THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

THE ESSENTIAL ROLE OF THE ALTERNATIVE SIGMA FACTOR, SIGMA(E) IN GRAM NEGATIVE BACTERIA

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SPRING 2016

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biochemistry and Molecular Biology
with honors in Biochemistry and Molecular Biology

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ABSTRACT

Due to increasing antibiotic resistance, new antibiotic development has become essential research. SigmaE (σ^E) is a potential target for antibiotic development, due to its activation when stress is induced in the cell envelope within most gram-negative bacteria. Though σ^E was found to be essential in the cell, mutations known as suppressors allow cells to grow in absence of σ^E. These suppressor mutations give insight to the role of σ^E. This thesis project involves the exploration of various suppressor mutations and their role as suppressors of σ^E. The goal of this thesis is to identify if mutations such as *prlF1* and *yccA11* and the overexpression of *prlF* are suppressors of σ^E. The *prlF1* mutation was originally found to relieve jamming of outer membrane protein secretion in *Escherichia coli* (1). The *prlF1* mutation was also found through sequencing of a strain that no longer required σ^E . Because σ^E monitors secretion of the outer membrane proteins (OMPs) and PrlF1 relieves hybrid jamming in the secretion machinery, it is hypothesized that PrlF1 may be a suppressor of σ^E for the same reason it is a suppressor of secretion problems. The suppression of "jamming" of outer membrane secretion performed by PrlF1 is done in Lon protease dependent manner (1). Therefore this thesis aims to identify if Lon protease is required for the *prlF1* suppression of σ^E. The overexpression of wild-type *prlF* has shown to relieve jamming in the secretion machinery, much like *prlF1* (2). Similarly *yccA11* also relieves lethality of jammed secretion machinery (3). Therefore, this thesis also aims to identify if *yccA11* and the overexpression of wild type *prlF* are suppressors of σ^E, due to their suppression of secretion problems in the outer membrane. Identifying the connection of these possible suppressors to σ^E will help to understand how the envelope is being maintained by σ^E, leading to better understanding of σ^E as an antibiotic target.

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ACKNOWLEDGEMENTS

I would like to thank Dr. Sarah Ades for mentoring me throughout the time working on my thesis. I would also like to thank Dr. Elena Sineva for her guidance in the early parts of my work and training me in many of the techniques I used throughout my thesis work.

Chapter 1

Introduction

1. 1 Antibiotic Resistance

1.1.1 Antibiotic Resistance History and Overview:

Antibiotics are one of the most successful therapeutic agents in the history of medicine. Antibiotic discovery has allowed the spread and incidence of bacterial disease to greatly decline, but as bacteria evolve many antibiotics are no longer effective. Antibiotic resistance has developed as a result of bacterial evolution and natural selection, leaving behind nearly untreatable infections. As the spread of antibiotic resistance grows in many developing nations, the area of antibiotic discovery becomes a pressing need. Bacteria such as *Staphylococcus aureus* and *Streptococcus pneumonia* show a public health threat, as they become resistant to even the most potent antibiotics.

As a result of the wide development of antibiotic resistance, 2 million people are infected and 23,000 die each year due to antibiotic resistant bacteria (4). Serious threats come from *Clostridium diffiicle,* Carbapenem-resistant *Enterobateriacea* (CRE), and drug-resistant *Neisseria gonorrhoeae* (cephalosporin resistance) (4). These resistant bacteria have the means to spread across the world, therefore they require immediate attention to limit transmission (4). The number of resistant bacterial infections rise each year, making antibiotic resistance an urgent public health issue that calls for the development of novel antibiotics (4).

1.2 The σ^E Stress Response

1.2.1 σ^E Overview

In bacteria, σ factors initiate RNA polymerase transcription on many DNA promoter sites. The primary σ-factor in bacteria regulates most genes during exponential growth. The primary σ- factor also directs transcription of genes that code for specific functions. The alternative σ-factors are often activated when there is an environmental change or molecular cues (5). The alternative sigma factor, SigmaE (σ^E) , partially controls extracytoplasmic stress (6). The σ^E stress response pathway is activated by changes or stress occurring in the cell envelope. Though this stress response plays an important role during times of stress, such as heat shock or outer membrane protein overexpression, it also play an important role in general cell physiology (8). This is clearly demonstrated for σ^E because it is essential for viability of some pathogens at both high and low temperatures (7)(10). Why σ^E is essential is not fully understood. It is currently known that σ^E is important because it controls genes that encode factors essential to the functioning of the periplasm and outer membrane (7). Possible explanations of why σ^E may be essential include: the σ^E regulon contains one or more essential genes or the loss of multiple non-essential genes within the regulon could be lethal (7). Understanding why σ^E is essential within the cell helps to better understand the fundamental biology of how σ^E is mediating the cell stress response through its regulon members. With this additional information, σ^E will become a better potential target for antibiotics due to its requirement for cell viability in some pathogens.

1.2.2 Heat Shock Response

During high temperatures, proteins within a bacterial cell can easily denature and aggregate, leaving a detrimental issue for the cell's secretion pathways. Within *Escherichia coli,* stress responses maintain homeostasis in the cytoplasm and cell envelope. These stress responses are highly maintained and regulated by two alternative sigma factors, σ^E and σ^{32} (11). The σ^{32} dependent stress response has been extensively research, but more is to be discovered about σ^E and its connection to genes that aid in its response to misfolded proteins.

Detection of a stress on the outer membrane is not directly detected by σ^E. RseA, an inner membrane protein with a single trans-membrane domain, binds to σ^E inhibiting its activity. RseB binds to the periplasmic domain of RseA and performs as a weak σ^E inhibitor. RseA, RseB, and σ^E are members of an operon that is transcribed by σ^E. This allows for tight regulation of σ^E activity (11).

During a response to stress in the cell envelope, three phases show different levels of σ^E activity. The first stage, initiation, shows increasing activity of σ^E, and it stays elevated through the second phase, adaptation. In the final stage, shut-off, σ^E shows a drop in activity level when the stress is removed. The level of σ^E activity in all three phases has been attributed to the degradation rate of RseA, showing that it is the primary means by which information is communicated to σ^E about cell envelope stress (11).

1.2.3 Porin Misfolding and Jamming of the Secretion Pathway

Because SigmaE is located in the cytoplasm, it cannot directly detect damage in the cell envelope. RseA resides in the inner membrane and binds to σ^E through its cytoplasmic N-

terminal domain. When unfolded porins accumulate, DegS cleaves RseA and initiates a proteolytic cascade that results in the complete degradation of RseA (11). Three proteases are needed to completely degrade RseA. The initial cleavage of RseA is performed by DegS, which cleaves the periplasmic domain of RseA. This exposes a fragment of RseA that is a substrate for RseP, which cuts the transmembrane region of RseA. This releases the cytoplasmic domain of RseA, which is still bound to σ^E. The final protease, ClpXP degrades the remaining piece of RseA and leaves free σ^E (9). When this cleavage occurs, σ^E will be available to bind to RNA polymerase and initiate transcription. DegS is the bridge of communication to σ^E because of its ability to notify σ^E when unfolded porins accumulate (11). DegS is regulated by binding its protease domain to the PDZ domain. When inhibition is removed, the DegS PDZ domain binds to the YZF motif on the C-terminus of most porins. This initiates the σ^E response to misfolded or unfolded porins because most correctly folded proteins have their c-terminal domain buried in the interface of the protein (12).

1.3 *prlF* Gene and *prlF1* Aid in Protein Secretion

Toxin-Antitoxin (TA) systems are a pair of genes located in one operon. One gene codes for a toxin, while the other codes for an antitoxin. The antitoxin inactivates a toxin through direct binding, or by binding DNA, repressing transcription of the toxin. TA systems have very different roles, but they generally can arrest growth in unfavorable conditions. One TA system contains the genes *prlF* and *yhaV*. The antitoxin, *prlF*, forms a tight complex with the toxin, *yhaV* (2).

Fusions of *lacZ* and signal sequences that target protein for secretion from the cytoplasm to the periplasm have been used to analyze outer membrane secretion in *E. coli*. These hybrid proteins allow for the monitoring of outer membrane protein export. The β-galactosidase activity is decreased when the hybrid protein is exported to the periplasm, due to the *lacZ* being transported to a noncytoplasmic location. Also, overexpression of these hybrid proteins can produce "jamming" in the secretion pathway and become lethal to the cell. An allele of *prlF, prlF1,* clears these jams in the secretion pathway. The 7 base pair duplication within *prlF1* causes a shorter gene product than *prlF* (13). This mutation causes a significant decrease in β-galactosidase activity due to the localization of the hybrid protein to the periplasm, suggesting that PrlF1 causes an increase in export of hybrid proteins. The same effect is seen when the gene product of *prlF* is overexpressed (2). This similar effect is seen because PrlF1 fails to inhibit transcription of its operon, which leads to an increase in PrlF (2).

This system of overexpression of PrlF and the presence of PrlF1 causes post-translational hyperactivation of the Lon protease. It is also known that Lon is required for PrlF1 to suppress secretion jamming (1). Currently the mechanism by which hyperactivated Lon protease causes *prlF1* phenotypes is unknown (2).

1.4 Role of *yccA11* in Relieving Hybrid Jamming

The Sec complex in *E.* coli is an evolutionarily conserved heterotrimeric membrane protein (Sec Y,E,G). *E.coli* targets proteins to the Sec complex in two ways. The first way being through post translation, which targets most OMPs and periplasmic proteins, and the second

being primarily inner membrane proteins that are co-translating through the Sec complex. When Sec complexes become physically jammed cell death can occur. During this jamming, essential translocator complexes are degraded by the protease FtsH. An inhibitor of FtsH, YccA, is able to counteract the destruction of the essential translocator components.

LamB-LacZ hybrid protein expression has proven to jam the secretion machinery used in the cell envelope (3). Overexpression of *yccA* is sufficient to relieve the lethality of jamming (3). An 8 amino acid deletion in the *yccA* gene gives the mutant, *yccA11.* This mutation binds to FtsH, but can not be degraded by the FtsH-mediated degradation (3). YccA11 is able to stimulate secretion of hybrid proteins and suppress the lethality of physical jamming and proteolytic destruction of SecY (3).

1.5 Suppressor Mutations of σ^E

It is still not understood why σ^E is essential within the cell (9). One approach to learning why σ^E is essential for *E.coli* growth is to investigate mutations within the cell that allow *E.coli* to grow without σ^E. These mutations, known as suppressors, can replace the critical functions of σ^E or provide a way for the cell to bypass σ^E. Three possible suppressors include: *prlF1, yccA11,* and the overexpression of *prlF.* These possible suppressors were hypothesized to be suppressors of σ^E because of their ability to suppress jamming in the secretion machinery of the cell envelope (3, 13, 14). The reasoning behind this hypothesis is due to the understanding that the loss of σ^E results in defaults in the secretion machinery (15). Therefore, because these suppressors fix or suppress these defaults, they will also suppress the need for σ^E within the cell.

Chapter 2

Materials and Methods

2.1 Materials

All media was LB medium. LB media consists of 10g/L tryptone, 5g/L yeast extract, and 5g/L NaCl

2.2 Strain Identities

Table 1 Strain Log

Strain	Parent Strain	Description	Genotype
A001		Lab wild type	*E. coli* MG1655
A002			*E. coli* MG1655 ΔrpoE::kan
A003			
A004		Lab wild type	*E. coli* MC1061
A0041	A004	Flp recombinase strain w/ lon knockout	*E. coli* MC1061 Δ*lon*::kan pCP20
A0042	A004	Flp recombinase strain w/ lon knockout	*E. coli* MC1061 Δ*lon*::kan pCP20
A00411	A0041	Flp recombinase product w/ lon knockout	*E. coli* 4 MC1061 Δ*lon* (unmarked #1)
A00412	AA0042	Flp recombinase product w/ lon knockout	*E. coli* MC1061 Δ*lon* (unmarked #2)
A005	A004	Suppressor + Strain	*E. coli* MC1061 *prlF1*$^+$
A0051	A005	Suppressor + Strain with recombination plasmid	*E.coli* MC1061 *prlF1*$^+$ *pKD46*
A006		PrlF deletion from Keio Collection	Δ*prlF::kan*

A007		PrlF deletion from Keio Collection	Δ*prlF::kan*
A012		(SEA 228) Lon deletion with PrlF1 suppressor	Δ*lon::kan prlF1*$^+$
A0121	A012	Flp recombinase product w/ lon knockout and PrlF1 suppressor	*E. coli* 45038 Δ*lon* (unmarked #1) *prlF1*$^+$
A0122	A012	Flp recombinase product w/ lon knockout and PrlF1 suppressor	*E. coli* 45038 Δ*lon* (unmarked #2) *prlF1*$^+$
A013		(CAG 45198) pKD3 +	
A014		(CAG 45197) pKD46 +	
A015		(SEA 6332)	*E.coli* MG 1655 ptrc99a
A018		(SEA 4114)	*E.coli nadB::*tet Δ*rpoE*:: kan
A020	SEA 607	Control Strain for Lon assay	*E.coli* Δ*ydcQ* (unmarked) Δ*lon*::kan
A021	SEA 6607	Control Strain for Lon assay	*E.coli* Δ*ydcQ* (unmarked) Δ*lon*::kan
A022	A001	PrlF Overexpression Control	*E. coli* MG1655 *pEbgC*
A023	A001	PrlF Overexpression Control	*E. coli* MG1655 *pPrlF*
A024	A004	PrlF Overexpression Control	*E. coli* 45038 *pEbgC*
A025	A004	PrlF Overexpression Control	*E. coli* 45038 *pPrlF*

2.3 P1 Lysate Isolation

E. coli containing the marker of interested was grown overnight (< 18 hours) in 5 mL of LB broth . Overnight cultures were diluted in LB 1:100 and grown at 30 or 37 ^{o}C until an optical density of 0.2 at 600 nm (OD_{600}). Calcium Chloride ($CaCl_2$) in 100th volume of the culture was added, and wild type phage in 100th volume of the culture was added. The culture was allowed to grow for 4-6 hours until it lysed. The culture was compared to a control culture, where no phage was added. 0.5 mL of Chloroform ($CHCl_3$) was added to the clear lysed culture. The whole culture was vortexed and then centrifuged at 4^{o}C and 5,000 x g for 10 minutes. The supernatant was transferred to a fresh tube with 0.5mL of $CHCl_3$. The final Pl Lysate was stored at 4^{o}C.

2.4 P1 Transduction

The *E. coli* strain of interest was grown overnight (<18 hours) in 5 mL LB broth and any appropriate antibiotic concentration (10µg/mL Tetracycline (Tet), 20µg/mL Chloramphenicol (Cam), or 20µg/mL Kanamycin (Kan)) at 30 or 37° C. 1 mL of the culture was centrifuged at 13.1 K rpm for 1 minute in an Eppendorf tube. The pellet was resuspended in 500 µl of 10mM $MgSO_4$, 5 mM $CaCl_2$. Three tubes were prepared with differing amounts of P1 lysate and cell culture. The cell control tube was prepared with only 100µl of the cell culture. The phage control was prepared with only 100µl of P1 lysate. The experimental tube was prepared with 100µl of cell culture and 10µl, 50µl, or 100µl of P1 lysate. All tubes were incubated at 37°C for 30 minutes. LB broth (1mL) was added to each tube. The tubes were incubated again at 37°C for 90 minutes. The tubes were centrifuged at 13.1K rpm for 1 minute. The cell pellets were resuspended in 100µl LB broth. The tube with only phage was not resuspended. 100 µl from each tube was plated on the perspective selective antibiotic using a glass rod spreading technique.

2.5 Plasmid DNA Preparation

An overnight culture of the strain containing the plasmid of interest was grown in 5 mL of LB broth with the correct plasmid selection antibiotic. The overnight sample was diluted 1:100 using 50mL of LB broth and 500 µl of sample. The diluted sample was grown to an OD_{600} of 2.0. Using an Omega bio-tek E.Z.N.A Plasmid DNA Midi Kit, the plasmid DNA was isolated. The 50 mL culture was centrifuged at 4,0000 x g for 10 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 2.25 mL of Solution I/RNase A.

Adding 2.25 mL of Solution II to the resuspended cells created a clear lysate. 3.2mL of Solution III was added to the clear lysate and a precipitate formed. The precipitate was centrifuged at 15,000 x g for 10 min at 4ºC. The supernatant was carefully removed and added to a HiBind DNA Midi Column in a 15 mL collection tube. The column and added supernatant was centrifuged at 4000 x g for 3 minutes. The column was washed with 3mL of HBC Buffer and centrifuged at 4000 x g for 3 minutes. The column was then washed twice with DNA Wash Buffer and centrifuged at 4000 x g for 3 minutes. Elution buffer was added to the column and allowed to sit for 3 minutes at room temperature. The column was centrifuged into a clean collection tube at 4000 x g for 5 minutes. The plasmid DNA was stored at -20ºC.

2.6 Preparation of Competent Cells

2.6.1 Heat Shock Competent Cells

An overnight culture of the strain of interest was grown in 5mL of LB broth with the appropriate concentration of antibiotic. The overnight culture was diluted 1:100 with 200µl of culture into 20mL of LB Broth. The culture was incubated in a shaker at 30ºC until OD_{600} 0.5 – 0.6. The culture was cooled on ice for 5 minutes and then centrifuged 3500 rpm and 4ºC for 10 minutes. The bacteria pellet was resuspended in 2mL of cold Transformation and Storage Solution (TSS). The competent cells were aliquated in the amount of 100µl into tubes to be stored at -80 ºC.

2.6.2 Electrocompetent Cells

An overnight culture of A005 was grown in 5mL of LB broth with 100µg/mL ampicillin (<18 hours). A 100µl inoculation of the overnight culture was made into 50mL of 10mM L-arabinose, 100ug/mL ampicillin LB broth. The culture was grown at 30ºC to an OD_{600} of 0.6. The culture as centrifuged for 15 minutes at 5,000 rpm. The supernatant was removed and the cells were resuspended in 50 mL of ice cold sterile H_2O. The resuspended cells were then centrifuged for 15 minutes at 5,000 rpm. The supernatant was removed and the cells were resuspended in 25 mL of ice cold sterile H_2O. The resuspended cells were then centrifuged for 15 minutes at 5,000 rpm. The supernatant was removed and the cells were resuspended in 5mL of ice cold 10% glycerol. The resuspended cells were then centrifuged for 15 minutes at 5,000 rpm. The supernatant was removed and the cells were resuspended in 0.1 mL of ice cold 10% glycerol. The final resuspension was used to make 40µl aliquots (17).

2.7 Transformation : Heat Shock Method

A frozen aliquot of competent cells of interest were allowed to thaw on ice. 100µl of competent cells and 1µl of plasmid DNA were incubated on ice for 10 minutes. The cells were heat shocked at 37ºC for 2 minutes. Immediately after the heat shock 100µl of LB broth was added to the sample and incubated at 30ºC for 1 hour. The sample was centrifuged at 13.1 K rpm for 1 min and resuspended in 100µl of LB broth. The resuspension was plated on the appropriate selective antibiotic.

2.8 Electroporation

A 40µl aliquot of A0051 electrocompetent cells was pipette mixed with 1µl of PrlF1 PCR product and incubated on ice for 1 minute. The mixture was added to an electroporation cuvette and pulsed. Immediately after pulsing the mixture, 1mL of LB broth was added to the cuvette. Cells were transferred to a culture tube and incubated at 37 ºC while shaking for 2.5 hours. 500 µl of culture was centrifuged for 1 min at 13.1K rpm and 100µl of supernatant was left for plating. The cells were plated on 20 µg/mL chloramphenicol LB agar and incubated overnight at 37ºC.

Chapter 3

Results

Part I: Random Suppressor Mutations

3.1 Generation of Random Suppressor Mutations

Random suppressor mutations allowing *E. coli* to grow without the *rpoE* can be selected through P1 transduction. Two wild type strains were used to isolate suppressor mutations, MG1655 and MC1061. Both strains are derivatives of the *E.coli* K12 lab strains. Three P1 lysate constructs were used to remove *rpoE* from the two wild-type strains: nadB::Tet Δ*rpoE*::Kan, nadB::Tet Δ*rpoE* (unmarked #1 or #2), and Δ*rpoE*::Kan (*Figure 1)*. Each of the lysates was used in a P1 transduction to remove *rpoE.*

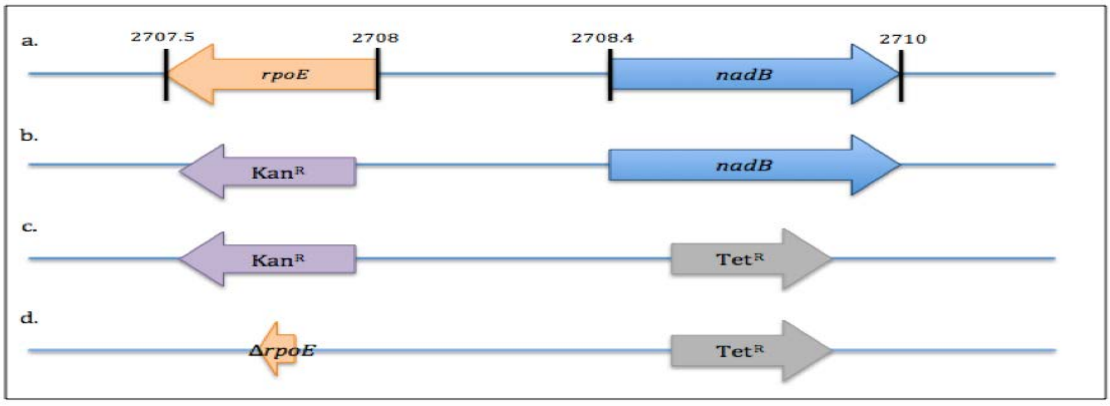

Figure 1: P1 Lysate Constructs
a. Wild-type *rpoE* and *nadB* position in *E.coli* genome, b. Δ*rpoE*::Kan phage construct, c. Δ*rpoE*::Kan *nadB*::Tet phage construct, d. Δ*rpoE*(unmarked) *nadB*::Tet phage construct.

When using the Δ*rpoE*::kan P1 lysate no resistant mutants are seen at 24 hours. At 48 hours an average of 4.8 colonies arose for MG1655 and 18.57 colonies for the MC1061 (*Figure 3*). Spontaneous mutants arose at 48 hours and could be isolated. These mutants were verified as having a Δ*rpoE* genotype by plating on 50 µg/mL X-gal or by PCR *(Figure 2)*. X-gal was able to identify Δ*rpoE* mutants because the lacZ gene is under the control of a SigmaE-dependent promoter in both strains. Blue colonies indicate wild-type *rpoE* and white colonies indicate Δ*rpoE* mutants *(Figure 2b)*. Δ*rpoE* mutants can be verified by PCR, using primers within the *rpoE* gene. PCR products with a length of 327 base pairs indicate a Δ*rpoE* genotype, where products with a length of 903 base pairs indicate wild-type *rpoE (Figure 2a)*.

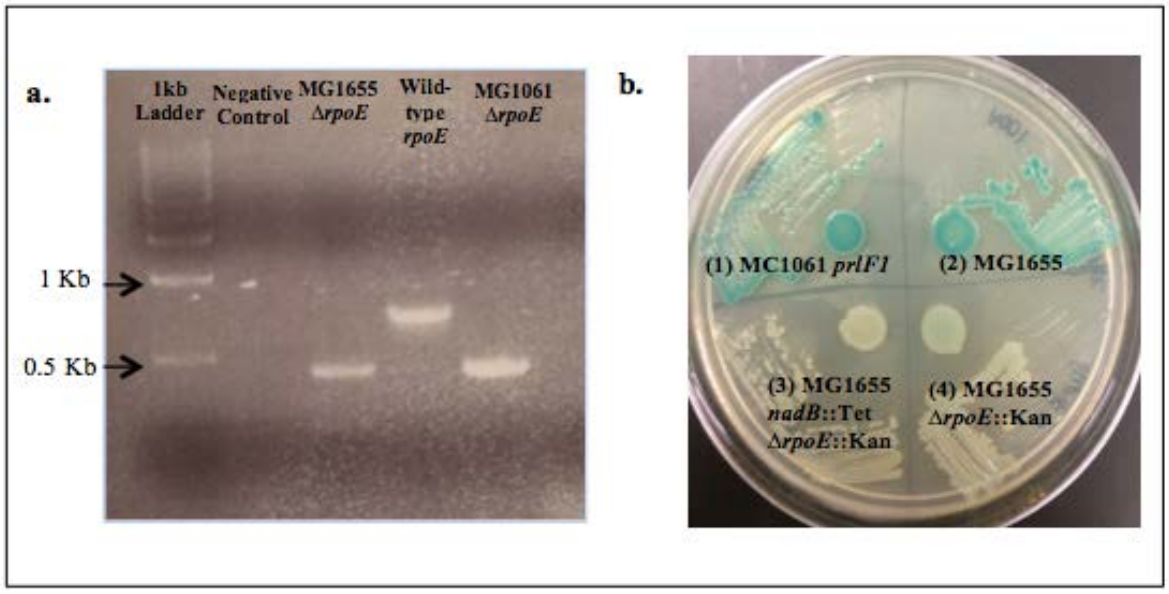

Figure 2: *rpoE* Knockout Verification Methods
a. PCR Method of verification. MG1655 Δ*rpoE* and MG1061 Δ*rpoE* product of 327 bp size and wild-type *rpoE* of 903 bop size. **b.** X-gal method of verification. (1) MC1061 *prlF1* with wild-type *rpoE*, (2) MG1655 with wild-type *rpoE,* (3) MG1655 *nadB*::Tet Δ*rpoE*::Kan with *rpoE* knockout, (4) MG1655 Δ*rpoE*::Kan with *rpoE* knockout.

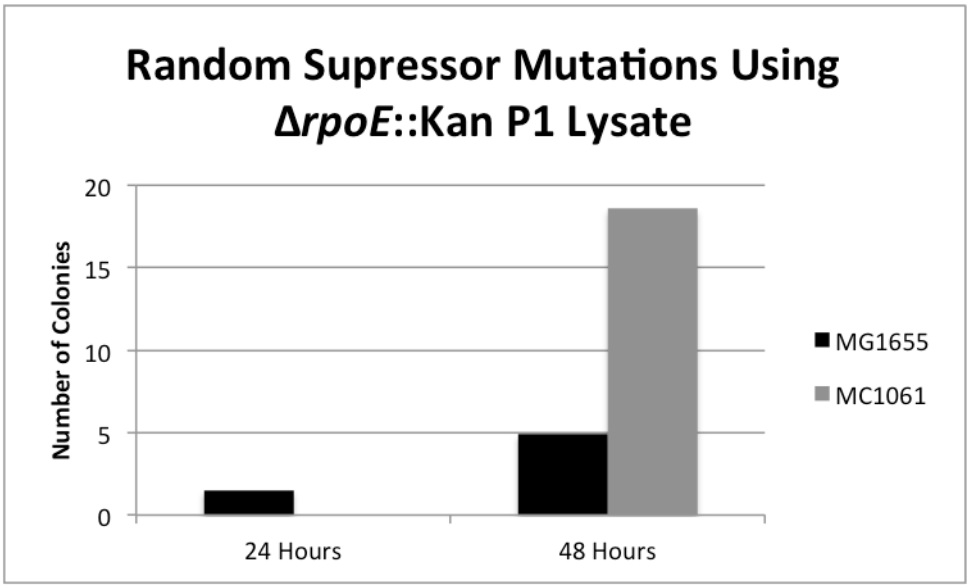

Figure 3: Generation of Random Suppressor Mutations Using Δ*rpoE*::Kan P1 Lysate
Pl Transduction was performed using a Δ*rpoE*::Kan P1 Lysate. Quantification was done by colony count at 24 and 48 hours. 48 hours is typical for suppressor mutations to surface. MG1655 is A001 and MC1061 is A004.

Using the Δ*rpoE* (unmarked) *nadB*::tet P1 lysate, 38 mutant colonies arose at 24 hours, but not all colonies are Δ*rpoE*. The cells were patched onto 50µg/ml X-gal, 10 µg/ml Tetracycline LB agar plates. Of 12 mutants made from the Δ*rpoE* (unmarked) *nadB*::tet phage, 11 were white cells on X-gal (*Table 2)*.This confirms that these colonies are Δ*rpoE* mutants.

The final lysate used to select for suppressor mutants was *nadB*::tet Δ*rpoE*::kan. Cells that were transduced with this phage show a high product of tetracycline resistant colonies, but some of these cells still have intact *rpoE.* By plating on kanamycin, it was identified if strains had a Δ*rpoE* genotype. Cells growing on both tetracycline and kanamycin were Δ*rpoE* mutants. This is known as a linked marker. If *rpoE* is essential in a strain a cross over will occur between *rpoE* and *nadB.* This will give tetracycline resistant colonies but not kanamycin resistant colonies. If *rpoE* is not essential in the strain the colonies will be resistant to both tetracycline and kanamycin, because a crossover event will not occur (*Figure 4)*. By plating 42 isolates on

kanamycin, only 6 isolates grew and were Δ*rpoE*. This was verified by plating each isolate on 50µg/mL X-gal (*Table 2)*.

Genotype	Type of P1 Lysate		
	Δ*rpoE::Kan*	**Δ*rpoE (unmarked), nadB::*Tet**	**Δ*rpoE*::Kan, nadB::Tet**
wt *rpoE*	0	1	36
Δ*rpoE*	4.8	11	6

Table 2: Summary of Random Suppressor Mutations
P1 Transduction results were assessed for intact *rpoE* using X-gal or PCR. Three lysates were used to knockout the *rpoE* gene showing varying results. Transduction colonies were chosen at both 24 and 48 hours.

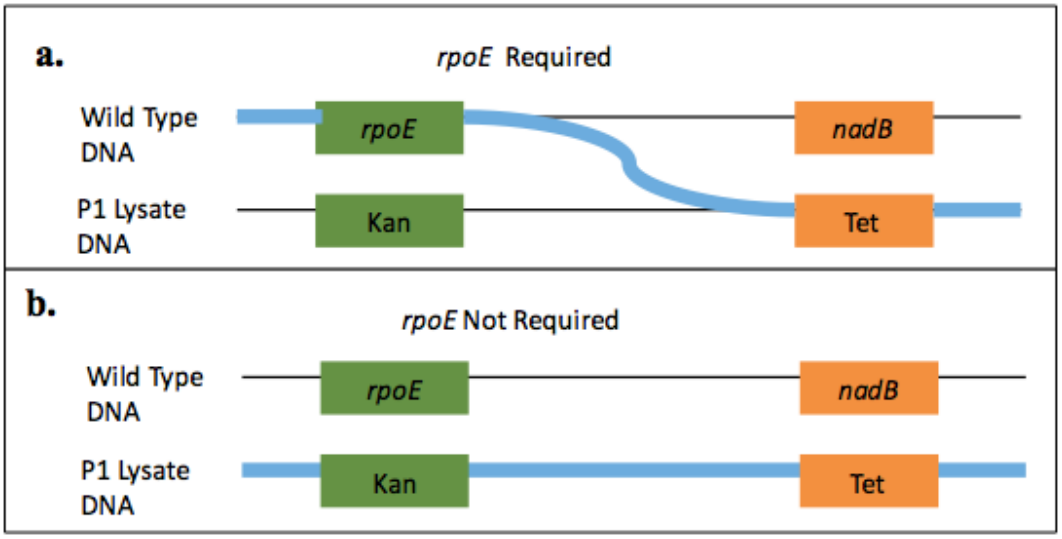

Figure 4: Possible Co-Transduction Recombination Results
nadB::Tet Δ*rpoE*::Kan P1 lysate construction. If rpoE is non essential cross over will not occur **(a)**. If *rpoE* is essential cross over will occur.(**b).**

Part II: The Suppression Effect of *prlF1* and the Overexpression of *prlF*.

3. 3: *prlF1* as a Suppressor Mutation

PrlF1 was previously found to mediate and relieve cell envelope secretion jamming (1). Due to this finding it was hypothesized that for the same reason prlF1 is a suppressor of σ^E for the same reason. If *prlF1* is a suppressor of σ^E , then strains containing *prlF1* should grow without σ^E. When *rpoE* is removed from strains containing *prlF1,* Δ*rpoE* mutants should arise in a shorter time than strains with wild-type *prlF*. The initial experiment to examine if *prlF1* is a suppressor mutation, was done through a P1 transduction using various Δ*rpoE* phage. Wild-type strains (MG1655 and MC1061) and *prlF1* strains (MC1061 *prlF1)* were transduced with Δ*rpoE* P1 lysates and colonies were counted at 24 hours. When *rpoE* is removed using a Δ*rpoE* :: Kan phage, the suppressor strain containing *prlF1* showed growth earlier and in higher numbers than compared to wild type *(Figure 5)*. Strains containing *prlF1* showed approximately 30 colonies at 24 hours, whereas MC1061 showed none and MG1655 shows 1-2 colonies at 24 hours. When the transduction plates were allowed to grow for 48 hours, spontaneous suppressor mutations surfaced on the wild-type plates. This resulted in MG1655 showing 5 colonies, MC1061 showing 18-19 colonies, and *prlF1* showing significantly more with 56 colonies at 48 hours.

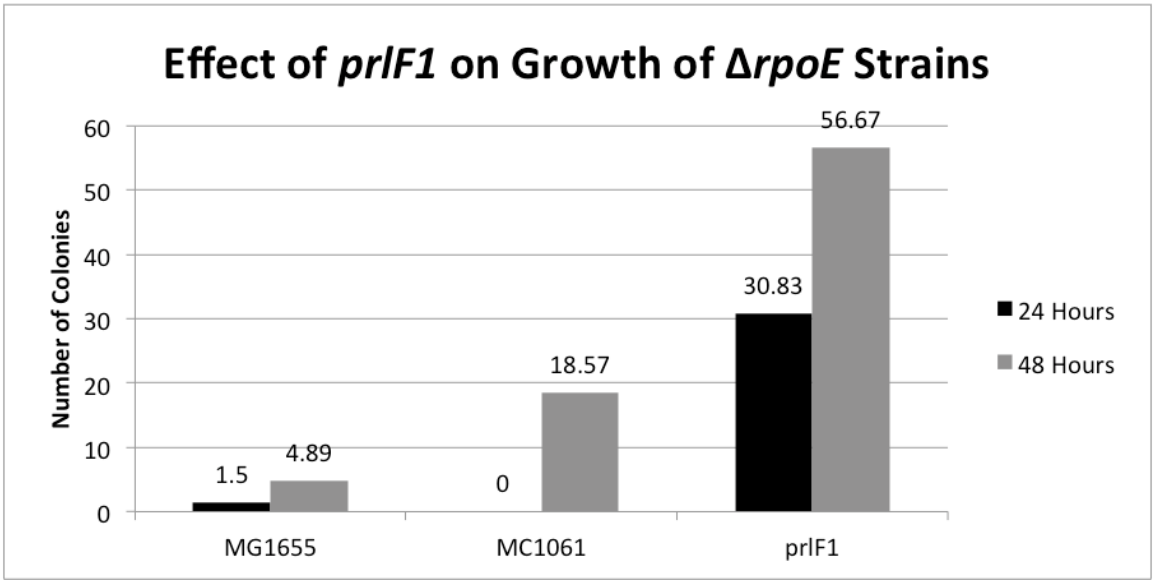

Figure 5: The Suppression Effect of *prlF1*
P1 transduction with Δ*rpoE*::Kan P1 lysate against a wild type and suppressor background. Quantification was done by colony count at 24 and 48 hours. A001 is a MG1655 wild type strain, A004 is a MC1061 wild type strain, and A005 is the *prlF1*$^{+}$ suppressor strain. Colony count numbers are based on an average of 7 trials.

3.4 Is *lon* Required for the *prlF1* Suppression Effect?

The *prlF1* mutation that suppressed jamming in the cell envelope secretion acted in a Lon protease (Lon) dependent manner (1). Due to this requirement it was hypothesized that Lon protease would also be required for the *prlF1* suppression of σ^{E}. If Lon is required for the suppression effect, then *prlF1* strains without the gene coding for Lon (Δ*lon)* will not grow without σ^{E}. A Δ*lon*::Kan strain was used from the Keio Knockout Collection to create a P1 Lysate. The *lon* gene was then removed from MG1655 (A001), MC1061 (A004), and a strain containing *prlF1* (A012) by P1 transduction using the Δ*lon*::kan phage. The kanamycin marker was removed from each of the Δ*lon* strains using a flp recombinase plasmid, pCP20. Then a linked marker P1 lysate, *nadB::*tet Δ*rpoE*::kan, was used to determine if rpoE could be removed. If *rpoE* was not required, the colonies will be kan resistant and tet resistant. If rpoE is required

then colonies will be only tet resistant (*Table 3)*. Strains containing *prlF1* and wild-type *lon* showed both tetracycline and kanamycin resistant colonies in a 15:10 ratio. The 10 colonies that grew on kanamycin had a Δ*rpoE* genotype. The *prlF1* Δ*lon* strain did not show growth on kanamycin. The strain showed an average of 41 colonies on tetracycline and 0 colonies on kanamycin. The control strains of MC1061, Δ*lon,* wild-type *prlF* also showed no growth on kanamycin, but grew an average of 11 colonies on tetracycline.

	prlF1	***prlF1* Δ*lon***	**MC1061 Δlon**
Tetracycline	15	41	11
Kanamycin	10	0	0

Table 3: Effect of Δ*lon* on *prlF1* Suppression Effect
Strains with Δ*lon* phenotype are assessed to identify if *lon* is required for the *prlF*1 suppression effect by performing *rpoE* knockout by P1 Transduction. Tetracycline resistant colonies identify *nadB* knockout and Kanamycin resistant colonies identify *rpoE* knockouts. A005 (*prlF1),* A0121 (*prlF1*Δ*lon*) , A0122 (*prlF1*Δ*lon*) , A00411 (wt *prlF* Δ*lon*), A00421 (wt *prlF* Δ*lon*). All experiments were performed by Alexa Hughes.

3.5 The Identification of *prlF1* as a Gain of Function Mutation

The removal of *prlF* can identify if *prlF* is essential in the cell, while showing if *prlF1* is a gain of function mutation. To prove that *prlF* is not essential in the cell, a Δ*prlF*::Kan P1 lysate was used to remove *prlF* from MG1655. Over 100 colonies grew as a result of the transduction in 5 trials. Using the Δ*prlF*::Kan strain (A006 and A007) another P1 Transduction was performed and compared to wild-type. This transduction was performed with a Δ*rpoE*::cam P1

lysate. The Δ*prlF* strain showed an average of 126.5 Δ*rpoE* colonies and the MG1655 wild-type strain showed an average of 114.5 Δ*rpoE* colonies (*Figure 6)*. The transduction products were plated on 20µM Chloramphenicol and 50µM X-gal. All of the transduction products showed white colonies, confirming they were Δ*rpoE.*

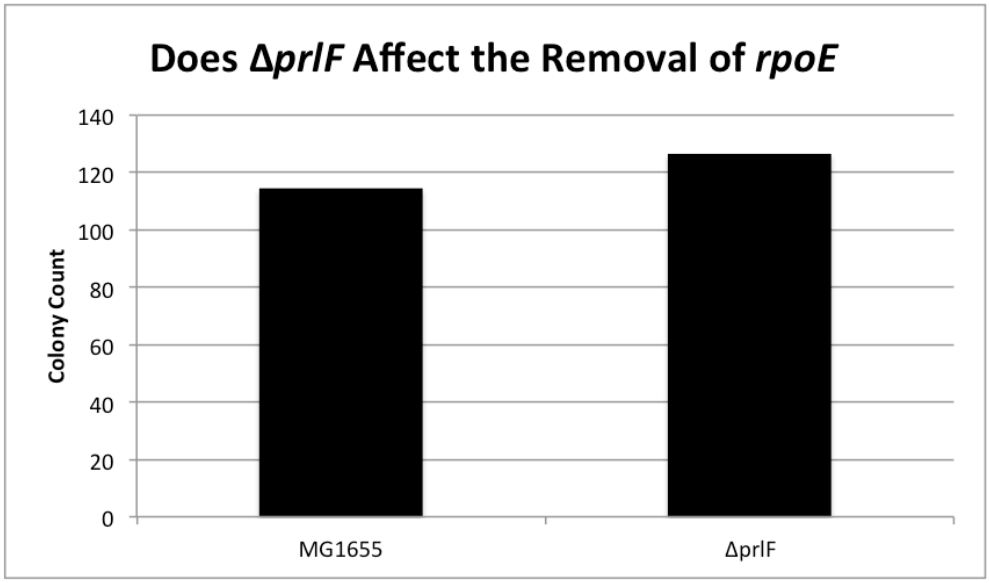

Figure 6: The Removal of *rpoE* in Δ*prlF* vs MG1655
P1 Transduction Results using a Δ*rpoE*::cam P1 lysate. Quantification was done by colony count and the results show an average of two trials. MG1655 (A001), Δ*prlF* (A007).

Part III The Suppression Effect of Overexpression of *prlF*

The overexpression of *prlF* has been shown to suppress secretion stress toxicity by increasing cell viability when there is jamming of the cell envelope transporting system (14). Because of this increased viability, it was hypothesized that the overexpression of *prlF* would be a suppressor of in σ^E. If overexpressing *prlF* suppresses the need for SigmaE, then when the *prlF* gene is overexpressed SigmaE can be removed from the strain. In order to overexpress *prlF* a plasmid with an IPTG-dependent promoter directing expression of *prlF,* from the ASKA

library, was transformed into MG1655 and MC1061. For comparison purposes a plasmid containing an unrelated gene, *ebgC,* was also overexpressed in MG1655 and MC1061. The overexpression of the genes induced by plating on 1 mM IPTG. Using a Δ*rpoE* (unmarked) *nadB*::Tet P1 lysate and a Δ*rpoE*::Kan *nadB*::Tet P1 lysate, *rpoE* was moved through P1 transduction. To check for intact *rpoE* the selective tetracycline plates contained 50μg/mL X-gal. The constructs made in MC1061 failed to grow after several trials. The MG1655 constructs showed no difference between overexpression of *prlF* and *ebgC*, nor did the constructs have any difference when compared to MG1655 without a plasmid (*Figure 7).* MG1655 and MC1061 *prlF1* were only plated on 10μg/mL Tet, 50μg/mL X-gal so no data is seen for IPTG. The MG1655 strain produced an average of 8.3 white colonies and 1 blue colony. MC1061 *prlF1* consistently showed higher colony counts than the other strains, with an average of 31.7 white colonies, showing that it is a suppressor of the need for *rpoE*. The MG1655 strain overexpressing *ebgC* showed an average of 7.3 colonies on IPTG and 4.7 without IPTG. The MG1655 strain overexpressing *prlF* showed an average of 13.3 colonies on IPTG and 11.3 without IPTG. When overexpressing both *ebgC* and *prlF* all of the colonies were white, indicating Δ*rpoE* mutants.

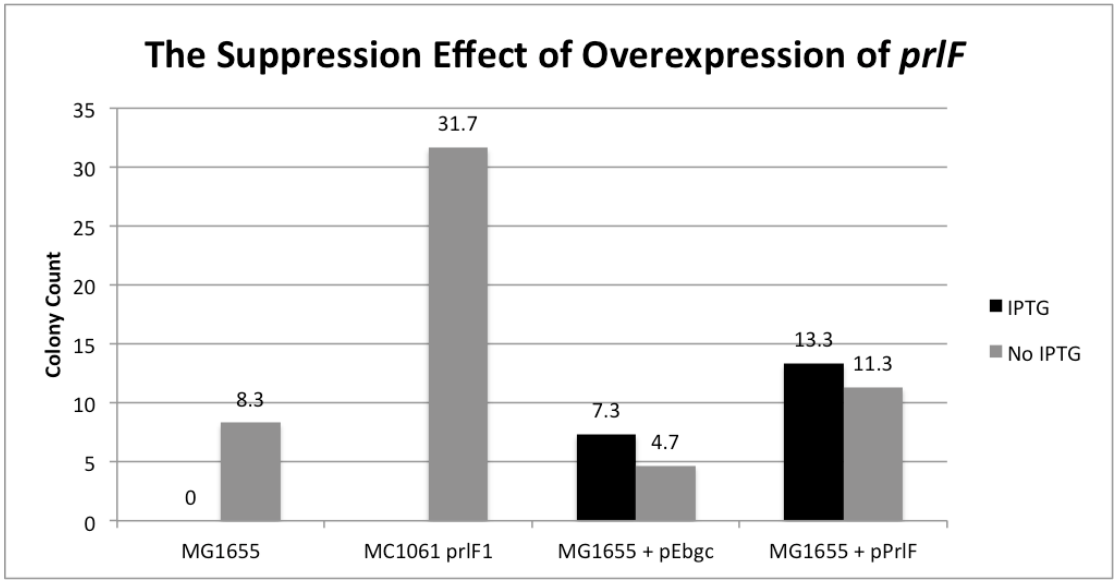

Figure 7: The Suppression Effect of Overexpresion of *prlF*
P1 transduction performed using *nadB*::Tet Δ*rpoE*::kan P1 lysate into MG1655 (A001), MC1061 *prlF1* (A005), MG1655 pEbgc (A022), and MG1655 pPrlF (A023). Transduction products were selected on 10µg/mL Tetracycline, 50µg/mL X-gal. MG1655 and MC1061 *prlF1* were not plated on IPTG. Colonies were counted at 24 hours. The colony count values are based on an average of 4 trials.

Part IV: The Suppression Effect of YccA11

Because of the effect that YccA11 relieves jamming in the cell envelope secretion machinery, and reduced degradation of SecY, it was hypothesized that the *yccA11* mutation may be a suppressor for SigmaE as well. If *yccA11* is a suppressor then *rpoE* would be easily removed from strains containing the mutation. In order to test the suppression effect of *yccA11* the *yccA11* mutation was moved into a MG1655 background. The *yccA11* mutation contains a kanamycin marker next to it, so a P1 lysate was made to move the mutation into MG1655. A Δ*rpoE* (unmarked) *nadB::*Tet P1 lysate was used to remove the *rpoE* gene. This was done by plating the transduction products on 10µg/mL Tetracycline and 50µg/mL X-gal. The strain

containing *yccA11* showed less growth compared to both controls, but mimicked the wild -type control more than the *prlF1* control The MG1655 strain showed 12 white colonies at 24 hours and 22 white colonies at 48 hours. The *prlF1* strain showed 38 white colonies at 24 hours and 63 white colonies at 48 hours *(Figure 8)*. Based on these colony counts the strain containing *yccA11* was more like the MG1655 wild-type strain in producing Δ*rpoE* mutants, compared to the *prlF1* suppressor strain.

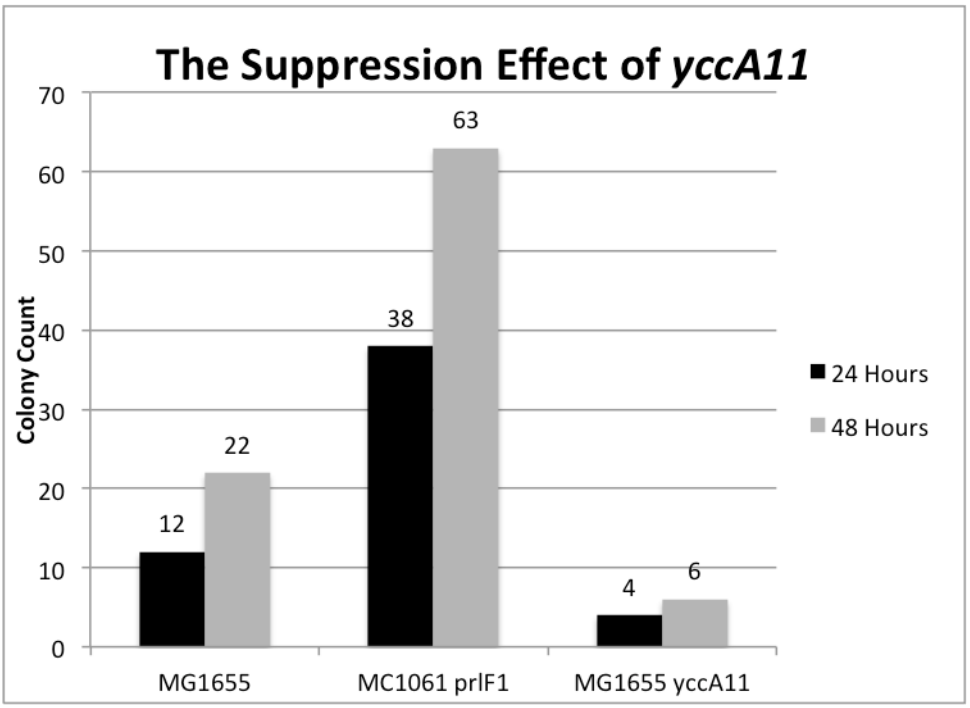

Figure 8: The Suppression Effect of *yccA11*
P1 Transduction products from *nadB*::Tet Δ*rpoE*::Kan P1 lysate. Colonies were counted at 24 and 48 hours after plating. Tetracycline and X-gal was used as a selective media. MG1655 is A001, MC1061 *prlF1* is A005, and MG1655 *yccA11* is A008-1.

Chapter 4

Discussion

This thesis shows that *prlF1* a mutation of the gene *prlF* is a suppressor for σ^E, that lon protease is required for the *prlF1* suppression of σ^E , and that the overexpression of *prlF* and the *yccA11* mutation are not suppressors of σ^E.

Using a P1 transduction method, various spontaneous suppressor mutations were found by removing *rpoE.* Most of these mutations arose after 48 hours of incubation in the presence of a selective antibiotic. Without these mutations the cells could not grow, which explains how no or only a few colonies were seen at 24 hours of incubation. The mutations allowed the cells to grow in absence of σ^E by either directly replacing the essential activity of SigmaE or by altering cell physiology so SigmaE is no longer required. Most likely, these suppressor strains would have defects in their cell envelope. These defects would be a future direction to classify these suppressors based on their survival in the presence of cell envelope stress. Methods such as plating the suppressor strains on SDS/EDTA to disrupt the outer membrane integrity were tested, but lacked significant or consistent results. Repeating this experiment as well as testing plating efficiencies at different temperatures would give insight into possible categories for these suppressor mutations.

The mutation in the *prlF* gene, *prlF1,* shows efficient suppression of σ^E. After using many P1 lysates to remove *rpoE, prlF1* has proven to be a suppressor of sigma E by showing earlier and more abundant growth of Δ*rpoE* mutants when compared to strains containing wild-type *prlF*. Using a linked marker of *nadB*::tet *rpoE*::Kan, the Δ*rpoE* mutants were selected for

on kanamycin after initial selection on tetracycline. This allowed the experiment to compare the *prlF1* strain to wild type in creating Δ*rpoE* mutants, and identifying if σ^E is essential in wild type versus *prlF1* mutants. Experiments done with only Δ*rpoE*::kan P1 lysate were time dependent and more likely to have additional suppressor mutations other than *prlF1* if grown for 48 hours. These Δ*rpoE* mutants were verified by PCR. Colonies collected at 24 hours and then tested by PCR were consistently Δ*rpoE* mutants rather than having intact wild-type *rpoE.* Through these experiments, it was identified that σ^E is less essential to cells that contain the *prlF1* mutation compared to wild type. This conclusion was based on the finding that *rpoE* could be more easily removed from cells containing *prlF1*.

All of the *prlF1* experiments were done in the same background, MC1061, that the mutation was found in. This was not ideal because there are additional mutations within the *prlF1* strain that may increase its suppression effect. To avoid these mutations, *prlF1* was to be moved into a new wild type background (MG1655). The attempts to move *prlF1* into an MG1655 background were unsuccessful. The method used was a gene disruption that allowed a antibiotic marker to be placed next to a gene of choice (18). The *prlF1* mutation was to have a chloramphenicol marker placed next to it by removing the uninvolved gene, *garD.* Due to technical reasons I was not able to replace garD with the chloramphenicol resistance gene. For future directions to move *prlF1* into MG1655, a gene near the *prlF1* gene will be removed using a P1 lysate from a strain within the Keio Collection (15). From this strain a new P1 lysate will be made that contains *prlF1* closely linked to a nearby gene knockout. This will function much like the relationship between *rpoE* and *nadB.* All transduction products will be verified by PCR.

Lon protease aids cells in surviving from DNA damage and developmental changes induced by stress. Lon has shown to be required for the *prlF1* suppression of cell envelope

secretion defects (1). The understanding if *lon* is required for the *prlF1* suppression effect helps to understand how *prlF1* is allowing cells to grow in absence of σ^E. In this thesis, the experiments involving Δ*lon* were done in partnership with Alexa Hughes. The linked marker used in these experiments identifies cells that are Δ*rpoE* by selecting for kanamycin resistant mutants. The *prlF1* strain containing wild-type *lon,* showed that 10/15 mutants were Δ*rpoE*, whereas all the Δ*lon* strains showed zero Δ*rpoE* mutants. It can be concluded that Lon is required for the *prlF1* suppression effect, because the *prlF1* Δ*lon* strains showed no Δ*rpoE* mutants. This was comparable to the wild-type *prlF* Δ*lon* strain, which has no suppressor mutations. Therefore the *prlF1,* Δ*lon* strain is acting like the wild-type strain and producing 0 Δ*rpoE* mutants. If Lon was not required, then the results for the *prlF1* Δ*lon* strains (A0121 and A0122) would be comparable to the *prlF1* strain (A005), which grew 10 Δ*rpoE* mutants.

To better understand the type of suppression effect *prlF1* is performing, the *prlF* gene was removed from the cell. The removal of *prlF* with a Δ*prlF*::kan P1 lysate showed a high number of colonies. This shows that removing the *prlF* gene is not lethal to the cell and the gene is not required for growth. Using the Δ*prlF*::Kan strain, *rpoE* was removed and showed similar growth when compared to wild-type. MG1655 showed an average of 114.5 colonies and the Δ*prlF* strain showed an average of 126.5 colonies when *rpoE* was removed with a Δ*rpoE*::cam P1 lysate. This indicates that the *prlF1* mutation is a gain of function mutation, rather than a loss of function mutation. If the suppression effect was due from a loss of function, removing *prlF* would have shown significantly more growth when *rpoE* was removed, similar to the results associated with *prlF1*. Because the growth was the same between the wild-type strain and the Δ*prlF* strain, it can be concluded that *prlF1* is a gain of function mutation.

The overexpression of *prlF* has been proven in the past to increase cell viability during stress conditions by enhancing the cell envelope secretion machinery (14). To test the effect of *prlF* overexpression and its role as a possible suppressor of σ^E, the gene had to be placed on an expression plasmid. Originally the gene was to be moved into a ptrc99a plasmid using restriction enzymes. After the plasmid failed to ligate to the *prlF1* PCR product several times, a new approach was attempted. The ASKA library gives a complete set of *E. coli* K-12 ORF (19). The ASKA expression plasmid coding for *prlF* was used to overexpress the gene, as well as an expression plasmid coding for *ebgC* to be used as a control. Expression of these two genes was induced through the IPTG promoter and performed when the cells containing the plasmid were plated on 1mM IPTG. After using both the linked marker (*nadB::*Tet, *rpoE* :: Kan) and the Δ*rpoE* (unmarked) *nadB*::Tet P1 lysates it could be concluded that the overexpression of *prlF* is not a suppressor of SigmaE. These conclusions can be made because there was very little difference between the wild-type results and the *prlF* overexpression results. All of the Δ*rpoE* mutants were confirmed by plating the transduction selection on X-gal. All colonies from all transductions were white other than one wild-type *rpoE* mutant produced by MG1655. MG1655 gives 8.3 Δ*rpoE* mutant colonies at 24 hours. The MG1655 + pPrlF gave 13.3 Δ*rpoE* mutant colonies on IPTG and 11.3 Δ*rpoE* mutant colonies without IPTG at 24 hours. These numbers are not significantly different. When comparing to the *prlF1* strain, which gives 31.7 Δ*rpoE* mutant colonies at 24 hours, it can be confirmed that the overexpression of *prlF* is not a suppressor of SigmaE.

YccA11 is known to be sufficient in relieving LamB-LacZ hybrid jamming in the SecY pathway to the cell envelope. Due to this suppression effect on jamming, it was hypothesized that YccA11 was a suppressor of SigmaE. The results of a P1 transduction disprove that

hypothesis. After the *yccA11* mutation was moved into MG1655, a P1 transduction using the Δ*rpoE*(unmarked) *nadB*::Tet was used to remove *rpoE.* The results of the YccA11 strain were comparable to the wild-type (MG1655) results. MG1655 showed 3x as many colonies as YccA11 at 24 hours and *prlF1* showed over 9x the amount of colonies as YccA11 at 24 hours. These results prove that YccA11 is not a suppressor of SigmaE, because the results are most comparable to the wild-type strain. The YccA11 strain does not show a shorter time of developing Δ*rpoE* mutants and is not comparable to the *prlF1* results, proving that YccA11 is not a suppressor of Sigma E.

In this thesis I have isolated suppressor mutations of σ^E, studied and identified the suppression effect of the *prlF1* mutation, identified that Lon protease is required for the *prlF1* suppression effect, and shown that overexpression of *prlF* and the *yccA11* mutation are not suppressors of σ^E . These findings will help to have a better fundamental understanding of σ^E. Hopefully this information will help to further the understanding of the stress response in gram negative bacteria and the many possibilities the cell may replace the SigmaE response or change its physiology so SigmaE is no longer essential.

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Undergraduate Research Assistant
Dr. Brad Wyble Cognitive Psychology Lab (August 2013- May 2014)

Awards and Scholarships

Ronald Venezie Scholarship

- This award is chosen by the biochemistry department and awarded to outstanding undergraduate students in the Eberly College of Science who also are enrolled in the Schreyer Honors College.

American Society of Microbiology Summer Undergraduate Fellowship

- This is a highly competitive fellowship aimed for students pursuing a Ph.D. in Microbiology. Fellows conduct research at their home institutions and present their research results at ASM Microbe the following year

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- This award is chosen by the biochemistry department and awarded for summer research projects.

Work Experience

Teaching Assistant (Spring 2016) University Park, PA
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- Assisting head professor in lab discussions, demonstrations, and preparatory work.

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Penn State Math Department, Supervisor: Beata Wysocka

- As a grading assistant, I graded homework assignments for two introductory Calculus classes.

Summer Tour Guide (Summer 2015) University Park, PA
Penn State Admissions, Supervisor Kerven Moon

- As a Summer Tour Guide, I gave 1.5 hour tours of Penn State's campus once or twice a day.

Professional Memberships

- Alpha Epsilon Delta: National Health Pre-professional Honors Society
- National Honors Society for Dance Arts
- National Society of Collegiate Scholars

Activities and Leadership

President/*Trip Leader*, Global Medical Brigades (August 2012- present)

- This organization helps to create holistic and sustainable communities in Central American countries, by developing many programs in rural communities to increase health and economic status.
- Brigades: Panama (2013), Nicaragua (2014), Nicaragua (2015), Honduras (2016)

Co-President, National Honors Society of Dance Arts (August 2013-Present)

- This organization is a dance honors society geared to create a professional environment for students to choreograph and perform.

Treasurer, Ambitions Dance Organization (August 2013-May 2014)

- This multicultural dance organization is dedicated to creating an excepting and growing environment for all dancers of any skill set and culture.

Academic Achievement Chair, Alpha Xi Delta: Beta Lambda Chapter (2014- 2015)

- This chair position was created to tutor and mentor sisters in any academic area.

Committee Member/Dancer, Penn State IFC/ Panhellenic Dance Marathon (2013-Present)

- OPPerations Committee (2013-2014) Air Bands Chair: choreographed a dance for my committee to compete in the OPP Sprit event, Air Bands
- Rules and Regulations Committee (2014-2015) Administration Chair: formulate meeting recaps, collect money, and organize events before THON weekend.
- Dancer (2015-2016): A dancer in THON stands for 46 hours without sitting or sleeping, in support of families battling pediatric cancer.

Team Member, Penn State Western Equestrian Team (2015)

- This athletic club team travels in the Pennsylvania region competing in shows focusing on the western style.