The Envelope Stress Response in Sedimentation-Resistant Escherichia Coli

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THE ENVELOPE STRESS RESPONSE IN SEDIMENTATION-RESISTANT

ESCHERICHIA COLI

by

NEEL KETAN SHAH

A thesis submitted in partial fulfillment of the requirements
for the Honors in the Major Program in Biomedical Sciences
in the College of Medicine
and in the Burnett Honors College
at the University of Central Florida
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Thesis Chair: Sean D. Moore, Ph.D.
ABSTRACT

Previous research discovered the existence of sedimentation-resistant mutants of *E. coli*. Genomic studies revealed that these mutants resisted sedimentation due to independent modifications to genes that influenced the Rcs signal transduction pathway, causing increased secretion of an exopolysaccharide capsule comprised primarily of colanic acid. The Rcs system is responsible for detecting envelope stressors; consequently, ampicillin and osmotic stress were used to perturb the cellular envelope and study the response of the mutants compared to wild-type cells. It was found that the overproduction of colanic acid in the mutants confers some resistance to envelope stress; however, the mutants still behaved similarly to wild-type cells. The doubling times of the strains grown in sodium chloride solutions were calculated. A wavelength scan from 400 nm to 800 nm was performed on strains grown in different salt concentrations to determine if there were significant differences in light scattering between the wild-type and mutant cells. Further analysis was performed that, along with the doubling time data, suggested that wild-type cells may have turned on genes for capsule production in response to being grown in high salt concentrations. Additional research could be conducted to test this hypothesis, perhaps through the quantification of colanic acid through a methyl pentose assay for wild-type cultures grown with high salt concentrations. The idea that wild-type cells could digest colanic acid as a carbon source when lacking resources was also investigated with different preparations of colanic acid. One preparation of colanic acid showed promising results, which could indicate that bacteria are able to digest their capsule in a novel method to produce energy when starved. Again, additional investigation should be conducted to confirm these results. Other future
experiments could study the metabolome of these mutants to determine if they have increased quantities of alarmones related to biofilm formation.
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LIST OF ACRONYMS/ABBREVIATIONS

C02 – weak mutant
C05 – medium mutant
C06 – strong mutant
CA – colanic acid
cps – capsular polysaccharide synthesis
ESR – envelope stress response
LB – Luria Bertani broth
Lpp – outer membrane protein; mutated in medium mutant
OD – optical density
Rcs – regulator of capsule synthesis
yrfF – IgaA homolog; mutated in the strong mutant
I. INTRODUCTION

1.1 Background

Prior research identified multiple strains of *Escherichia coli* that oppose sedimentation, even at heightened magnitudes of applied force [1]. After further investigation, it was found that these distinctive bacteria were able to remain suspended throughout a liquid culture as a result of mutations [1]. Some of the *E. coli* were pelleted in the solution; however, a large majority still remained in suspension, which could be observed outwardly due to the turbidity of the media [1]. The mutant strains exhibited secretion of an exopolysaccharide comprised of colanic acid from the cell envelope that was found to be integral in their ability to resist sedimentation [1]. The secreted exopolysaccharide was anchored to the envelope of the cell [1]. This linkage increased the hydrodynamic drag force the bacteria experienced when in a fluid and, subsequently, the bacteria’s motion was impeded without the direct use of energy [1]. The mutant strains exhibited a shared mucoid phenotype but were each caused by different mutated genes [1]. Despite the different genes that caused the mucoid phenotype, they all were found to affect the same signal transduction pathway [1].

This discovery was particularly significant because it could expose a new way that bacteria can adapt to resist external changes in their environment; consequently, these bacteria could thrive in situations in which their presence is potentially detrimental. The envelope that was produced could protect bacteria from the human immune system by preventing interactions with the targeting antibody [1]. One mutant was even found to shed the exopolysaccharide into the surrounding solution, which could be a method by which bacteria can avoid opsonization [1]. Different cell envelope stressors, like antibiotics and high salt environments, were tested to
determine if the exopolysaccharide offered protection. Studies were also conducted to determine if the morphology of the mutants differed significantly from wild-type (WT) cells. Finally, an experiment was performed to determine if the exopolysaccharide could provide an alternative carbon source that could be utilized by the wild-type cells during times of starvation. The response of the cell to physical stressors is an important regulatory quality that needs to be studied in different organisms. Learning if the exopolysaccharide can be used as fuel could expose innovative means by which bacteria can adapt to survive and proliferate.

Industrial applications have been considered, as well, contingent on synthetic production of the exopolysaccharide, as well as its attachment to other surfaces [1]. The suspension of particles in certain mixtures involved in manufacturing processes can eliminate a major output of energy required for blending; for example, waste treatment facilities would not need to have massive apparatuses solely to churn the components of the storage receptacles [1]. Immunological functions have also been anticipated [1]. As aforementioned, the exopolysaccharide could prevent the binding of antibodies to bacterial epitopes, or the capsule could even be shed to rid the bacteria of molecular markers; however, if the mechanism of action is elucidated, a countermeasure could be proposed to target harmful bacteria that are capable of evading phagocytes of the immune system [1].

1.2 *Escherichia coli*

*E. coli* is a prokaryotic, Gram-negative bacterium that is shaped like a rod and is typically about one to two micrometers in length [2]. It is a facultative anaerobe that has evolved to live in the mammalian gut; thus, its optimal temperature for growth is 37°C [3,4]. The organisms in this experiment, however, were always grown at a temperature of 30°C. This made them more
convenient to work with because growth was slowed, allowing the bacteria to reach stationary
phase later. Growing the cells at 30°C was also done because the mutants being studied
displayed temperature-sensitive alleles [5]. 30°C was the permissive temperature that allowed
expression of the mutant genes; conversely, 37°C was the non-permissive temperature that
cause the mutants to behave like wild-type cells despite the presence of mutant alleles [5]. *E.
coli* is frequently used as a model organism in molecular biology and genetics studies because of
its well-known properties and rapid generation time; additionally, its ability to transfer DNA
through transduction and transformation is valuable to researchers [3]. *E. coli* is also very
durable, versatile, and easy to handle, which makes it a prime organism for laboratory studies
[3].

Many strains of *E. coli* are harmless to humans, though pathogenic strains do exist [2].
The relationship between *E. coli* and humans is important to analyze because of the varying
types of symbiotic relationships that can occur, ranging from commensal to pathogenic [2]. The
K-12 strain is commonly used as a reference, and mutants from the BW30270 strain, a K-12
derivative, that exhibit the mucoid phenotype were examined [2]. It has been found that these
mutants are able to grow on solid media in a manner that resembles the formation of a biofilm.
While it is known that *E. coli* are able to produce a biofilm when growing in such environments
as the lower intestines of mammals, the mutant biofilm-like phenotype is noteworthy because of
its dissimilarities to the wild-type phenotype [3].

1.3 Antibiotic Resistance

Antibiotic resistance typically refers to the inherited ability of bacteria to rapidly acquire
mutations that allow them to survive in the face of elevated concentrations of antibiotics,
regardless of the time of treatment [6]. Bacterial resistance is quantified through a metric called the minimum inhibitory concentration (MIC), which is the smallest concentration of an antimicrobial agent that will prevent visible growth of a microbe after it has been allowed to incubate overnight [6,7].

One common form of bacterial persistence is the development of a biofilm [8]. The mutant *E. coli* displayed a biofilm-like layer with a mucoid appearance, as well as an increase in biomass. The similarities between the morphologies is meaningful because biofilms are intrinsically resistant to antimicrobials [8]. Having an exopolysaccharide layer surrounding the mutant strains could allow them to be more resistant to antibiotics than wild-type cells.

Several mechanisms exist to account for this inherent characteristic; for example, the width of the biofilm and the shielding matrix of polymers could physically prevent certain antimicrobial agents from pervading [8]. Another hypothesis suggests that some cells are starved of nutrients, which causes them to grow at a slow rate [8]. Bacteria that experience limited growth are not as susceptible to these harmful agents [8]. One other speculation is that the bacteria in a biofilm could potentially have reduced susceptibility due to a protective phenotype that is expressed as a biological response to growing on a solid surface [8]. Bacterial infections that are persistent due to the construction of biofilms are difficult to eradicate [8]. If the mechanism of action for the secretion of the exopolysaccharide is determined, it could have further positive effects by potentially revealing a method to treat this type of bacterial infection.

1.4 Ampicillin

Ampicillin is a bacteriostatic penicillin derivative that can affect the envelope of both Gram-positive and Gram-negative bacteria [9]. The mechanism of action involves the binding
and inactivation of proteins on the inner membrane of the cell wall of bacteria that are integral in creating cross-linkages of peptidoglycan chains for increased structural integrity [9]. When ampicillin binds to the penicillin-binding proteins, bacterial cell wall synthesis is interrupted, which eventually leads to lysis [9]. Because of its efficacy and cost-effectiveness, ampicillin will be used to determine if the sedimentation-resistant bacteria show altered growth patterns compared to the wild-type.

1.5 Osmotic Stress

Bacterial cell envelopes are permeable to a large number of solutes, as the cells must be capable of regulating the intracellular environment when posed with extracellular challenges [10]. If extracellular solute concentrations are high, bacteria can face dehydration, which can ultimately result in cell death [10]. When placed in an environment with a high solute concentration, the bacterial inner membrane can be pulled away from the cell wall due to water loss [10]. This increase in distance between the cell wall and inner membrane can be detected by bacterial stress-sensing systems and can trigger a protective response like capsule production [10]. In this experiment, sodium chloride was used to induce osmotic stress in the wild-type and mutant strains.

1.6 Colanic Acid

Due to the occurrence of pathogenic bacteria that are resistant to antibiotics, new treatments options have been considered; for instance, bacteriophage therapy, a remedy in which phages infect bacteria to kill them [11]. Bacteriophage therapy has many advantages, but it has led to the advent of phage-resistant bacterial mutants [11]. These mutants could prevent phage
infection by not allowing the phage to adsorb to the surface of the cells as a result of the presence of an extracellular matrix [11].

It has been found that one of the components of the extracellular matrices produced by these strains of *E. coli* is colanic acid, an exopolysaccharide [11]. Colanic acid has backbone with three repeating units of D-glucose, L-fucose, and L-fucose; additionally, this backbone has branches comprised of three units of D-galactose, D-glucouronic acid, and D-galactose [12].

![Figure 1. Colanic acid structure.](image)

Colanic acid is comprised of a backbone with three repeating units of D-glucose, L-fucose, and L-fucose. Branches with three units of D-galactose, D-glucouronic acid, and D-galactose extend from this backbone. This image was modified from Kessler, 2018.

Colanic acid production is regulated by a cluster of genes in the *wca* operon in *E. coli*; additionally, the transcription of these genes is controlled by the Rcs (regulator of capsule synthesis) phosphorelay system [13]. The Rcs system has an integral part in the further steps of biofilm assembly, and colanic acid allows the bacteria to form a defensive casing, which is crucial to note [14]. In the case of biofilms, the group of bacteria as a whole is protected; however, in the case of the mutant strains being studied, the secretion of the exopolysaccharide
allows each individual bacterium to be shielded through the production of a capsule. Capsules have been found to increase resistance to antibiotics; therefore, it is possible that secretion of colanic acid from the cell can allow the cells to be less affected by antibiotics [15]. It has been found that the mutant strains overexpress colanic acid during stationary phase. Some of the physical stressors that cause an upregulation in colanic acid production include osmotic stress and cell envelope perturbation [11]. Studying the relationship between physical stressors and the secretion of colanic acid may yield further insight about the mechanism that the mutant strains of *E. coli* use to resist sedimentation.

### 1.7 Envelope Stress Responses

As a result of interacting with a constantly changing external environment that can pose threats, bacteria need to have the ability to modify and repair their cell envelope [16]. Envelope stress responses (ESRs) are the reaction of bacterial cells to alleviate injuries or deficiencies of the cell wall through alteration of transcription [16]. The Rcs phosphorelay system senses harm to the lipopolysaccharide (LPS) layer caused by cationic antimicrobial peptides (CAMPs) and responds by increasing the production of exopolysaccharides [16].

Two-component systems (TCS) and phosphorelays in bacteria have a representative sensor kinase at a histidine residue and a response regulator at an aspartate residue [14]. Phosphorylation of the effector subsequently alters the regulation of a specific protein that is involved in the response; moreover, this change in protein management can affect transcription [14]. In the BW30270 strain of *E. coli*, RcsC acts as a sensor and RcsB acts as an effector of a TCS that regulates the expression of the exopolysaccharide colanic acid [17].
Recent studies have also shown that small RNAs (sRNAs) could also quickly regulate multiple ESRs when cells are exposed to several concurrent stressors [16]. The sRNA RprA is a highly stimulated component of the Rcs phosphorelay system in response to outer membrane damage [16]. The function of RprA is to increase the production of $\sigma^S$—it accomplishes this by removing a structure in the rpoS mRNA that hinders translation [16]. The protein $\sigma^S$ is significant because it is the major controller of general stress, and it encourages bacteria to transition from planktonic growth to biofilm development [16]. This transition, originally caused by some type of membrane damage, links the biofilm-like production of the exopolysaccharide with the resulting resistance to sedimentation and, in turn, with adverse immunological effects due to potential bacterial resistance against antibiotics.

1.8 Mutant Strains – C02, C05, and C06

Multiple mutant strains were discovered, and it was found that they all displayed a mucoid phenotype; however, the strength of the phenotype varied. The phenotype was classified on a relative scale from weak to medium to strong. Three mutant strains were selected for these experiments—the C02, C05, and C06 mutants. These particular mutants were representative of the weak, medium, and strong phenotypes, respectively. It was discovered that the weak mutant had a tendency to shed the colanic acid it was producing, while the medium and strong mutants kept colanic acid attached to the cells.

The genome of the BW30270 strain of E. coli used in this experiment was sequenced prior. The weak, medium, and strong phenotypes were discovered to be caused by different mutations. In the weak mutant, C02, it was found that the rcsC protein had a missense mutation at position 840, wherein leucine was substituted with arginine. The rcsC protein is a component
of the Rcs signal transduction pathway that can ultimately affect transcription [14]. RcsC is a transmembrane protein located across the inner membrane of *E. coli* with a sensor kinase domain and a receiver domain [14]. RcsC can autophosphorylate after receiving a signal from RscF, an outer membrane protein that responds to cell envelope stressors [14]. The Rcs signal transduction system regulates the expression of genes associated with colanic acid capsule production via the *cps* (capsular polysaccharide synthesis) operon [18]. In the medium mutant, C05, it was found that the major outer membrane lipoprotein Lpp had a deletion that removed 14 amino acids from lysine at position 26 through alanine at position 39. Lpp is the most abundant protein in *E. coli* and helps tether the outer membrane to the peptidoglycan layer through a covalent linkage, which increases the structural integrity of the cell [19]. Lpp interacts with the stress-sensing protein RcsF; furthermore, it has been demonstrated that the Rcs signal transduction pathway is directly related to the length of the Lpp lipoprotein [19]. When the length of Lpp was artificially increased, signaling was inhibited [19]. When the length of RcsF was increased to compensate, signaling was restored [19]. The deletion of 14 amino acids from the structure of Lpp suggests that the medium mutant could be more sensitive to cell envelope stressors, which could result in increased colanic acid secretion. In the strong mutant, C06, it was found that there were three mutations causing the mucoid phenotype. Two of these mutations affected genes for diguanylate cyclases responsible for producing cyclic di-3′,5′-guanylate, an important ubiquitous bacterial secondary messenger that can regulate biofilm formation [20]. Despite these two mutations, the phenotype of the strong mutant can be attributed mainly to one other mutation. The primary mutation is a missense mutation in the *yrfF* protein at position 564, wherein alanine is substituted with proline. YrfF is a transmembrane protein of the inner membrane and is a homolog of the
protein IgaA, which is responsible for suppressing activation of the Rcs signal transduction pathway [21]. Because yrfF is altered, Rcs proteins are not controlled as tightly, which results in an overproduction of colanic acid secreted from the cell.

**Figure 2. Rcs signal transduction pathway.** Lipoprotein lpp and RcsF work together to detect outer-membrane stress. RcsF sends a signal to RcsC if stress is detected. RcsC autophosphorylates at a histidine residue. The phosphate group is then transferred to an aspartic acid residue, followed by another histidine residue on RcsD. RcsD serves as the link between RcsC and RcsB. RcsB is phosphorylated at an aspartic acid residue and works in
conjunction with RcsA to upregulate the \textit{cps} operon and RprA sRNA. This results in increased colanic acid secretion and a transition from planktonic growth to biofilm formation. YrfF is an analog of IgaA, which serves to suppress the Rcs phosphorelay system. This image was modified from Majdalani and Gottesman, 2005.

![Diagram of colanic acid secretion](image)

**Figure 3. Colanic acid secretion.** The \textit{cps} operon synthesizes proteins that attach colanic acid to the cell envelope. WcaJ begins to assemble CA in the cytoplasm. An enzyme moves the polymer to the outer face of the inner-membrane. WcaD attaches CA polymers to form a long chain. The CA chain is extruded through the outer-membrane channel Wza with the help of Wzc. In the weak mutant, CA is hydrolyzed and shed; in the medium and strong mutants, CA remains attached to the cell envelope. This image was modified from Ranjit and Young, 2016.

1.9 Light Scattering

Light scattering occurs when particles of light strike matter and deviate from their original trajectory due to the matter through which the light passes [22]. Light can either be scattered or absorbed. Scattering can reveal characteristics of an object’s shape or its constituent materials [22]. It is known that the amount of light scattering that occurs in a solution with
microbes is proportional to the accumulated biomass [22]. Light with wavelength of 600 nm is frequently used to study the growth of microbes and measure the concentration of bacterial cells because most of the light is scattered [22]. Bacterial cells have marginal amounts of absorption of light at 600 nm, so it is useful when light scattering measurements are desired [22]. In most cases, a direct relationship between biomass and light transmission can be formed; however, sometimes cell physiology and the production of various metabolites can influence light scattering [22]. In this experiment, an EPS coating of colanic acid exists around the mutant strains [22]. In spite of this, the 600 nm wavelength can still be effectively used to measure light scattering because colanic acid is an optically transparent substance. Two main types of light scattering exist, dependent on the size-to-particle ratio: Rayleigh scattering and Mie scattering [22].

Rayleigh scattering predominantly occurs with very small substances that are usually less than one-tenth the size of the wavelength irradiating the object [22]. In this model, light scattering is not dependent on the angle at which scattering is measured [22]. Due to the small size of the particles, there is no preferential deviation of the light in any one direction [22]. Rayleigh scattering does not apply to *E. coli* well, as the cells are larger than the wavelength being used [22].

Mie scattering predominantly occurs with large objects that are greater than the size of the wavelength irradiating the object [22]. In this theory, light scattering has a strong angular dependence [22]. The angle of incident light can affect the intensity of light scattered at the angle at which it is measured [22]. As a result, angular dependence can be used in some systems to determine the architecture of the material being studied. Bacterial cells are larger than the
wavelengths used to measure light scattering; consequently, Mie theory has a better fit to explain bacterial light scattering. Still, Mie scattering does not model bacterial light scattering perfectly because bacterial cells are slightly smaller than particles ideal for Mie scattering. The light scattering of mammalian cells is frequently modeled well by Mie scattering [22].

Another factor that contributes to how intensely light is scattered are the refractive indices of the medium and the particle through which light passes [22]. If cells are present in the medium, light must pass through them, which can cause it to bend and produce a different light scattering intensity than if cells were absent and light were passing through the medium alone [22]. Larger refractive indices result in a larger scatter pattern.

1.10 Preliminary Research

The BW30270 strain of E. coli was used to cultivate the mutant strains used in this experiment. The BW30270 strain is an RPH+ FNR+ relative of the MG1655 substrain. DNA from cells of this strain was purified, and the genome was sequenced. Several lineages of bacteria that displayed the different strengths of the mucoid phenotype were found, ranging from weak to medium to strong on a relative spectrum. The C02, C05, and C06 mutants were found to be excellent representatives of the weak, medium, and strong phenotypes, respectively, and were thus used for the experiments performed. Based on the genomic analysis and prior experimentation performed, specific mutations that cause the mucoid in each of the phenotype categories were elucidated.

Investigation has shown that the mutant strains of E. coli are able to maintain their suspension in the centrifuged fluid without actively using energy [1]. The current hypothesis is that the exopolysaccharides present on the outer surfaces of the cell increase hydrodynamic drag
the cells experience within the solution, which prevents them from pelleting [1]. The position of the mutant bacteria can be altered by subjecting them to extreme centrifugal force; however, they still do not completely form a pellet [1]. The bacteria loosely aggregate against each other, rather than forming a solid mass [1].
II. MATERIALS AND METHODS

2.1 Research Objectives

The experiment aimed to study the link between membrane stressors and colanic acid secretion from sedimentation-resistant bacteria. The envelope stressors in question included a bacteriostatic antibiotic and osmotic stress. A growth study with a parental wild-type and three different mutants was performed, and the results were compared to determine if the membrane stressors caused varying amounts of colony formation and levels of colanic acid expression. To test for antibiotic resistance, the mutants were placed in a 96-well plate with increasing concentrations of the antibiotic. The optical density at 600 nm (OD₆₀₀) of each well was read in a spectrophotometer at five-minute intervals for twelve hours, and a graph was generated to show the growth pattern. To test for resistance to water loss caused by osmotic stress, the mutants were placed in a 96-well plate with increasing concentrations of salt in Luria-Bertani (LB) broth with 0.2% glycerol. The same procedure was followed to create a graph that displayed the growth pattern of the mutants in these conditions. After the growth rate experiment was conducted, the doubling time was calculated; additionally, the final density was analyzed to see if there were significant differences in the amount of growth seen in each sample. Once the differences in growth were quantified, the results were normalized, and a statistical test was performed to determine if the four samples had significantly dissimilar morphologies. A separate experiment was done to understand if the wild-type cells could utilize extracellular colanic acid as a carbon source to produce energy.
2.2 Mutant Strain Growth

*E. coli* strain BW30270 was used to generate the mutants [1]. The cells were plated on LB-glycerol (0.2%) agar and incubated for 16-18 hours at 30°C [1]. An isolated colony from the plate was used to inoculate 10 mL of LB broth in a 125 mL Erlenmeyer flask with 0.2% glycerol [1]. The flask was placed in a shaker at 220 rpm at 30°C for 16-18 hours [1]. The bacteria reached stationary phase of growth, and 1 mL of the mutant was pipetted into a microcentrifuge tube and spun at 3250 × g for 10 minutes [1]. 750 µL of the supernatant were transferred into a freezer stock tube with 250 µL of 50% glycerol and 50% DI water to achieve a final concentration of 12.5% glycerol [1]. The freezer stocks were vortexed; subsequently, 200 µL of the stock solution were transferred into new 125 mL Erlenmeyer flasks with 10 mL LB-glycerol (0.2%) [1]. The same protocol was followed to grow successive generations, and the incidence of the mucoid phenotype was registered when a solid pellet was no longer identifiable after centrifugation [1]. The mutants from that generation were plated on LB-glycerol (0.2%) agar and grown in an incubator at 30°C for 16-18 hours [1]. For this experiment, new freezer stocks of the wild-type, weak mutant, medium mutant, and strong mutant were created.

2.3 Growth Study – Ampicillin

To determine if the weak, medium, and strong mutants displayed potential resistance to antibiotics due to the exopolysaccharide coating that forms a capsule around them, a growth study was performed. First, the range of antibiotic concentrations to use in the experiment was determined by researching typical minimum inhibitory concentrations for *E. coli*. It was determined that a two-fold serial dilution of the antibiotic would be best for the growth study, with concentrations ranging from 3.125 µg/mL to 50 µg/mL. The antibiotic used for the test was
ampicillin. One positive control included LB-glycerol (0.2%) broth and the wild-type, weak, medium, or strong cells. Normal growth was expected. Another positive control included LB-glycerol (0.2%) broth with 100 µg/mL ampicillin and the wild-type, weak, medium, or strong cells. Because 100 µg/mL ampicillin is an inhibitory concentration, no growth was expected. The negative control was plain LB-glycerol (0.2%) broth with no inoculum. Again, no growth was expected if no contamination occurred.

The two-fold serial dilutions of ampicillin were made from a 1000X stock that was made prior. The stock solution of ampicillin was made by dissolving 1 g of sodium ampicillin into 10 mL of 50% ethanol and 50% DI water. The stock solution had a final concentration of 100 mg/mL (1000X) ampicillin in 50% ethanol and 50% DI water. When the final concentrations of ampicillin were achieved for each sample, the concentration of ethanol decreased to a value considered negligible, thus not inhibiting growth.

The wild-type and the three mutants were streaked onto an LB-glycerol (0.2%) agar plate. An isolated colony was selected for each of the four types of cells and was used to inoculate 1 mL of LB-glycerol (0.2%) broth. The wild-type, weak, medium, and strong cells were grown in 9 mL glass test tubes for 16-18 hours at 30°C in a shaker at 220 rpm. The liquid cultures were diluted in a 1:10 ratio with LB-glycerol (0.2%) broth in a 1 mL cuvette, and the OD₆₀₀ was determined. The values were normalized to a 1:100 dilution of the culture with the lowest OD₆₀₀ in LB-glycerol (0.2%) broth to ensure that each well used in the growth study was inoculated with the same quantity of bacteria.

Because the edges of the 96-well plate created an environment different from the wells in the center of the plate, the center wells were used for the study. The surrounding cells were filled
with 200 µL of DI water, 200 µL of the positive control, or 200 µL of the negative control. The wells in the center were divided into quadrants so the wild-type and each mutant had triplicates of the five selected concentrations of ampicillin. 500 µL of the wild-type, weak, medium, or strong cells from the normalized cultures and 500 µL of the appropriate ampicillin concentrations were thoroughly mixed in microcentrifuge tubes. Each of the experimental wells in the center of the plate was filled with 200 µL of each solution with the bacteria and ampicillin from the microcentrifuge tube. The solution with the bacteria and antibiotic was vortexed before addition to the wells, and the solution in the well was pipetted up and down for proper mixing. Care was taken to avoid the formation of bubbles in the wells, which could have potentially altered the measured OD$_{600}$. This procedure ensured reproducibility of the results. A depiction of the organization of the 96-well plate is shown below.
Figure 4. **96-well plate set-up for growth study with ampicillin.** Wells A1 through D1 contained a positive control of LB inoculated with either the wild-type or one of the mutant strains with no ampicillin. Wells A12 through D12 contained a positive control of LB with 100 µg/mL ampicillin. Wells E1 through H1 contained a negative control of plain LB. The four mutant strains were separated into quadrants on the plate, and triplicates of each concentration of ampicillin were tested. The green wells contained the WT, the orange wells contained the weak mutant, the blue wells contained the medium mutant, and the gray wells contained the strong mutant.

Once the wells were filled, the plate was placed into a spectrophotometer. The conditions of the growth study were set to a temperature of 30°C with continuous shaking. The OD$_{600}$ was measured every five minutes for a period of 12 hours.

2.4 Growth Study – Osmotic Stress

To determine if the wild-type, weak, medium, and strong cell types displayed potential resistance to osmotic stress and dehydration as a result of the exopolysaccharide coating, another
growth study was performed. For this experiment, sodium chloride concentrations were varied to create different environments in which the cells were grown. The concentrations of sodium chloride that would be used were based on the concentration used in the standard LB-glycerol (0.2%) broth, which had a salt concentration of 5 g/L. In the experiment, two-fold increments were used to create final concentrations ranging from 2.5 g/L to 20 g/L sodium chloride in the LB-glycerol (0.2%) broth. One positive control included LB-glycerol (0.2%) broth with 5 g/L sodium chloride and the wild-type, weak, medium, or strong cells. Normal growth was expected. Another positive control included LB-glycerol (0.2%) broth with 100 g/L sodium chloride and the wild-type, weak, medium, or strong cells. Because 100 g/L sodium chloride is an inhibitory concentration of salt, no growth was expected. The negative control was plain LB-glycerol (0.2%) broth with 5 g/L sodium chloride and no inoculum. No growth was expected if no contamination occurred.

The dilutions of sodium chloride had to be made carefully because the LB-glycerol (0.2%) broth in which the bacteria were grown already contained 5 g/L of sodium chloride; as a result, to make the desired final concentrations of salt, ranging from 2.5 g/L to 20 g/L, a different procedure was followed. Stocks of LB-glycerol (0.2%) broth were made with 0 g/L, 5 g/L, 15 g/L, and 35 g/L sodium chloride. All other components of the LB-glycerol (0.2%) broth were kept at their normal concentrations.

The wild-type, weak, medium, and strong mutants were streaked onto an LB-glycerol (0.2%) agar plate from the freezer stocks. An isolated colony from each of the four types of cells was used to inoculate 1 mL of LB-glycerol (0.2%) broth with 5 g/L sodium chloride. The wild-type, weak, medium, and strong cells were grown in 9 mL glass tubes for 16-18 hours at 30°C in
a shaker at 220 rpm. The liquid cultures were diluted in a 1:10 ratio with LB-glycerol (0.2%) with 5 g/L sodium chloride in a 1 mL cuvette, and the OD\textsubscript{600} was determined. The values were normalized to a 1:100 dilution of the culture with the lowest OD\textsubscript{600} in LB-glycerol (0.2%) broth to ensure that each well used in the growth study was inoculated with the same quantity of bacteria.

Because the edges of the 96-well plate created an environment different from the wells in the center of the plate, the center wells were used for the experiment. The surrounding wells were filled with 200 µL of DI water, 200 µL of the positive controls, or 200 µL of the negative control. The wells in the center were divided into quadrants so the wild-type and each mutant had triplicates of the four selected concentrations of sodium chloride. 500 µL of the wild-type, weak, medium, or strong cells from the normalized cultures and 500 µL of the appropriate LB-glycerol (0.2%) broth with varying sodium chloride concentrations were thoroughly mixed in microcentrifuge tubes. Each of the experimental wells in the center of the plate was filled with 200 µL of each solution with the bacteria and LB-glycerol (0.2%) broth from the microcentrifuge tube. The solutions with the bacteria and varying sodium chloride concentrations were vortexed before addition to the wells. The solutions in the wells were pipetted up and down to ensure thorough mixing. Care was taken to avoid formation of bubbles in the wells, which could have potentially altered the measured OD\textsubscript{600}. This procedure ensured reproducibility of the results. A depiction of the 96-well plate is shown below.
Figure 5. 96-well plate set-up for growth study with NaCl. Wells A1 through D1 contained a positive control of inoculated LB with 5 g/L NaCl. Wells A12 through D12 contained a positive control of LB with 100 g/L NaCl. Wells E1 through H1 contained a negative control of plain LB. The four mutant strains were separated into quadrants on the plate, and triplicates of each concentration of sodium chloride were tested. The green wells contained the WT, the orange wells contained the weak mutant, the blue wells contained the medium mutant, and the gray wells contained the strong mutant.

Once the wells were filled, the plate was placed into a spectrophotometer. The conditions of the growth study included a temperature of 30°C with continuous shaking. The OD$_{600}$ was measured every five minutes for a period of 12 hours. The growth rate of each sample was calculated to determine if there was a significant difference. The final density of each sample was compared in two ways. One method determined if the type of cell used had significant differences in
resistance to osmotic stress, while the other determined if the salt concentration used caused significant differences in the amount of growth.

2.5 Statistical Analysis of Light Scattering

After the osmotic stress test was performed, the wild-type, weak, medium, and strong cells that were grown in the 2.5 g/L (low), 5 g/L (normal), and 20 g/L (high) sodium chloride solutions were of interest. To determine if the mutants differed optically from the wild-type and from each other, a wavelength scan was performed. First, the wild-type, weak, medium, and strong mutants were streaked onto an LB-glycerol (0.2%) agar plate. Isolated colonies were selected for each type of cell and were used to inoculate 1 mL of LB-glycerol (0.2%) broth with the low, normal, and high concentrations of salt. The cultures were grown in 9 mL glass tubes and set in a shaker for 16-18 hours at 30°C and 220 rpm. After the cultures had reached stationary phase, 100 µL of each culture were pipetted into three wells of a 96-well plate. Triplicates of each sample were used to increase the reliability of the data. The plate was placed into a spectrophotometer, and an endpoint wavelength scan was performed from 400 nm to 800 nm for each sample.

The data collected were adjusted to the average optical density measured at 600 nm for the wild-type cells, as this was the usual wavelength used to measure the growth of each sample in the prior growth experiments. By adjusting the data and normalizing at 600 nm, it was found that the extremes of the wavelength scan, close to 400 nm and 800 nm showed a splay. To determine if the differences in final density of the four cell types in the low, normal, and high salt concentrations were statistically significant, a two-sample unpaired t-test was performed at each wavelength. Prior to the t-test being performed, an f-test was performed to check if the data was
homoscedastic or heteroscedastic. The f-test results showed that a homoscedastic t-test was appropriate for each data set at each wavelength. In each grouping of low, normal, and high salt concentrations, multiple comparisons were made. The weak, medium, and strong mutants were compared to wild-type; the medium and strong mutants were compared to the weak mutant; and the strong mutant was compared to the medium mutant. The p-values for each wavelength between 400 nm and 800 nm was calculated to determine if there were statistically significant optical differences due to colanic acid secretion between the four types of cells.

2.6 Minimal Medium with Addition of Colanic Acid

Another hypothesized use of the secreted exopolysaccharide colanic acid was as a potential carbon source during times of starvation. To test this hypothesis, MOPS minimal medium was made from a Teknova MOPS EZ Rich Defined Medium Kit. The modified MOPS medium contained 10X MOPS Buffer, 0.132 M K$_2$HPO$_4$, and sterile H$_2$O. Glucose was introduced into the medium and served as the only carbon source that the *E. coli* could use for energy. This medium was filter sterilized with a 0.2-micron filter. Glucose concentrations in the MOPS medium ranged from 0% to 0.2%. The MOPS medium with 0% glucose was used as a positive control, and DI water was used as a negative control to check for contamination. By using different concentrations of glucose, the concentration at which growth was inhibited by lack of carbon could be determined by a drop in the final density of the solution. A concentration at which growth was inhibited by a dearth of carbon yet had enough viable cells was desired.

The wild-type cells were used for this experiment. The wild-type cells were streaked onto an LB-glycerol (0.2%) agar plate from a freezer stock. An isolated colony was selected and used to inoculate 5 mL of the MOPS medium with no glucose. A stock solution of 20% sterile glucose
was used to create serial dilutions of the concentrations of glucose with the MOPS medium lacking the inoculum. 500 µL of the inoculated MOPS medium with no glucose and 500 µL of the MOPS medium with glucose were mixed in 9 mL glass tubes to achieve the necessary final concentrations of glucose in samples with cells present. The glass tubes were placed in the shaker for 16-18 hours at 30°C and 220 rpm.

After the cultures had grown, 100 µL of each sample were placed into wells of a 96-well plate. The plate was placed in a spectrophotometer, and the OD$_{600}$ was measured to determine relative growth at each concentration of glucose. Because the WT cells in the MOPS medium with 0.1% glucose showed a lower OD$_{600}$ than the WT cells in the MOPS medium with 0.2% glucose, it was determined that 0.1% glucose was a low enough concentration to be a limiting factor. As all of the glucose had been used up in the sample, the same culture was used for the next phase of the experiment.

Five preparations of colanic acid were used in the next moiety of the investigation. These solutions of colanic acid were dialyzed against 1) 50 mM MES and 100 mM NaCl; 2) 0.5 mM EDTA; 3) isopropanol; 4) isopropanol, 50 mM MES, and 100 mM NaCl; and 5) ethanol, 50 mM MES, and 100 mM NaCl. 100 µL of the WT cells that were grown in the original MOPS medium with 0.1% glucose were added to 9 mL glass tubes; additionally, 100 µL of either MOPS with 0% glucose, MOPS with 0.2% glucose, or each of the different colanic acid preparations were added to the same tubes. The tube with the WT cells and 0% glucose was used as a positive control, while a different tube with 200 µL of DI water was used as a negative control. The tubes were placed in a shaker for 16-18 hours at 30°C and 220 rpm. Because the solution with 0.2% glucose added had a final concentration of 0.1% glucose, it was expected to
reach approximately the same OD$_{600}$ as the original sample of cells grown in MOPS medium with 0.1% glucose. After the cultures had sufficient time to grow, the OD$_{600}$ of each sample was measured.
III. RESULTS

3.1 Growth Curves – Ampicillin

Five concentrations of ampicillin in LB-glycerol (0.2%) broth were tested with the four strains of *E. coli*. Some outlier values can be seen in each of the graphs, perhaps caused by machine error. Overall, the mutant strains demonstrated slightly different behavior from the wild-type—they appeared to reach higher optical densities in the lowest concentration of ampicillin. This indicated that the exopolysaccharide coating of colanic acid present on the mutant strains likely conferred resistance to ampicillin; however, once the ampicillin concentration was increased, the mutant strains could not overcome the inhibitory effects on cell wall synthesis and acted in a manner similar to wild-type. This is an important physiological finding, though, as it indicated that significantly higher concentrations of antibiotics compared to normal might be required to eradicate an infection caused by bacteria that can similarly display this mucoid phenotype.
3.125 μg/mL Amp

6.25 μg/mL Amp

12.5 μg/mL Amp

25 μg/mL Amp

50 μg/mL Amp

- WT
- Weak
- Medium
- Strong
Figure 6. \( \text{OD}_{600} \) data from growth study with ampicillin. The WT and mutant strains were grown in varying concentrations of ampicillin. The \( \text{OD}_{600} \) was measured every five minutes for 12 hours.

3.2 Growth Curves – Osmotic Stress

To further observe the growth of the bacteria when subjected to envelope stress, four concentrations of sodium chloride in LB-glycerol (0.2%) broth were tested with the four mutant strains. An unexpected decrease in the optical density was found in all samples between 10 and 12 hours, which could have been a result of machine error. The growth rate of each sample appeared relatively similar during exponential phase, but the strains reached different final optical densities as more growth was permitted.
Figure 7. **OD<sub>600</sub> data from growth study with NaCl.** The WT and mutant strains were grown in varying concentrations of sodium chloride. The OD<sub>600</sub> was measured every five minutes for 12 hours.

### 3.3 Log<sub>2</sub> Transformation of Growth Curves - NaCl

To better understand the growth pattern of the wild-type and mutant strains in the different salt concentrations, a log<sub>2</sub> transformation of the data was performed. The log<sub>2</sub> transformation helped reduce some of the noise in the data, which allowed clearer observations with respect to growth rate.
Figure 8. Log₂ transform of OD₆₀₀ data from growth study with NaCl. The log₂ of the data from the growth study with different salt concentrations was performed.

Based on the log₂ transformations, it was seen that the strains did have comparable growth rates in each of the tested salt concentrations. The doubling time for each strain in each concentration of sodium chloride was calculated using the log₂ transformation graphs. Once the OD₆₀₀ was transformed, a derivative plot was used to find the region with the steepest slope. A linear regression of the time interval with the highest slope was performed. The inverse of the
slope of this line was used to calculate doubling time. An example calculation is shown below with the wild-type cells grown in LB-glycerol (0.2%) broth with 5 g/L sodium chloride.
A) Log₂ Transform of WT 5 g/L NaCl (Normal LB)

B) First Derivative of Log₂ Transform of WT 5 g/L NaCl (Normal LB)

C) Log₂ Transform of WT 5 g/L NaCl (Normal LB)

\[ Y = 1.958X + 8.119 \]
Figure 9. Log$_2$ transformation example. A log$_2$ transformation was used to calculate doubling time for each strain. (A) The log$_2$ of the OD$_{600}$ of the wild-type cells grown in LB-glycerol (0.2%) broth with 5 g/L sodium chloride was graphed. (B) The first derivative was found to determine the region of the graph with the steepest slope. The region from 45 to 100 minutes had the largest slope. (C) A linear regression was performed on the log$_2$ values between 45 and 100 minutes. The equation was plotted, and the slope of the line was used to calculate doubling time.

3.4 Doubling Times of Strains Grown with NaCl

The inverse of the slope from the linear regression was multiplied by 60 to find the doubling time in minutes. An example calculation is shown below.

$$\frac{1}{1.958} = 0.5107$$

$$0.5107 \times 60 \text{ minutes} = 30.64 \text{ minutes}$$

The doubling time for the wild-type cells grown in LB-glycerol (0.2%) with 5 g/L sodium chloride was 30.64 minutes. The calculated doubling times for each of the remaining strains in their respective salt concentrations are shown in the table below. Overall, the strains appeared to grow fastest in the 5 g/L NaCl (normal LB) solution; furthermore, the highest salt concentration (20 g/L) caused the slowest growth. In the low salt (2.5 g/L) and normal salt (5 g/L) conditions, the wild-type cells grew the fastest of any strain; conversely, the wild-type cells grew the slowest in the moderately high salt (10 g/L) and high salt (20 g/L) conditions of any strain.
Figure 10. Doubling times with NaCl. The doubling times with error for each strain grown in LB-glycerol (0.2%) with different concentrations of sodium chloride.

Table 1. Doubling times with NaCl. The doubling times in minutes for each strain grown in each concentration of sodium chloride were calculated using the log₂ transformation.
3.5 Final Density from Growth Curves - NaCl

The strains reached different optical densities after the 12-hour growth period. The final OD$_{600}$ that each culture attained was graphed. Small differences were observed between the mutant strains and the wild-type strain in each salt concentration, with the most pronounced differences occurring in the solution with 10 g/L sodium chloride.

![Final Density Graph]

Figure 11. Final OD$_{600}$ of strains grown with NaCl. The mutant strains reached densities similar to the wild-type culture in each of the four salt concentrations tested.

3.6 Wavelength Scan of Strains Grown with NaCl

The findings from the ampicillin and osmotic stress growth studies were of interest because of the similarities of the mutants to the wild-type cells. The mutant strains may have experienced a slight resistance to ampicillin and osmotic stress, but not to a great extent. Despite
the known presence of the colanic acid secretions, the mutants behaved in a manner very similar to wild-type; consequently, an analysis of the light-scattering properties of each strain was performed to explore differences in morphology. The average optical density of light wavelengths from 400 nm to 800 nm was graphed for each strain that was grown in the low salt (2.5 g/L NaCl), normal LB (5 g/L NaCl), and the high salt (20 g/L NaCl) conditions. The mutant strain graphs were then normalized to the OD<sub>600</sub> of the WT strain in their respective salt concentrations.
Average OD in 2.5 g/L NaCl LB

Average OD in 5 g/L NaCl LB (Normal LB)

Average OD in 20 g/L NaCl LB

Average OD in 2.5 g/L NaCl LB Adjusted to WT OD_{600}

Average OD in 5 g/L NaCl LB Adjusted to WT OD_{600}

Average OD in 20 g/L NaCl LB Adjusted to WT OD_{600}

- WT • Weak • Medium • Strong
**Figure 12. Wavelength scan.** The wild-type and mutant strains were grown in the low, normal, and high salt concentrations in LB-glycerol (0.2%) broth. A wavelength scan was performed to measure the optical density of the samples from 400 nm to 800 nm. The results were normalized according to the OD$_{600}$ of the wild-type strain in each salt condition. The red boxes highlight a splay was seen in the normalized graphs at the extreme wavelengths.

The differences in the average OD could have been caused by different quantities of bacteria in the inoculum for the starter culture; thus, it was important to normalize the results to compare the light-scattering behavior appropriately. 600 nm was chosen as it was the standard wavelength used to measure the optical density of the cultures in previous experiments. By normalizing, it was observed that a splay between the mutants and the wild-type strains occurred close to 400 nm and 800 nm. Intriguingly, convergence of the wild-type and mutant strains at the extremes was noted in the normalized graphs as the salt concentration increased.

### 3.7 Statistical Analysis of Light Scattering

A two-sample unpaired t-test was performed to determine if the differences between the wild-type and mutant strains at the extremes of the wavelength scan were statistically significant. Each mutant strain was compared to wild-type, as well as to the other mutant strains. The graphs below divide the plots adjusted to the WT OD$_{600}$ in half for more clarity. The left side shows the wavelengths from 400 nm to 600 nm, while the right side shows the wavelengths from 600 nm to 800 nm.
Figure 13. Two-sample unpaired t-test of means for OD$_{400}$ and OD$_{800}$. A t-test was performed to find statistically significant differences in the optical densities of each strain grown in the low, moderately high, and high salt concentrations. Each mutant strain was tested against the wild-type, as well as against the other mutants. The level of significance was set at $\alpha = 0.05$. Tests that yielded statistically significant results are displayed on the graph, while the p-values of these tests are displayed in a table on the graphs. One asterisk (*) indicates a p-value < 0.05; two asterisks (**) indicates a p-value < 0.01.

To find all wavelengths at which statistically significant results existed, more two-sample unpaired t-tests were performed. The p-values of each test were plotted against the wavelength that was tested. Because the original OD values were normalized to the WT OD$_{600}$ value, the graphs approached a p-value of 1 at a wavelength of 600 nm.
Figure 14. P-values from t-tests across wavelengths 400 nm to 800 nm. Each mutant strain was compared to wild-type, as well as to the other mutant strains, for every wavelength between 400 nm and 800 nm. The p-values were graphed. The dotted black line near the bottom of every graph represents the level of significance set at $\alpha = 0.05$.

3.8 Minimal Medium with Addition of Colanic Acid

The wild-type strain was grown in varying concentrations of glucose to find the amount that would still produce a large number of viable cells while depleting all available glucose. The
OD\textsubscript{600} was measured after 16-18 hours to quantify growth in each sample and select the desired culture.

<table>
<thead>
<tr>
<th>OD\textsubscript{600}</th>
<th>WT MOPS 0% glu</th>
<th>WT MOPS 0.025% glu</th>
<th>WT MOPS 0.05% glu</th>
<th>WT MOPS 0.10% glu</th>
<th>WT MOPS 0.20% glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.057</td>
<td>0.122</td>
<td>0.244</td>
<td>0.518</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. OD\textsubscript{600} data from WT grown in MOPS minimal medium with glucose.** The OD\textsubscript{600} was measured to determine which culture had a large number of cells and used up all glucose present. 0.10% glucose was the culture selected because the OD\textsubscript{600} decreased with reference to 0.20% glucose.

The WT culture grown in 0.10% glucose was used to inoculate different preparations of colanic acid. The new cultures were incubated for 16-18 hours, and the final OD\textsubscript{600} was measured to see if significant growth occurred.

<table>
<thead>
<tr>
<th>OD\textsubscript{600}</th>
<th>WT MOPS 0% glu</th>
<th>WT MOPS 0.10% glu</th>
<th>WT MOPS CA Prep. 1</th>
<th>WT MOPS CA Prep. 2</th>
<th>WT MOPS CA Prep. 3</th>
<th>WT MOPS CA Prep. 4</th>
<th>WT MOPS CA Prep. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.273</td>
<td>0.034</td>
<td>0.094</td>
<td>0.028</td>
<td>0.034</td>
<td>0.243</td>
<td></td>
</tr>
</tbody>
</table>

CA Prep. 1: Dialyzed against 50 mM MES, 100 mM NaCl
CA Prep. 2: Dialyzed against 0.5 mM EDTA
CA Prep. 3: Dialyzed against isopropanol
CA Prep. 4: Dialyzed against isopropanol, 50 mM MES, 100 mM NaCl
CA Prep. 5: Dialyzed against ethanol, 50 mM MES, 100 mM NaCl

Table 3. OD\textsubscript{600} data from WT grown in MOPS minimal medium with glucose or CA. The starter culture that was grown with 0.10% glucose was used to inoculate samples with 0% glucose, 0.10% glucose, and five different preparations of CA.

CA Prep. 5 showed promising results, as the solution achieved an OD\textsubscript{600} of 0.243, which was comparable to the OD\textsubscript{600} of 0.273, which was achieved by the cells grown in 0.10% glucose. Curiously, CA Prep 5 fostered higher growth after being dialyzed against ethanol, 50 mM MES, and 100 mM NaCl, while CA Prep 4 did not after being dialyzed against isopropanol, 50 mM MES, and 100 mM NaCl. CA Prep 1, which was dialyzed against 50 mM MES and 100 mM NaCl also did not allow significant growth compared to CA Prep 5.
IV. DISCUSSION

4.1 Findings and Conclusions

Several mutant strains of *E. coli* that resisted sedimentation were discovered through past research. These mutant strains exhibited a shared mucoid phenotype, caused by the oversecretion of the exopolysaccharide colanic acid. The mucoid phenotype existed on a spectrum, but three prominent mutants, representative of the weak, medium, and strong phenotypes, were chosen for further experimentation. Little information is currently known about these peculiar strains. It was found that they independently developed mutations in different genes, which caused the expression of this phenotype. All mutations that were discovered affected proteins related to or part of the Rcs signal transduction pathway, which is responsible for regulating capsule synthesis [14].

Capsule synthesis can be used to protect bacterial cells from envelope stressors that they may encounter [15]. For this reason, two different types of envelope stressors were tested: ampicillin and osmotic stress. Ampicillin is a bacteriostatic antibiotic that interferes with cell wall synthesis [9]. Its mechanism of action is to bind to penicillin-binding proteins and inhibit the final step in constructing the cell wall, leading to cell lysis [9]. Excess sodium chloride in solution can cause osmotic stress by drawing water out of the cells [10]. The cell wall of bacteria is responsible for maintaining turgor pressure [10]. Dehydration causes the inner membrane to pull away from the cell wall, a phenomenon that can trigger the Rcs signal transduction pathway, leading to capsule production [17].

Growth studies were performed to determine if the colanic acid coating present on the mutant strains conferred some additional resistance to the envelope stressors being tested. Five
concentrations of ampicillin, increasing two-fold from 3.125 µg/mL to 50 µg/mL, were utilized in one growth study. Four concentrations of sodium chloride in LB-glycerol (0.2%) broth, increasing two-fold from 2.5 g/L to 20 g/L, were utilized in a different growth study. It was found that there were slight differences in the behavior of the mutant strains compared to wild-type; for example, the mutant strains grew to higher optical densities in the culture with 3.125 µg/mL ampicillin. The mucoid phenotype may have given the mutant strains some resistance to envelope stressors; however, the differences between the strains in each growth study was not significant enough to warrant declaring that the mutant strains were explicitly different from wild-type in the manner in which they reacted, aside from the test condition with 3.125 µg/mL ampicillin.

After the experiment with varying salt concentrations, further calculations were performed to determine more information about the strains. A log₂ transformation of the growth curves was performed. The transformed curves were then used to find the doubling time for each strain in each salt concentration. Interestingly, the doubling times of the wild-type cells in 2.5 g/L NaCl and 5 g/L NaCl were the fastest, while the mutants grew slower; however, the doubling times of the wild-type cells in 10 g/L NaCl and 20 g/L NaCl were the slowest, while the mutants grew faster. A potential explanation for this occurrence could be that osmotic stress from high sodium chloride concentrations promoted the expression of genes for colanic acid secretion. As the salt concentration increased from normal LB, the doubling time for the wild-type strain also increased. The wild-type cells would not grow as quickly because resources would be dedicated to ensuring the cells’ survival through production of colanic acid to create a protective capsule. On the other hand, the mutant strains that already had colanic acid present on the cell envelope
would not need to reallocate as many resources to capsule production and, therefore, could grow at a more rapid pace. The final optical densities of each strain in each salt concentration were plotted and, again, not many large differences were found between the mutant strains and the wild-type. This suggested that, despite growing at different rates during exponential phase, the strains still managed to reach approximately equal cell concentrations.

Because of the similarities found between the mutant and wild-type strains from the growth studies, an analysis of how much light they scatter was performed to find any differences in morphology. A wavelength scan from 400 nm to 800 nm was performed on all of the strains in the low salt, normal salt, and high salt conditions. Once the average optical density was plotted, the data were normalized to the OD$_{600}$ of the WT cells in each salt concentration. Normalizing the data helped eliminate differences in optical density that could have been caused by having different quantities of cells in the sample. The normalized plots showed a splay at the extreme wavelengths examined; furthermore, it appeared as though the strains converged and demonstrated similar behavior as the salt concentration increased. This convergence supported the hypothesis that wild-type cells turned on genes to express colanic acid and form a capsule around the cell to protect it from osmotic stress caused by the excess sodium chloride.

To find if any statistically significant differences existed in the amount of light scattered, a two-sample unpaired t-test of means was performed for the strains at 400 nm and at 800 nm in each salt concentration. The level of significance was set at $\alpha = 0.05$. In the low salt solution, it was found that there were significant differences at both 400 nm and 800 nm between the WT and the medium mutant, as well as between the WT and strong mutant. Interestingly, there were no statistically significant differences found in the normal salt solution at either extreme of the
wavelength scan. In the high salt solution, it was found that a significant difference was present between the weak and strong mutants at 400 nm; in addition, weak mutant significantly differed from all three other strains at 800 nm, with the largest difference being between the weak and strong mutants. After learning this information, future growth studies could be performed at 600 nm, but with additional data collected at 400 nm and 800 nm.

The two-sample unpaired t-test of means was then conducted across all wavelengths to generate graphs of the p-values. These graphs not only showed all wavelengths at which statistically significant results were obtained but also the general pattern that each t-test followed. Based on these graphs, it can be confirmed that the WT strain differed the most from the medium and strong mutants at the extreme wavelengths tested in the low salt condition. It can also be confirmed that no statistically significant results were seen in the normal salt condition. The p-values at the extremes for the high salt condition, however, did not paint an entirely accurate picture. The p-value at the 400 nm endpoint was below 0.05 for only the comparison between the weak strain and the strong strain. The p-value at the 800 nm endpoint was below 0.05 for all comparisons between the weak strain and another strain. Overall, it appeared that the trend was that the weak mutant differed from the other three strains at both extremes.

The final experiment performed was based on the hypothesis that the secreted colanic acid could be a novel mechanism for bacteria to store carbon in a functional method. If true, it would allow the bacteria to digest colanic acid to produce energy during times of starvation. The WT strain was grown in minimal medium with varying concentrations of glucose to find an amount that would slightly inhibit growth. This was done to ensure all glucose in the sample was utilized. The concentration selected was 0.10% glucose; subsequently, this culture was used to
inoculate different preparations of colanic acid to determine if growth could occur. Solutions with no glucose and 0.10% glucose were also inoculated to form a basis of comparison. Five different preparations of colanic acid that were dialyzed against varying solutions were used: 1) 50 mM MES and 100 mM NaCl; 2) 0.5 mM EDTA; 3) isopropanol; 4) isopropanol, 50 mM MES, and 100 mM NaCl; and 5) ethanol, 50 mM MES, and 100 mM NaCl. Significant growth was not observed in four out of five preparations; however, the fifth preparation that was dialyzed against ethanol, 50 mM MES, and 100 mM NaCl showed encouraging results. The optical density of this culture was measured to be 0.243; the optical density of the culture regrown with 0.10% glucose was measured at 0.273 in comparison.

4.2 Future Research

The sedimentation-resistant mutants that were studied in this experiment seem to give their cells slight added protection against envelope stressors due to the overproduction and secretion of colanic acid. The behavior of the mutants did mimic the behavior of the wild-type cells in many ways, so these mutants should be studied in greater detail to find more differences. A metabolomics experiment could be performed to see if the strains have significantly different levels of cyclic di-3',5'-guanylate, especially because the strong mutant has mutations in diguanylate cyclases [20]. Cyclic di-3',5'-guanylate is utilized as part of a pathway that can regulate biofilm formation [23]. A recent link between cyclic di-3',5'-guanylate and (p)ppGpp, an important alarmone involved in the bacterial stringent response, has been proposed, suggesting that both signaling molecules can lead to an increase in antibiotic resistance [23].

The amount of colanic acid present in cultures of wild-type strains grown with high concentrations of sodium chloride could be quantified to determine if they did indeed switch on
genes for capsule production. Because L-fucose is a constituent of colanic acid, a methyl pentose quantification protocol could be followed to determine the amount of colanic acid present in the wild-type cultures [1].

The preparation of colanic acid dialyzed against ethanol, 50 mM MES, and 100 mM NaCl should also be studied further to determine if wild-type cells can truly use the colanic acid as a carbon source. The mutant strains should also be studied with a similar protocol to determine if the external colanic acid could be brought back into the intracellular environment to be metabolized for energy. If the hypothesis is confirmed, a completely novel method by which bacteria could reserve carbon could be uncovered.
V. REFERENCES


http://stars.library.ucf.edu/honorstheses/332


