *rcsA*. Meaning, the most resistant mutants were able to sustain growth better after deletion compared to less resistant mutants. Again, Mutant 1501 retains its ability to grow in the presence of amp, even upon deletion of *rcsA* (Figure 5a), while colonies observed for Mutant 1503 begin to decrease in number. Additionally, it is not until the concentration of amp is increased to 10 μg/ml, when the more resistant strains, such as Mutant 1501, begin to decrease in number after the deletion (Figure 5b). This implies that deleting *rcsA* in Mutant 1501 does not affect growth as much. *rcsA* appears to be more important for the resistance of Mutant 1503.

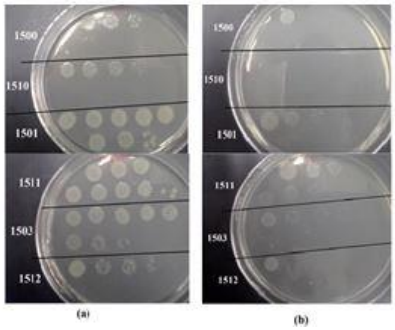

Figure 5: Spot Titer Results upon Deletion of *rcsA*
(a) Plating of wt (1500), mutant 1501, and mutant 1503 strains with corresponding Δ*rcsA* transductants (1510, 1511, 1512) on LB+AMP at a concentration of 5 μg/ml. (c) Plating of wt, mutant 1501, and mutant 1503 strains with corresponding Δ*rcsA* transductants on LB+AMP at a concentration of 10 μg/ml. Each spot was a taken from a serial dilution of an overnight culture for the specified strains.

Spot Titer Determinations for Deletion of *rcsF*

The deletion of *rcsF* yielded similar results as the deletion of the first two components. As was the case with the MICs, it appeared that the results for deletion of *rcsF* was in between *rcsB* and *rcsA*; meaning, there was a decrease in the number of colonies, more so than deletion of *rcsA*, but not as much upon deletion of *rcsB* (Figure 4). This decrease was more apparent with strains that were not as resistant, for example Mutant 1503 compared to Mutant 1501 (Figure 6). Differently, upon increase from 5 μg/ml to 10 μg/ml, there was a significant decrease in the number of colonies for almost all the mutants. The spot titer determinations for Mutants 1501 and 1503 are shown below as an example (Figure 6).

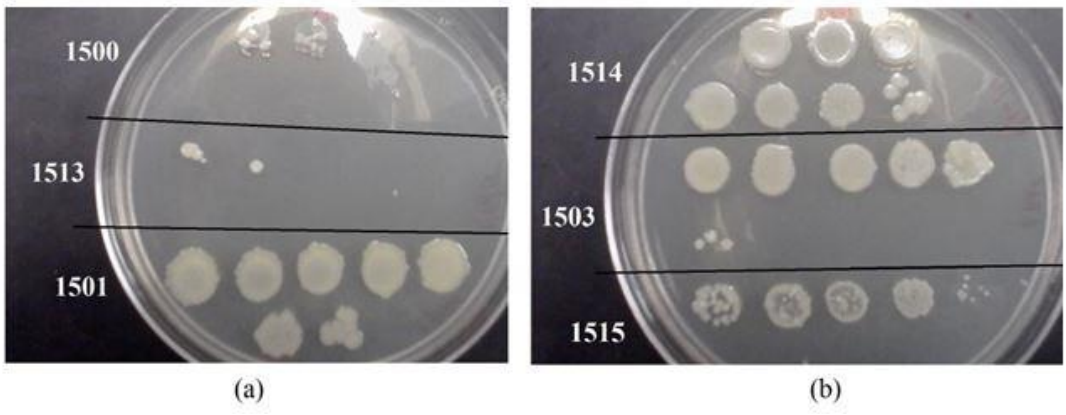

Figure 6: Spot Titer Results upon Deletion of rcsF
(a, b) Plating of wt (1500), mutant 1501, and mutant 1503 strains with corresponding Δ*rcsF* transductants (1513, 1514, 1515) on LB+AMP at a concentration of 5 μg/ml. Each spot was a taken from a serial dilution of an overnight culture for the specified strains.

Dependence on the Rcs Phosphorelay for Resistance to Ampicillin

The results thus far have been looking at the individual components on their own. When the data is combined into one graph, it is easier to visualize which component the cells are relying on most – whether it is *rcsB*, *rcsA*, or *rcsF* (Figures 7, 8, 9 and 10). For example, with

regards to Mutant 1503, there is a significant drop in the number of colonies between the transition from a concentration of 5 μg/ml amp to 10 μg/ml amp upon deletion of *rcsF (*Figure 7). It is not until another increase in concentration to 15 μg/ml that there is a significant decrease in the number of colonies upon deletion of *rcsA*, and yet another increase to 20 μg/ml amp until we see that decrease in the number of colonies with *rcsB* deleted. This suggests that, for Mutant 1503, there is more dependence on *rcsF* than *rcsA*, and more dependence on *rcsA* than *rcsB*. A similar trend followed for Mutant 1501 (as shown in Figure 7), but we don't see the Δ*rcsB* strain decreasing in the number of colonies, even after the addition of 20 μg/ml amp – which again goes back to the fact that Mutant 1501 is only partially dependent, while Mutant 1503 is completely dependent. For the remaining mutants tested, the same trend of dependence on each individual Rcs component stayed consistent, with some strains more sensitive to amp than others. Of the 10 mutants tested, only Mutants 1503 (Figure 7), 1518 (Figure 8), and 1519 (Figure 9) showed complete dependence on the Rcs phosphorelay as the strains lacking each component decreased to little or no growth before the corresponding Mutant strain. The results for Mutant 1517 (Figure 8) are uninterpretable since all strains, including the mutant, decreased to little or no growth after amp has increased to 10 μg/ml. With these results, it is not clear if the Mutant 1517 strain is dependent on any of the components. For the remainder of the mutants, Figures 7, 8, 9, and 10 display partial dependence on the pathway because only some and not all of the strains lacking each chosen Rcs component decreases in the number of colonies with increasing concentrations of amp. The general trend for each is the same, however. Each Δ*rcsF* strain decreases in the number of colonies before either of the other components. Therefore

suggesting that, regardless of partial or complete dependence, the most dependence is seen with *rcsF*.

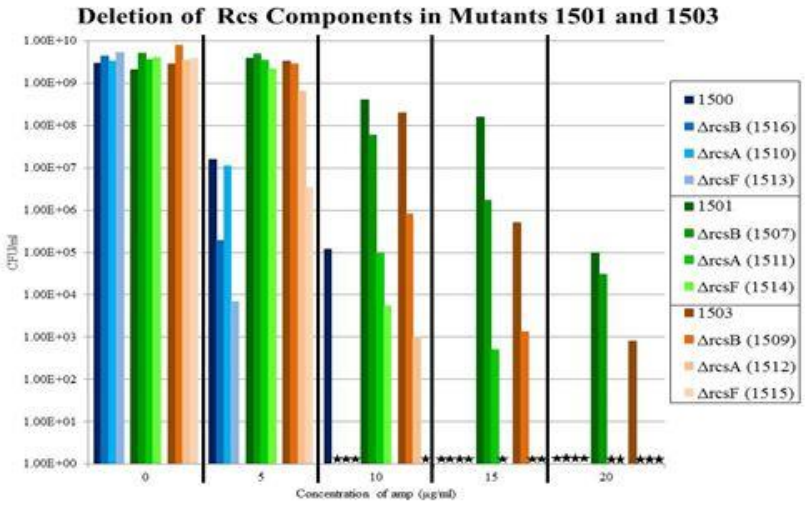

Figure 7: Cfu/ml Results for Deletion of Rcs Components in Mutants 1501 and 1503
The colony counts for WT (1500) and its corresponding transductants (1516, 1510, 1513) shown in blue. Mutant 1501 with its corresponding transductants (1507, 1511, 1514) shown in green. Mutant 1503 with its corresponding transductants (1509, 1512, 1515) shown in orange. Bars with stars at the bottom indicate there was little to no growth for that particular plate.

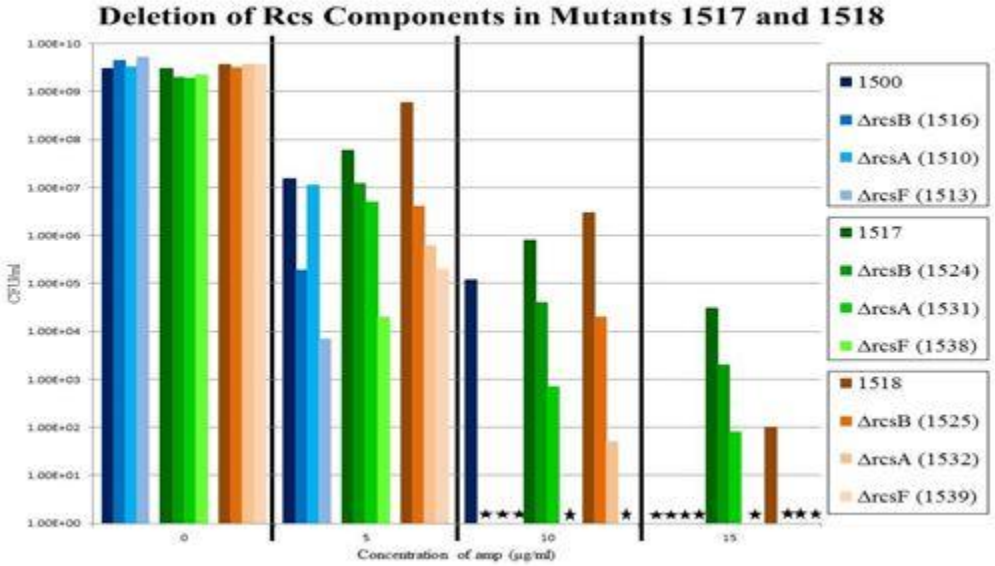

Figure 8: CFU/ml Results for Deletion of Rcs Components in Mutants 1517 and 1518
The colony counts for WT (1500) and its corresponding transductants (1516, 1510, 1513) shown in blue. Mutant 1517 with its corresponding transductants (1524, 1531, 1538) shown in green. Mutant 1518 with its corresponding transductants (1525, 1532, 1539) shown in orange. Bars with stars at the bottom indicate there was little to no growth for that particular plate.

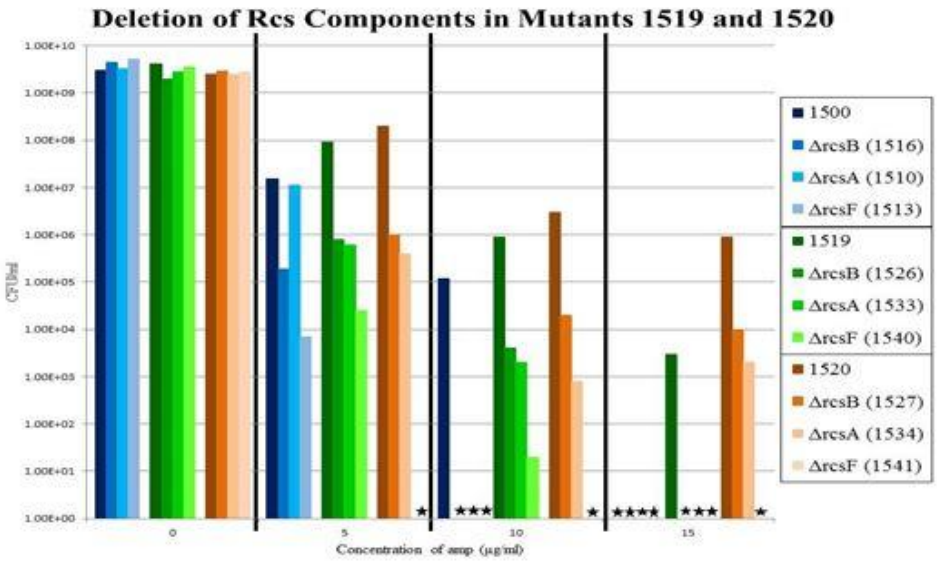

Figure 9: CFU/ml Results for Deletion of Rcs Components in Mutants 1519 and 1520
The colony counts for WT (1500) and its corresponding transductants (1516, 1510, 1513) shown in blue. Mutant 1519 with its corresponding transductants (1526, 1533, 1540) shown in green. Mutant 1520 with its corresponding transductants (1527, 1534, 1541) shown in orange. Bars with stars at the bottom indicate there was little to no growth for that particular plate.

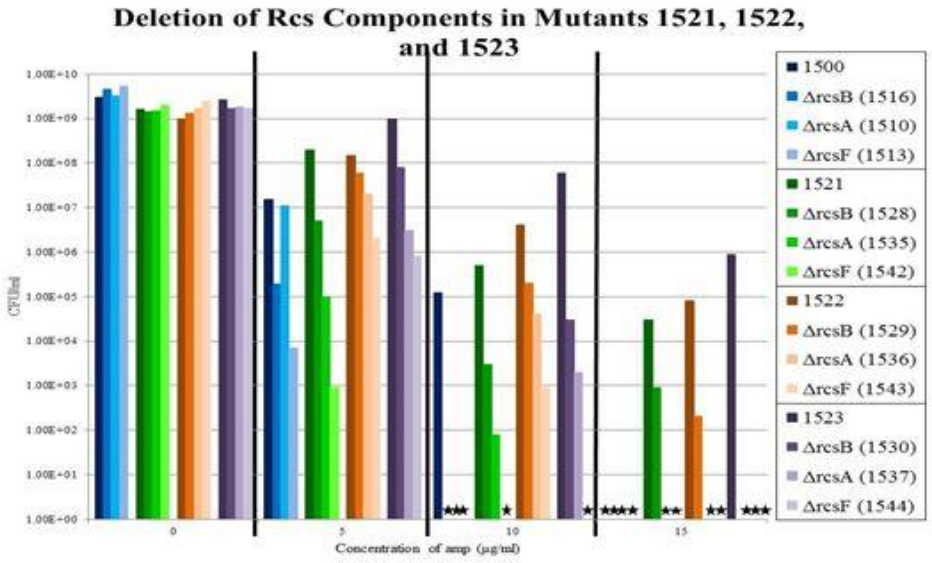

Figure 10: CFU/ml Results for Deletion of Rcs Components in Mutants 1521, 1522, and 1523
The colony counts for WT (1500) and its corresponding transductants (1516, 1510, 1513) shown in blue. Mutant 1521 with its corresponding transductants (1528, 1535, 1542) shown in green. Mutant 1522 with its corresponding transductants (1529, 1536, 1543) shown in orange. Mutant 1523 with its corresponding transductants (1530, 1537, 1544) shown in purple. Bars with stars at the bottom indicate there was little to no growth for that particular plate.

Ecological Aspect of Antibiotic-Induced Stress Response

The Manipulation of Timing and Intensity

The first aim in the second series of experiments for this thesis, the manipulation of each ecological factor, was to determine how intensity and timing related to one another before incorporating the manipulation of frequencies. It was important to establish the right times in the growth curve to add the drug and the right amount of antibiotic to add because we didn't want to kill all the bacteria or not add enough drug to see an effect.

To start out, time points of 0 hours, 3 hours, and 6 hours for beginning, middle, and end, respectively, were chosen based on the growth curve of the WT strain of *E. coli* used. In addition to the time points, an initial intensity of 15 μg/ml was chosen, based on the preliminary MIC of the WT, 10 μg/ml. WT cells were aliquoted into a 96 well plate and grown up over the course of 8 hours. At each time point, 15 μg/ml amp was added to the indicated well.

Initial trials with these conditions show that timing has an important effect for the outcome of the response (Figure 11a). Based on the initial data with the first set of conditions, it was decided that 0, 3, and 6 hours were not ideal time points to use for the addition of ampicillin. Instead, time points were shifted closer a point in the growth curve when cells were still growing in exponential phase; which were 0 minutes, 1 hour 30 minutes, and 3 hours for beginning, middle, and end, respectively. In addition to the change in time points, a different intensity was used because it was decided that 15 μg/ml was too strong of a dosing size. Therefore, for the next set of conditions, 10 μg/ml amp was added instead (Figure 11b).

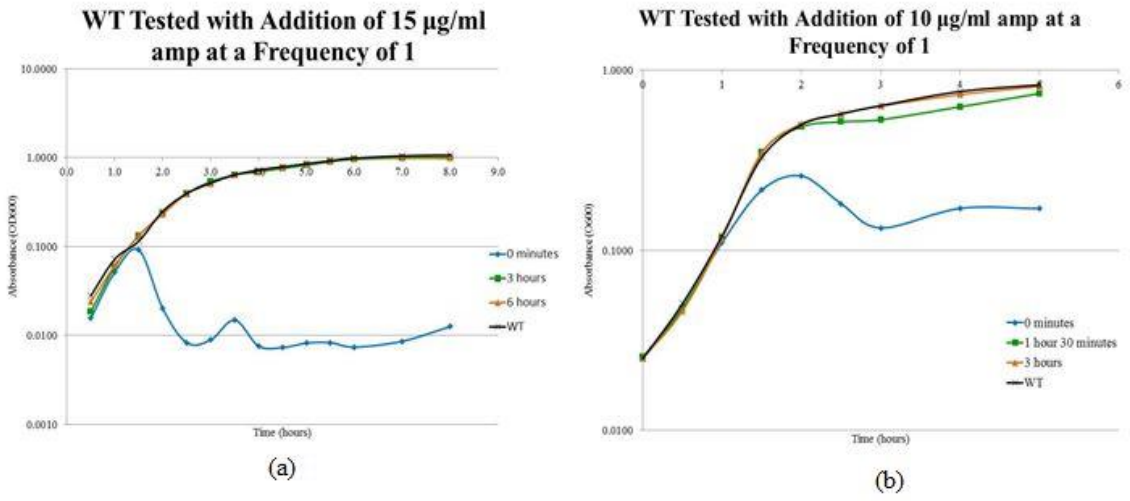

Figure 11: Manipulation of Timing at a Frequency of 1
Figure 11 panels (a, b) show the manipulation of time and intensity, while keeping frequency constant. The time points and intensities for each were chosen based off trials and errors, (a) 15 µg/ml amp was added at 0 hours, 3 hours, and 6 hours and (b) 10 µg/ml amp was added at 0 hours, 1 hour 30 minutes, and 3 hours

After preliminary testing, it was decided that time points of 15 minutes (beginning), 1 hour and 15 minutes (middle), and 2 hours and 15 minutes (end), yielded data with the most interpretable results. The next step in these series of experiments was to understand the effect of varying intensity as a function of timing. To accomplish this, WT cells were grown in LB in a 96 well plate and treated with 5, 10, 15, or 20 µg/ml amp added at the beginning, middle, or end of exponential phase (15 minutes, 1 hour 15 minutes, and 2 hours 15 minutes, respectively) in designated wells. The cells were allowed to grow for a total of 4 hours and optical density measurements were taken every half hour. At the end of the 4 hours, spot titer determinations were carried out for each well in order to determine how many viable bacteria remained at the end of the experiment. The average of 3 different trials with the set up described are shown in Figure 12. *E. coli* was more sensitive when treated at the beginning compared to the middle or end. When treated at the middle of the exponential phase, more amp was required to reduce

growth and the amount of amp added had a larger effect. Lastly, the results show there is little to no effect when amp is added to the end of the growth curve.

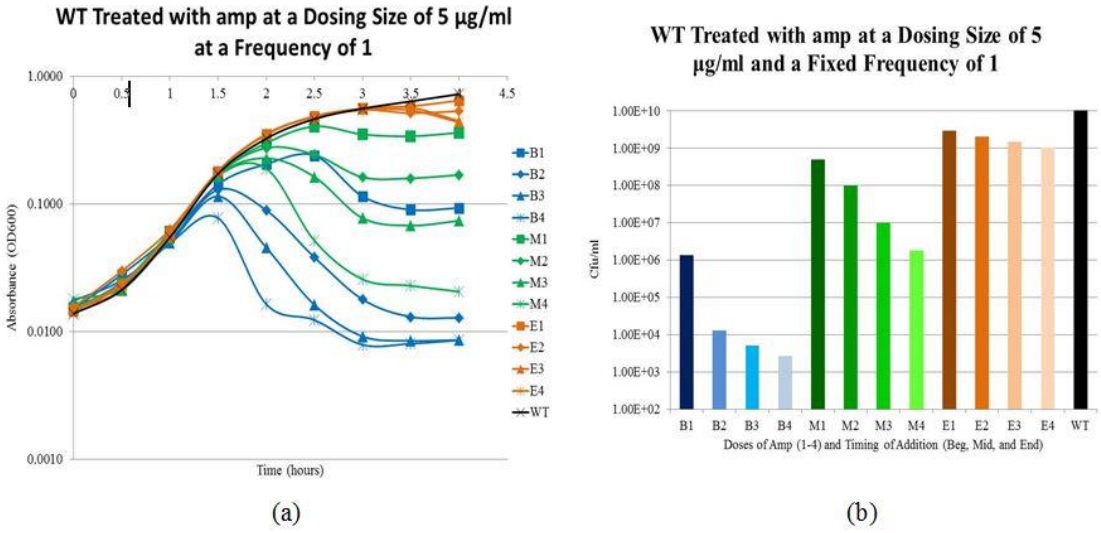

Figure 12: Timing is the Most Important Factor

Figure 12 (a) Shows the absorbance readings across 4 hours. Times of amp addition are specified by: B = beginning, M = middle, and E = end. Amount of amp added at each time points: 1 = 5 µg/ml, 2 = 10 µg/ml, 3 = 15 µg/ml, and 4 = 20 µg/ml. (b) Shows the colony forming units for the corresponding absorbance readings at the end of the 4 hours of growth.

The Manipulation of Frequency

Next, we examined the effect that frequency had on the bacteria's ability to survive in the presence of amp. Using a similar setup, we examined the four different intensities of 5, 10, 15, and 20 µg/ml amp at the same time points of 15 minutes, 1 hour 15 minutes, and 2 hours 25 minutes but added amp at 2 or 3 different time points in the growth curve instead of giving amp all at one time point. The addition of multiple frequencies involved taking the intensity and splitting it depending on the frequency being tested. For example, at a frequency of 2 and an intensity of 5 µg/ml, the first 2.5 µg/ml was added at the first time point and the remaining 2.5 µg/ml was added at the second time point. Likewise, for a frequency of 3 and an intensity of 5

μg/ml, the amount added at each time point was 1.25 μg/ml amp. Ultimately, the final concentrations of amp ended up being the same as the first section, which were 5, 10, 15, and 20 μg/ml amp.

By varying the frequency, we were able to test whether adding the drug all at once or in multiple additions affected growth. Based on Figures 13 and 14, timing still has the most important effect on the antibiotics ability to decrease the number of cells, but the variation in intensity becomes more apparent with increased frequency. In Figure 13, we see that when amp is added at higher intensities at the BM or BE, it is able to effectively kill the cells. Especially, when compared to addition at the ME, regardless of the amount added. This data still supports the argument that timing is the most important factor because when we see the number of cells that decrease with the addition at higher intensities, it is only when that addition involved adding a the B. This is further supported when looking back at a frequency of 1 (Figure 12). The spit addition at BE of 15 μg/ml amp total is equivalent to giving 7.5 μg/ml at the B only. In Figure 12, addition of 10 μg/ml results in an absorbance reading just above 0.01, which is equivalent to giving the split addition shown in Figure 13. This suggests that when given at the BE, it is only the amount at the B that has a significant impact of the cells ability to survive, since the absorbance did not decrease any further. Additionally, adding amp at the end only had no effect, even when the intensity was 20 μg/ml. This further suggests that the second 7.5 μg/ml addition of amp at the E of the BE split frequency does not have any significant contribution to the antibiotics ability to decrease the number of growing cells.

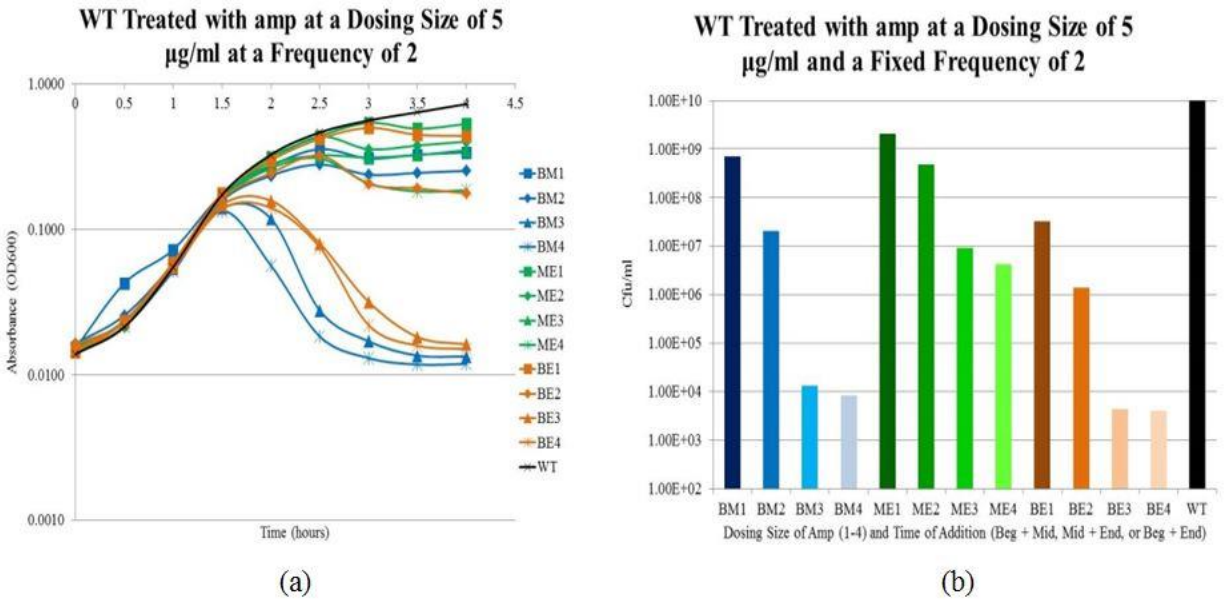

Figure 13: Variation in Intensity Becomes More Apparent with Increased Frequency
(a) Shows the absorbance readings across 4 hours at a frequency of 1. Times of amp addition are specified by: BM = beginning + middle, ME = middle + end, and BE = beginning + end. Amount of amp added at each time points was accomplished by: 1 = 2.5 µg/ml at first time point and 2.5 µg/ml at second time point, 2 = 5 µg/ml at first time point and 5 µg/ml at the second time point, 3 = 7.5 µg/ml at the first time point and 7.5 µg/ml at the second time point, and 4 = 10 µg/ml at the first time point and 10 µg/ml at the second time point. (b) Shows the colony forming units for the corresponding absorbance readings at the end of the 4 hours of growth.

Another way to further support the importance of timing and intensity at multiple frequencies is by comparing the results when adding the same total amount of amp at different times and different frequencies. For example, Figures 14a and 14b show the absorbance readings at 10 µg/ml final concentration of amp. Figures 14c and 14d show 15 µg/ml final concentration of amp. At 10 µg/ml, the addition at the B was more effective than that same amount at any other time points or combination of time points. For example adding 5 µg/ml at the B and an additional 5 µg/ml at either the M or E to give a final concentration of 10 µg/ml was not as effective. It is not until 15 µg/ml amp is added that we see a difference in the ability of the drug to effectively kill at additional frequencies. The results show that it is comparable to give a final concentration of 15 µg/ml amp in 1, 2, or 3 additions at the B, BM, BE, and BME. In other

words, the cells respond the same way when exposed to 15 µg/ml amp either all at once or split into 2 or 3 separate exposures when the beginning is playing a role.

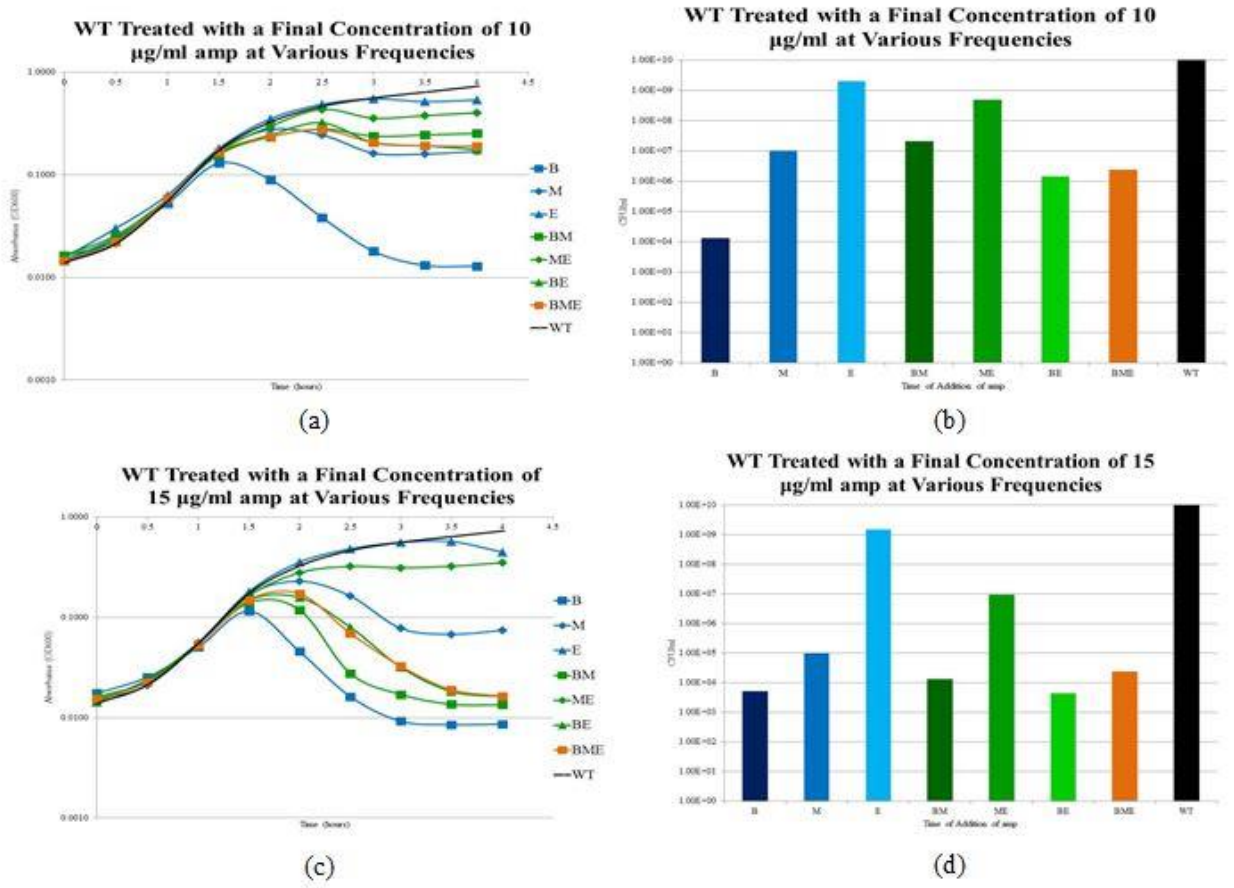

Figure 14: Manipulation of Frequency at Fixed Intensities

(a) Shows the absorbance readings at varying frequencies for a final concentration of 10 µg/ml amp. Frequencies of 1 are in blue, accomplished by adding 10 µg/ml amp at B, M, or E. Frequencies of 2 are in green, accomplished by adding 5 µg/ml at the first time point and 5 µg/ml at the second time point. Frequency of 3 in orange, accomplished by adding 3.3 µg/ml at each time point B, M, and E. (b) Shows the colony forming units that correspond with (14a) after 4 hours of growth. (c) Shows the absorbance readings at varying frequencies for a final concentration of 15 µg/ml amp. Frequencies of 1 are in blue, accomplished by adding 15 µg/ml amp at B, M, or E. Frequencies of 2 are in green, accomplished by adding 7.5 µg/ml at the first time point and 7.5 µg/ml at the second time point. Frequency of 3 in orange, accomplished by adding 5 µg/ml at each time point B, M, and E. (d) Shows the colony forming units that correspond with (14c) after 4 hours of growth.

Discussion

Very rarely will organisms experience situations they do not feel stressed. Antibiotic treatment can be viewed as a stress for bacteria, and their ability to respond to the stress affects the outcome of the treatment. The misuse of antibiotics is a major reason for the recent increase in antibiotic resistance in many infectious strains of bacteria. By understanding how bacteria respond to an antibiotic-induced environment, the knowledge can be gained to help solve the issue of antibiotic resistance and allow us to better control the amount of antibiotic usage in the future.

The first target looked at in this series of experiments was the Rcs phosphorelay. It was originally hypothesized that the cell envelope stress response pathway may contribute to the resistance seen by various mutants isolated on ampicillin. Upon deletion of the pathway, it was shown that a portion of the mutant strains were partially dependent on the pathway when exposed to an antibiotic-induced environment. There was a notable difference when comparing liquid MIC data to solid media via spot titer determination. When viewing MIC data alone, it appeared that the most important factor of the pathway was the *rcsB* gene, as six of the Δ*rcsB* strains showed a decrease in MIC compared to their corresponding parent mutant strains with *rcsB*. Upon deletion of *rcsA*, only two strains showed a decrease in MIC and deletion of *rcsF* resulted in a decrease in MIC for five strains. However, when viewing the solid MIC and the colony forming units for each strain, the *rcsF* gene appeared to be the most important factor, as that was the first that resulted in little or no growth compared to Δ*rcsA* and Δ*rcsB* strains. For some of the strains, plating solid media came up as expected when comparing to the liquid MICs, but for others, there were no connections between the two. For example, Mutant 1501 showed a

decrease in MIC upon deletion of *rcsF* (Transductant 1514) from 10 to 15 μg/ml. When plated on solid media, the number of Δ*rcsF* (Transductant 1514) colonies had decreased when plated on 15 μg/ml, as expected. Mutant 1503 however, showed no change in MIC upon deletion of *rcsF* (Transductant 1515), but did see a decrease after deleting *rcsB* (Transductant 1509). When plated on solid media at 10 μg/ml, the exact opposite occurred; *rcsF* (Transductant 1515) had decreased before *rcsB* (Transductant 1509). Originally, it was thought that the differing results from MIC to spot titer determination could be due to the experiments done at different times and the use of different stocks of ampicillin. All the MICs were done before plating, therefore a different stock was used for the two experiments. A problem with working with amp is that the ability of the drug to function diminishes with each freeze/thawing occurrence. Over time, amp hydrolyzes and results in products that are no longer active. With multiple freezing and thawing cycles, the concentration of active unhydrolyzed amp decreases. One explanation could be that once the original stock of amp was used for the MICs had completely hydrolyzed, the new stock used used for the spot titer determinations was active unhydrolyzed amp. However, that decrease in activity should affect all strains the same amount, theoretically, and the ratios should be the same. What could have happened was the use of different plates when measuring. Meaning, that the *rcsB* mutants were plated on one set of plates and the *rcsA* mutants were plated on a different set of plates. In the future, the parent, *rcsB*, *rcsA*, and *rcsF* mutants should all be on the same plates so that each one can be compared more easily to one another. Additionally, upon repeating these experiments, both the MICs and spot titer determinations should be performed at the same time, instead of separately.

The next approach than can be made with the Rcs phosphorelay data is with regards to cross-resistance. By testing cross-resistance, we can determine if the cells that have increased

resistance to amp are also resistant to other β-lactams. If not, then it implies that the resistance is specific to one β-lactam only. With these cross-resistant strains, the experiment can be repeated and the importance of the Rcs phosphorelay can be examined by deletions of the components in each cross-resistant strain. There have been previous experiments done in the lab where certain mutants resistant to one antibiotic have been plated on another antibiotic, in the attempt to isolate cross-resistant mutants. This idea was initially explored with amp and mecillinam (mec), however we were not able to successfully isolate any cross-resistant strains. We tried taking mutant strains resistant to mec and plating them onto different concentration of amp. The mec resistant strains were provided by another member of the lab, Jordan Antetomaso. The strains used were mutants that displayed different levels of resistance to mec. Some were isolated on 3 μg/ml mec and others were isolated on 0.3 μg/ml mec. Unfortunately, the strains tested showed no significant increase in resistance compared to the WT when tested with amp. Therefore, it was decided at the time that cross—resistance with mec was insignificant. However, there is the possibility of testing the other way around. Instead of taking strains resistant to mec and trying to grow on amp, there may be some level of cross-resistance if strains resistant to amp were grown onto mec. In other words, a strain that is resistant to mec may be susceptible to amp, while a strain that is resistant to amp may also be resistant to mec. This difference in resistance could be linked to the method by which the two different antibiotics work. Like amp, mec also targets PBPs that are involved in peptidoglycan synthesis. However, the range of PBPs that mec targets is not as broad as the range that amp targets. Amp targets multiple PBPs, resulting in cell lysis and death. Mec targets PBP2, a protein involved in elongation during cell growth (**28**). By inhibiting this PBP, the cells are left with a spherical morphology that prevents them from surviving . Perhaps the specificity of the targeting is linked to the difference in resistance.

The second series of experiments done was with regards to an ecological point of view and how manipulation of certain factors that regulate antibiotic-induced stress can change the response to that antibiotic-induced environment. To begin testing this, the relationship between timing and intensity needed to be understood before the addition of multiple frequencies could be taken into account. The establishment of proper time points and intensities used was critical for the design of the experiment. A majority of the data obtained was done mainly through trial and error. We didn't want to add too much drug too quickly to the point where nothing was growing, and thus no data to interpret. It was decided the best time points to use were: 0.15 hours (beginning), 1.15 hours (middle), and 2.15 hours (end). These time points fit the growth curve of the WT strain the best because the addition of amp was added while the cells were still in exponential phase and growing. By 2.15 hours, the exponential phase was beginning to taper off into stationary, which is why that time point was chosen as the "end." Besides establishing the time points, the intensity was also determined using preliminary trials. The MIC for the WT strain was 10 μg/ml amp. Based on the preliminary trials, it was decided that an established dosing size of 5 μg/ml amp would be used for the remainder of the experiments. With the proper time points and dosing size in mind, the intensity was varied from 1x to 4x the dosing size of 5 μg/ml at specific time points, while keeping the frequency fixed at 1 (Figure 12). The data obtained from these trials showed that timing was more important than intensity. In other words, adding the drug earlier rather than later was most effective at killing the cells and intensity did not matter as much. The addition of drug at the beginning for intensities of 10, 15, and 20 μg/ml amp showed essentially no difference in absorbance or colony forming units, suggesting that there is no difference in intensity for that time point, once 10 μg/ml amp was reached. However, while intensity was not as important as timing, there was a difference in growth at higher

intensities when amp was added to the middle of the exponential growth phase. This suggests that a higher concentration may needed at when added at the middle. Cells at the end of the growth curve did not appear to be affected at all, even at higher concentrations of amp. Therefore, at entry into stationary phase, the cells were essentially not treatable.

Intensity did, however, begin to show a difference after multiple frequencies were incorporated. In this event, the established dosing size was split according to the frequency at which the antibiotic-induced stress was being added. For example, at a frequency of 2, 5 μg/ml amp was added at the first time point and another 5 μg/ml amp was added to the same well at the second time point. Thus, the well ended with a total amount of amp that is equal to 2x the dosing size of 5 μg/ml, which was 10 μg/ml. Based on the results shown in Figure 13a, splitting the amount in half requires individual additions of 7.5 or 10 μg/ml (totaling 15 and 20 μg/ml, respectively). Intensities at individual additions of 2.5 or 5 μg/ml (totaling 5 and 10 μg/ml, respectively) is not enough to effectively kill the cells. Additionally, when added at higher intensities to time points of beginning and middle (BM) or beginning and end (BE) the antibiotic was able to effectively kill the cells compared to addition at middle and end (ME), regardless of the intensity used. This data suggests that when given at the BE, it is only the amount at the B that has a significant impact of the cells ability to survive. This idea is further supported when looking at a frequency of 1 – the addition of amp at the end, regardless of intensity used, had no effect. Thus, these observations support the previous argument that timing is the most important factor when compared to intensity.

The next approach that can be made with regards to manipulation of ecological factors is to change the model system used. For the results obtained through these series of experiments, individual cell populations of WT. Instead of using an individual population, we can instead mix

the populations with the two different strains, WT and a Δ*tolC* (dT) strain. In this experiment, the model is a more realistic representation of what occurs during an infection. At the start, there is a small fraction of the population that are resistant strains, and that fraction increases as the infection gets worse. The WT and dT strains were chosen because one is more susceptible to antibiotic treatment than the other. The dT strain is characterized as Δ*tolC*, which means it lacks the *tolC* gene. WT has a functioning *tolC*. The purpose of the *tolC* is it allows for the presence of an efflux pump utilized by the cells. The efflux pump is able to pump the antibiotic out of the cells when first encountered, thus protecting the cells from the antibiotic treatment. By lacking this pump, dT strains are more susceptible to amp than the WT.

In order to distinguish between the two when plated on solid medium, blue/white screening on Xgal will be utilized. The WT strain is characterized as Δ*lac* while the dT strain has a functioning *lac* gene. Within the *lac* gene, there is a specific sequence called the *lacZ*. When expressed, *lacZ* results in the protein called β-galactosidase (β-gal). When there is Xgal present in the medium, β-gal utilizes Xgal as a substrate and the result is a blue colony. If the *lac* gene is absent (Δ*lac*), β-gal cannot be synthesized from *lacZ* and the cells appear white when plated on Xgal medium. Therefore, blue/white screening can be a very useful tool when counting the number of WT colonies compared to the number of dT colonies that grow. Different ratios of 1:1, 1:10, and 10:1 of WT and dT can be created, respectively. For these experiments, the same factors can be manipulated and it will allow us to explore if the ecological factors affect the selection of the resistant strain over the susceptible strain.

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Education

2012 – present **The Pennsylvania State University, University Park, PA**
Schreyer Honors College, Eberly College of Science
Major: B.S. Biochemistry and Molecular Biology
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2008 – 2012 **Southside High School, Elmira, NY**
Graduated 2012 with Salutatorian Status and Honors

Thesis Title

Bacterial Responses to Antibiotic-induced Stress

Thesis Supervisor

Sarah Ades

Experience

2013 – Present **Research Assistant in Penn State Biochemistry Laboratory**
Studying how gram-negative bacteria respond to an antibiotic-induced stressful environment, and how changing or manipulating various factors of the disturbance event changes the response.

2014 – Present **Spring Undergraduate Research Exhibition at Penn State**
Poster presentations of my research done pertaining to Rcs Phosphorelay and Manipulation of Ecological Factors

2015 **Eberly College of Science Fall Exhibition at Penn State**
Poster presentation of my research done pertaining to Manipulation of Ecological Factors

2013 **Shadowing at Arnot Odgen Medical Center, Elmira, NY**
Shadowed Rose Byland, M.D., of the radiology department and Serge Dauphin, M.D., of the oncology department at the Falck Cancer Center

2010 **Shadowing at Roswell Park Cancer Institute, Buffalo, NY**
Shadowed a Post-Doctoral Research Affiliate, Evan Zynda, and his work pertaining to signaling pathways and cancer research

2015 – Present **Weis Markets**
Customer Service Desk Associate and Front End Monitor

2014 **Vector Marketing**
Sales Representative for Vector Marketing/CUTCO

2010-2011 **Elmira Pioneers Amateur Baseball Club**
Concessions, Souvenirs, and Cashier

Honors

2015	**Undergraduate Summer Erickson Discovery Grant** Awarded to undergraduates continuing summer research
2015	**Second Place at Spring Undergraduate Research Exhibition** Awarded 2nd place in Life Sciences category for research done pertaining to Rcs Phosphorelay
2012 – present	**Dean's List** Awarded to Undergraduates that earn above a 3.5 GPA for the corresponding semester within the Eberly College of Science
2012	**National Society of Collegiate Scholars (NCSC)**
2013	**Penn State Chapter Phi Eta Sigma Honors Society**
2012	**Salutatorian** Awarded Salutatorian status for 2012 graduating class at Southside High School
2011	**Bausch & Lomb Honorary Science Award, presented by University of Rochester** Awarded to a high school junior with the highest science average
2011	**AP U.S. History Excellence Award** Granted to a junior within the top 2% of the class