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The Stringent Response in Pseudomonas aeruginosa Influences the Phenotypes Controlled by the Gac/Rsm System

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The Stringent Response in *Pseudomonas aeruginosa* Influences the Phenotypes Controlled by the Gac/Rsm System

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by

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ABSTRACT

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Pseudomonas aeruginosa is a ubiquitous, opportunistic pathogen that causes acute and chronic infections. Infection is typically initiated via motile and virulent strains. After exposure to stressors, acute infections make both genotypic and phenotypic switches to a chronic, sessile strain. This is due to intricate regulatory networks directing gene expression in response to stressors. One network, GacA/GacS, has been established to control virulence factors. The stringent response of bacteria is mediated by alarmones produced primarily by RelA which responds to starvation.

To study the effect of the stringent response on the virulence switch. A series of experiments were run in both PAO1, a virulent strain, and PDO300, an acute strain, and RelA deletion mutants of each transcriptional fusions of GacA/GacA system were integrated in the wild-types and mutants. Alginate, swimming, twitching, and biofilm formation assays were performed on all. The preliminary data suggests that the stringent response influences the GacA/GacS system.

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This paper, electronic file, what have you, has been a work in progress for a long time. Through constant ups and downs in this cycle we call life the following people played, and continues to play, a critical role shaping my life and ideas. This is not an all-inclusive list, if you feel your name has been left out in error then please write it below on the line provided.

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CHAPTER 1. INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium that contains a wide variety of virulence genes that operate at different levels to cause disease, including the excretion of toxins through different secretion systems (Mulcahy et al. 2008). This bacterium is one of the leading etiological agents of nosocomial infections, which affect patients suffering from AIDS, people at intensive care units, patients in burn wards, and is the major cause of morbidity and mortality in patients with cystic fibrosis (Rosenfield et al. 2001).

One of the major complications for CF patients occurs in their lungs, where *P. aeruginosa* undergoes attachment to the surface via type IV pili, extracellular DNA, Psl, and Pel. (Allesen-Holm et al. 2006; Byrd et al. 2009). The latter two are extracellular polysaccharides that form a matrix that gives the colony a spatial structure that clusters bacteria within proximity of each other (Ma et al. 2009; Mann and Wozniak, 2012). Psl not only mediates strong attachment of the bacteria to surfaces but it also protects *P. aeruginosa* from host defenses by reducing neutrophil phagocytosis (Mishra et al. 2012). Pel has been shown in vitro recently to promote a specific, symmetric attachment where the rod-shaped bacteria lie flat on the surface (Cooley et al. 2013). Both the Psl and Pel polysaccharides are required for the formation of solid surface biofilms (Friedman and Kolter 2004; Colvin et al. 2011a; Colvin et al. 2011b; Jennings et al. 2015).

After the initial attachment and over the span of numerous years the *P. aeruginosa* genome develops mutations that cause this microorganism to undergo a phenotypic conversion from a non-mucoid to a mucoid morphology. This phenotype is characterized by the overproduction of alginate (Irie et al. 2010). Also called alginic acid, this is a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate and (C-5) α-L guluronate. The

chemical structure of this polysaccharide is seen in Figure 1. This complex polysaccharide links together with other monomers to form a thick mucoid biofilm that is very protective for *P. aeruginosa* (Flemming et al. 2010). Alginate helps protect the bacterial populations from not only antibiotics but also components of host immune system; it scavenges bactericidal reactive oxygen species, and interferes with complement activation, chemotaxis, and neutrophil and macrophage phagocytic killing (Franklin et al. 2011; Ghafoor et al. 2011; Mishra et al. 2012). The overproduction of alginate is not the only change that occurs during this phenotypic switch. It has also been shown that the virulence genes involved in attachment and colonization are turned off during the phenotypic switch, and as this occurs a new set of virulence genes are turned on (Alibaud and Kohler 2008). This gives *P. aeruginosa* the ability to control its environment by effectively eliminating any competitive bacteria that may be in its vicinity. Figure 2 is a graphical representation of the phenotypic changes that occur between PAO1 and PDO300.

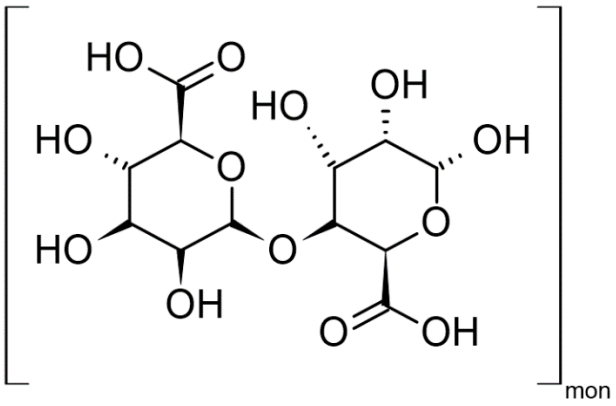


Figure 1. This figure shows the chemical structure of alginic acid (ACD/ChemSketch, version 2021.1.2)

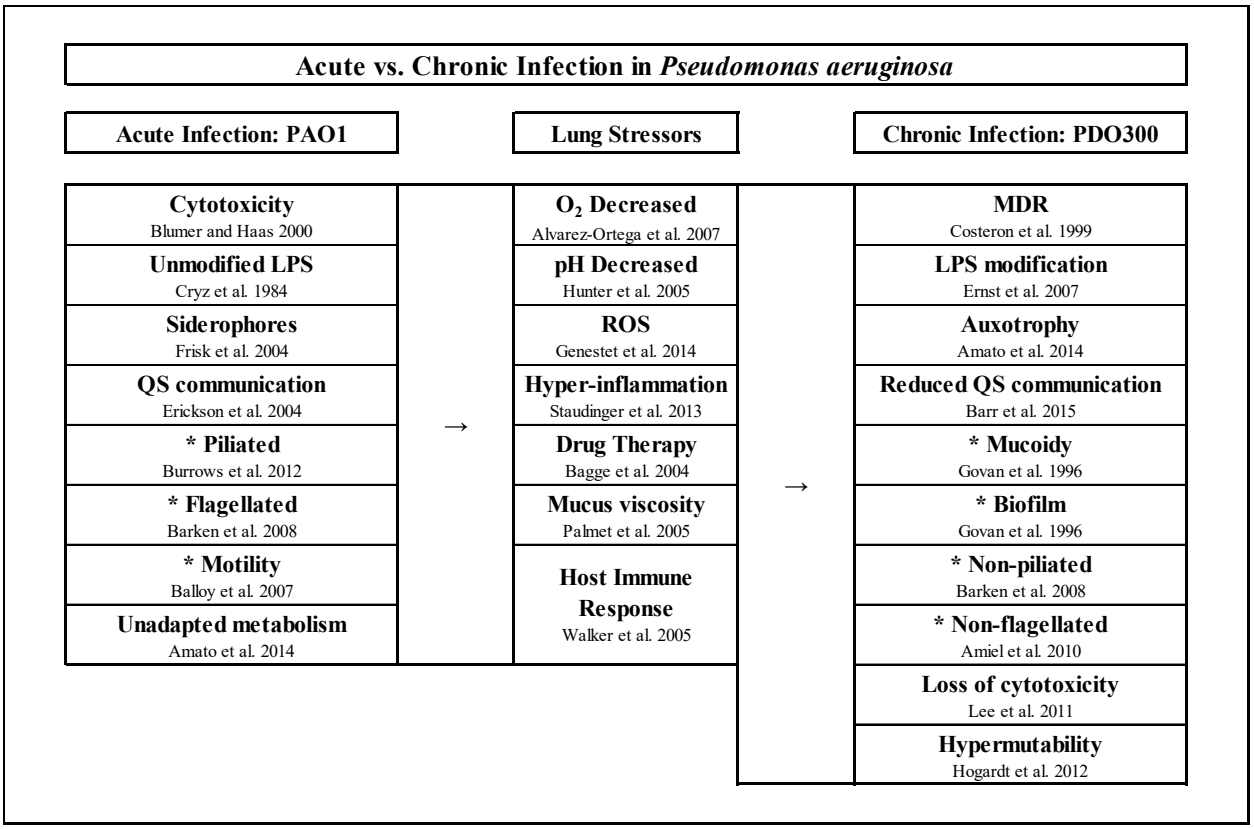


Figure 2. This table is a graphical representation of the phenotypic changes of virulence factors in *Pseudomonas aeruginosa*. As the prevalence of lung stressors occurs the mutations in the genome at *mucA* help hasten the change from acute to chronic infection. The items marked with an asterisk are studied in the thesis.

Understanding the biochemical pathways involved in the phenotypic conversion may be the key to helping those who suffer from this chronic infection of *P. aeruginosa*. However, as simple as this sounds the reality is that the pathways involved in both the phenotypic switch and the resulting change in virulence factors are complicated and still need to be ironed out.

Las, Rhl, and PQS: Quorum Sensing

What has been discovered is that the expression of not only alginate, but also various other virulence factors, is regulated by complex mechanisms known as quorum sensing, the stringent response, and the use of small RNA post-transcriptional regulators (Cooley et al. 2013). Quorum sensing involves the activation of specific genes at high cell densities in response to

chemical signals released by *P. aeruginosa* and will have a wide variety of outcomes (Gonzalez et al. 2006). There are three known quorum sensing (QS) systems in this bacterium. The first two systems, Las and Rhl, utilize N-acyl-homoserine lactones (AHL) as signaling molecules, while the third, PQS, uses a quinolone as the signaling molecule (Nadal-Jimenez et al. 2012). The PQS is chemically distinct from the two AHL systems, but it plays an integral part of the QS hierarchy (McKnight et al. 2000; Romling et al. 2012). The QS systems in *P. aeruginosa* are thought to regulate 6-10% of the bacterial genome, which suggests that these systems play an integral role in pathogenicity (Nelson et al. 2009; Williams and Camara 2009).

The current model of QS shows that the signal molecules are continuously produced at low cell densities. These molecules amass in the environment, in direct proportion to the growth of the bacterial population. At a certain concentration of AHL, the molecules will be able to bind to their respective receptors after penetrating the cell envelope; this will lead to a series of target gene regulations to be activated. This configuration of leveled regulation works to ensure that not only *P. aeruginosa* but the majority of bacteria that use QS systems can form organized communities that seek to exchange information with other cells to coordinate their activities (Lee and Zhang 2014). Among the processes regulated by QS are the synthesis of secondary metabolites, enzymes, and virulence factors, which allow bacteria to colonize various ecological niches (Lazdunski et al. 2004).

The AHL systems have specific molecules in common with each other. The first is the R protein, which is a transcriptional regulator. This protein will activate transcription in response to a specific signal produced by the I protein, the autoinducer synthase. The I protein will synthesize the signaling molecule that is recognized by the R protein. The recognition of this signaling molecule and the R transcriptional regulatory protein is specific to a single bacterial

species, meaning that the specific autoinducer molecules facilitate intraspecies communication, as opposed to interspecies (Federle and Bassler, 2003; Potvin et al. 2008).

The *las* system is comprised of LasR, which functions as the R protein, and LasI, which synthesizes the signal molecule N-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo-C12- HSL). The second system, *rhl*, is made up of RhlR and RhlI, which synthesizes the production of the signal molecule N-butanoyl-L-homoserine lactone (C4-HSL). The Las and Rhl systems are not autonomous, and they form a regulatory cascade in which LasR activates the expression of *rhlR* and *rhlI* (Lazdunski et al. 2004). A schematic representation of this pathway can be found in Figure 3.

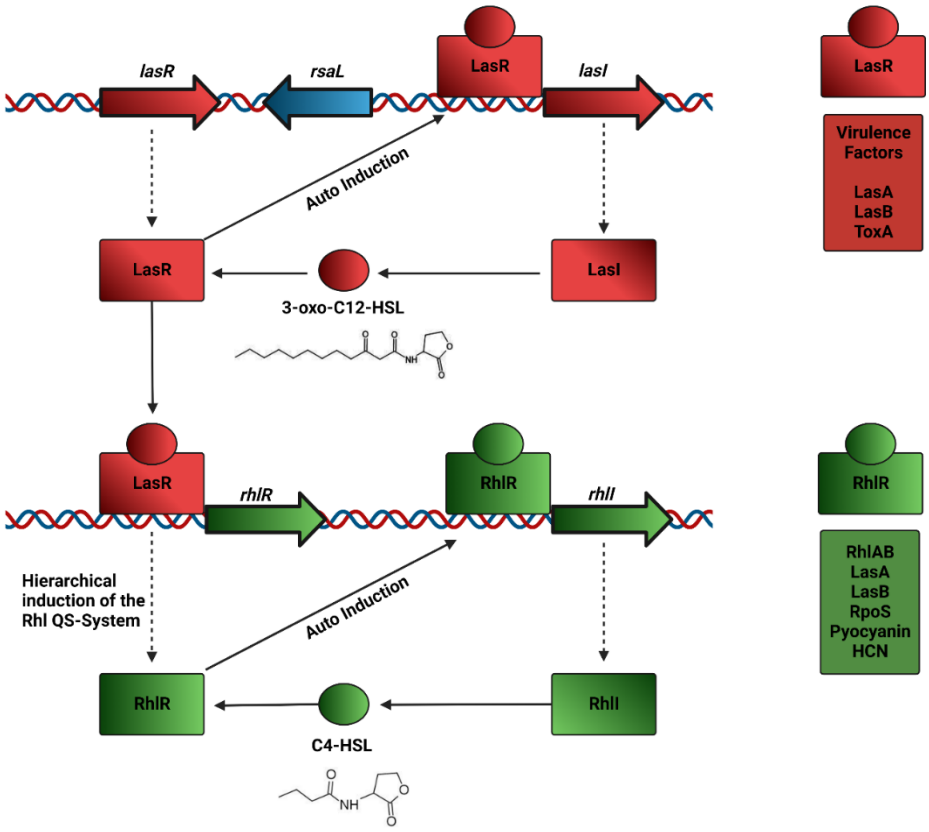


Figure 3. This schematic representation shows the Las and Rhl Systems in *Pseudomonas aeruginosa*

The *las* system has been shown to be involved in the regulation of various virulence factors, while the *rhl* system regulates a broad spectrum of *P. aeruginosa* genes. In CF patients, *lasR* transcripts have been detected in the sputum samples, and this accumulation correlated with *lasA* and *lasB* (elastase), *toxA* (exotoxin A), *arpA* (efflux pump), *lasI* and *rhlR* mRNA transcripts, indicating a functional link on the regulation of these genes (Jensen et al. 2007; Kessler et al. 1997). RhlR has been shown to bind to a specific upstream sequence of the *rhlAB*, which encodes a rhamnosyltransferase, gene independently of the presence or absence of C4-HSL. When C4-HSL is present, transcription of *rhlAB* is activated, while transcription is repressed in the absence of C4-HSL. C4-HSL has also been shown to regulate genes such as *lasB*, *rpoS*, *rhlA* and *rhlI*. Recent studies have suggested that the newly adapted Type 6 Secretion System is under the control of LasR (Rutherford 2012).

There is a third regulatory protein in the AHL QS systems called QscR that is thought to be an anti-activator. These anti-activators modulate the QS systems by interacting with the R proteins and effectively inhibit DNA binding. This inhibits transcriptional regulation. QscR has 32% sequence identity with RhlR and 29% with LasR. It also has both R protein regulatory domains, an AHL binding domain and a DNA binding domain. At the start of exponential growth phase when the concentration of AHL is low, QscR forms an inactive heterodimer with LasR and RhlR. This inhibits the expression of QS regulated genes. At the onset of stationary phase when the concentration of AHL has increased the formation of LasR homodimers bound to 3-oxo-C12-HSL, and RhlR bound to C4-HSL, is more prevalent, and this leads to the regulated expression of the target genes (Lazdunski et al. 2004).

The newest QS molecule to be discovered in *P. aeruginosa* is 2-heptyl-3-hydroxyl-4-quinolone, also known as PQS (Liang et al. 2012). It is a signaling molecule that plays a large

and integral role in the QS hierarchy. PQS is a diffusible signaling molecule that accumulates in the local environment and modulates gene expression. This system is intricately linked with the QS systems (Farrow and Pesci 2007). LasR regulates PQS production which is necessary for the transcription of the *rhlR* and *rhlI* genes. This creates a regulatory link between the *las* and *rhl* QS systems (McKnight et al. 2000; Lazdunski et al. 2004).

PQS is synthesized from anthranilate by the products of the *pqs* operon, *pqsABCDEH*. The molecule 4-hydroxy-2-heptyl-quinolone (HHQ) acts as the signaling molecule; it is released from and taken up by the bacterial cells (Choi et al. 2011). The product of the gene *pqsH* completes the conversion of HHQ to PQS, and this gene is positively regulated by LasR in complex with 3-oxo-C12- HSL. The production of PQS is also repressed by the Rhl system, which indicates there is a balance between the two systems (Wilder et al. 2011). PQS is produced maximally during the late stationary phase of growth, and under certain conditions, it is detectable at the onset of stationary phase. It can also be produced without LasR being present (Diggle et al. 2003).

PQS has been shown to act in the T cell signaling pathway by inhibiting cell proliferation and the release of IL-2. This suggests that the pathogen can influence the immune system to optimize its survival. The PQS signaling system is also able to exert antimicrobial activity and has been shown to promote biofilm formation (Toyofuku et al. 2010). The complex interactions between the Las/Rhl, PQS, and Gac/Rsm Two Component Regulatory System can be seen in Figure 4.

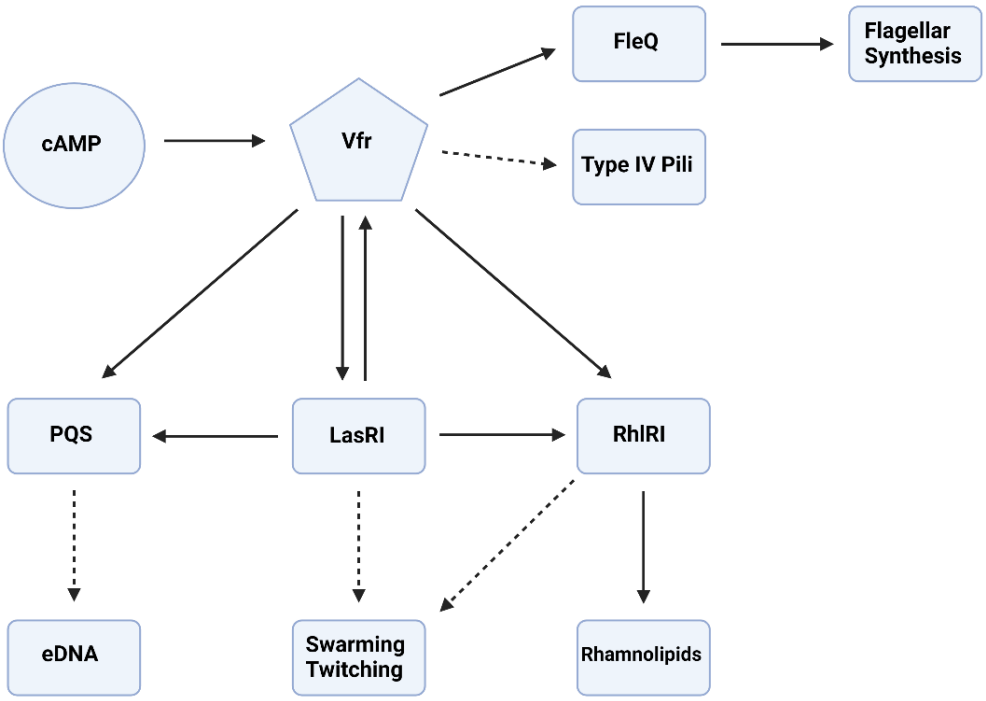


Figure 4. The interconnections between Las/Rhl, PQS, and Gac/Rsm Two Component Regulatory System acts to control various phenotypes in *P. aeruginosa*

Mucoid Conversion of P. aeruginosa by Gene Mutation in mucA

As stated earlier, the conversion of *P. aeruginosa* colonies from a non-mucoid to mucoid phenotype marks the transition to a persistent state, characterized by an accelerated pulmonary decline in CF patients (Lyczak et al. 2002). The inability of both the innate and acquired immune responses to clear the chronic infection establishes mucoid *P. aeruginosa* as a major pathogen in these patients. One study showed that 97.5% of CF patients were infected with *P. aeruginosa* by the age of three (Burns et al. 2001). This study also showed that the majority of the strains initially collected from the upper and lower airways of CF patients had non-mucoid phenotypes when grown in planktonic conditions. As time progresses, there is a change in the growth patterns and phenotype of the colonizing strains. Studies have shown that numerous

environmental conditions can cause the phenotypic switch in *P. aeuruginosa* (Mathee et al. 1999; McDaniel et al. 2015). The bacterial samples recovered from CF sputum often have mucoid morphologies and have a biofilm mode of growth.

The phenotypic switch from non-mucoid to mucoid in *P. aeruginosa* is largely due to the acquisition of stable mutations within at least two regions of the chromosome (Govan and Deretic 1996; Oliver et al. 2000); known as the *muc* loci. Over 80% of mucoid *P. aeruginosa* isolates from CF patients had mutations in *mucA* (Boucher et al. 1997). MucA is an anti-sigma factor found in the inner membrane whose amino terminus lies in the cytosol. MucA is responsible for direct contact with AlgT/AlgU/σ^{22} (Damron et al. 2009). AlgU is a member of the extracytoplasmic subfamily of RNA polymerase σ factors that are involved in the regulation of extracytoplasmic functions. The mutations in the *muc* loci result in an inactivated MucA. This inactivation of MucA leads to the deregulation of AlgU and, ultimately, the activation of AlgU dependent promoters; specifically, the promoters for alginate biosynthesis genes (Mathee et al, 1997; Qiu et al. 2007). A schematic representation of this pathway can be found in Figure 5.

The lab strain *P. Aeruginosa* PDO300 was originally created by Mathee et al. from the isogenic strain PAO1. These two strains only differ in the *mucA* allele and was constructed by homologous recombination using a plasmid harboring the mutated *mucA-22* from *P. aeruginosa* strain FRD1; which was isolated from a CF patient. The inactivation of *mucA* in the non-mucoid PAO1 causes the overproduction of alginate.

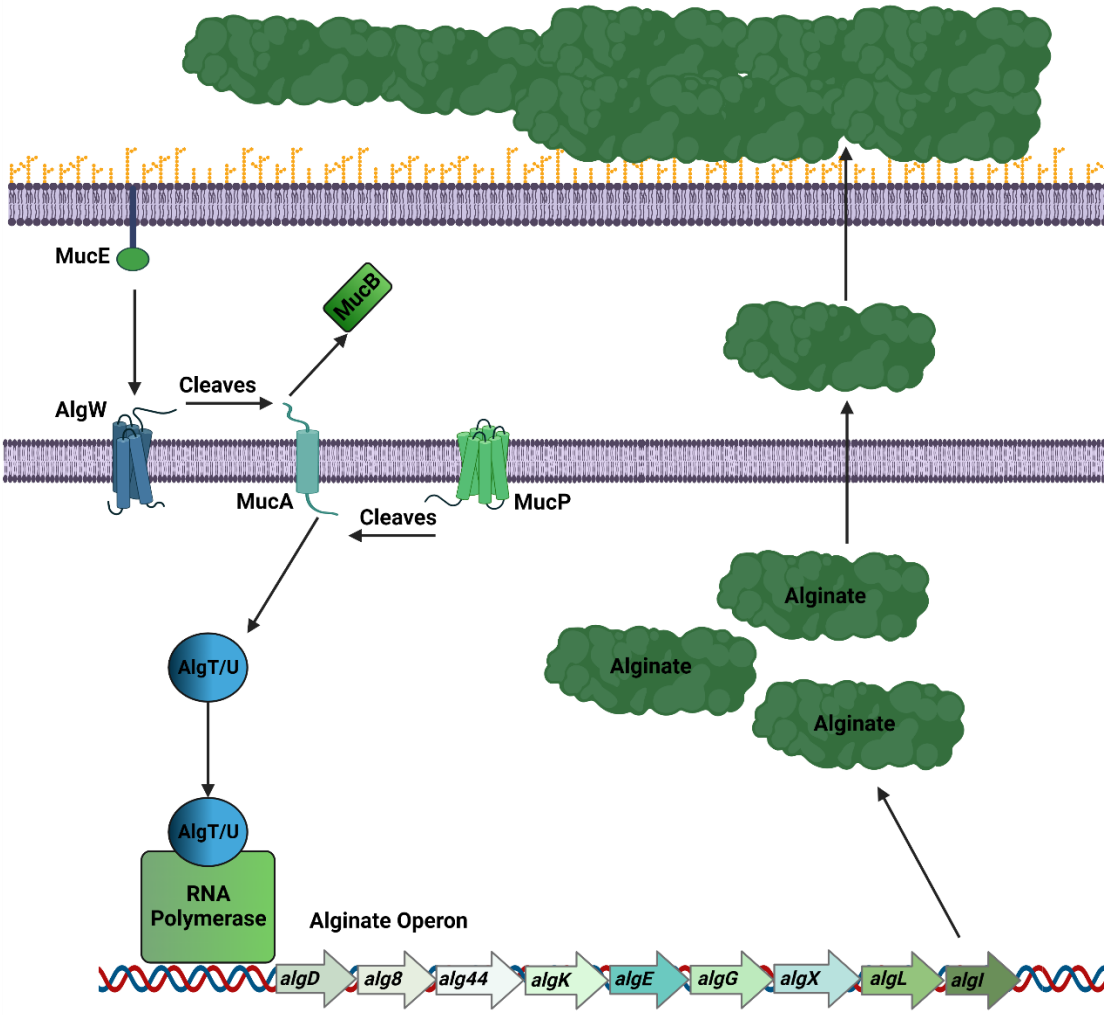


Figure 5. Schematic representation of expression of the alginate biosynthetic operon due to the mutation in *mucA*. Over time and exposure to external stimuli, *mucA* acquires two mutations in stable regions of its genome leading to an activation of AlgT/U promoters. In the schematic above, AlgT/U binds with RNA polymerase to transcribe the alginate operon leading to an increase in alginate production.

GacA/GacS: Two Co.mponent Regulatory System

Two Component System (TCS) signaling pathways are a major signaling mechanism found in bacteria that are used to monitor critical external and internal stimuli, including nutrient levels, concentration of ions and gases, temperature, redox states, and cell density. The TCS then turns these stimuli into an adaptive response. The TCS pathways share a conserved core

structure: a homodimerizing histidine kinase protein domain (which acts as a sensor molecule), and a cognate receiver domain (the response regulator). These two domains are coupled through a histidine-aspartatic acid phosphorelay mechanism where the receiver domain is phosphorylated upon stimulation of the sensor molecule (Huang et al. 2008). *P. aeruginosa* encodes seventy different TCS pathways, which corresponds to ~8% of its genome, and it appears that these different systems are insulated against harmful cross-phosphorylation between sensors and non-cognate response regulators (Goodman and Merighi 2009).

For *P. aeruginosa*, a number of these systems regulate the expression of genes necessary for transitioning from environmental reservoir to the host; this includes overcoming innate immune defenses and initiating the disease process. A central TCS is GacA/GacS, which positively controls the expression of *lasR* and *rhlR*, via the transcription of two small RNAs (sRNA), RsmZ and RsmY (Kay et al. 2006). GacS is the sensor kinase that upon receiving stimuli will phosphorylate the response regulator GacA to initiate downstream signaling.

The activation of GacS is under the control of two orphan TCS sensor kinases called RetS and Lost Adherence Sensor (LadS). These orphan TCS act as a genetic switch that jointly regulates the genes involved in acute and chronic infections. RetS is required for the Type 3 Secretion System (T3SS), repression of biofilm formation, and colonization. This kinase exerts its regulatory activity through a direct and specific interaction with GacS, where RetS binds to it with high affinity (Mikkelsen et al. 2001). This binding blocks GacS phosphorylation at the early step of signal transduction by disrupting the formation of GacS homodimers. This produces a non-productive GacS. Upon detection of an unknown signal, RetS will dissociate from GacS and the signal transduction to GacA will occur.

LadS is a hybrid sensor kinase that controls the expression of T3SS systems and biofilm-promoting polysaccharides. This kinase helps regulate the GacS/GacA phosphorylation pathway. The mode of action that it undertakes to regulate this pathway has yet to be determined (Taylor et al. 2002). A schematic representation of this pathway can be seen in Figure 6.

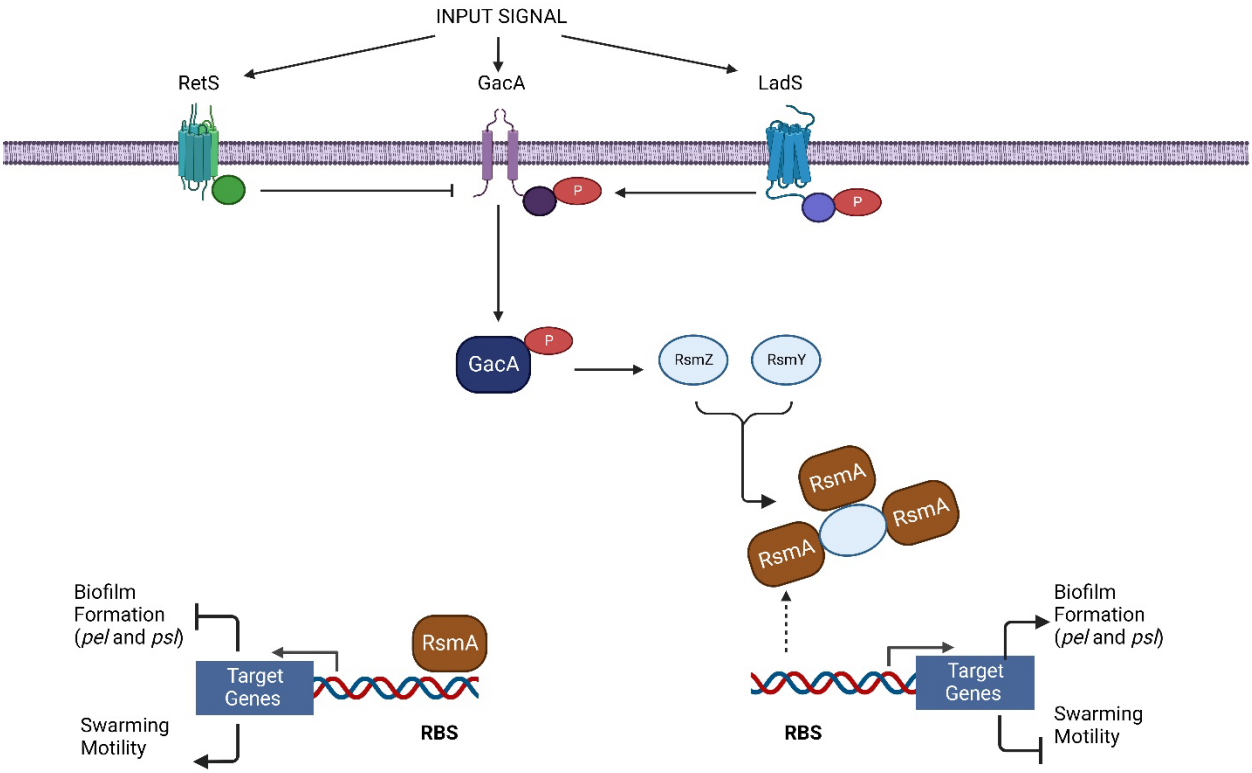


Figure 6. This schematic representation shows the Gac/Rsm pathway in *Pseudomonas aeruginosa*. As GacA is phosphorylated a cascade of events occurs resulting in the transcription of RsmY/Z and the binding of RsmA.

RsmA: A Small RNA Binding Protein

The phosphorylated GacA will activate the transcription of the two small RNAs, RsmZ and RsmY. These act as antagonists to the post-transcriptional regulator RsmA. These regulatory RNAs inactivate RsmA by directly binding to the protein and this alters the level of free RsmA available to bind to its target RNAs (Brencic and Lory 2009). RsmA acts by binding to specific

mRNA targets, stabilizing some and inducing the degradation of others. RsmA maintains positive regulation of the Type 3 Secretion System, bacterial motility, and the PQS signal biosynthesis while negatively regulating N-acylhomoserine synthesis (Mulcahy et al. 2008; O' Callaghan et al. 2011).

RelA and SpoT: The Stringent Response

The stringent response is a means that bacteria have evolved to deal with nutritional stress. When bacteria face amino acid starvation the protein known as RelA (Battesti and Bouveret 2009) enhances the production of nucleotides guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp). The synthesis of the alarmones is performed by combining adenosine triphosphate and guanosine triphosphate (Figure 7). Along similar lines, the protein SpoT can act as both a hydrolase and synthase and is thought to produce the alarmone (p)ppGpp under carbon starvation conditions and membrane stress (Boes et al. 2008). In normal conditions, SpoT acts in an anti-agonistic manner to RelA.

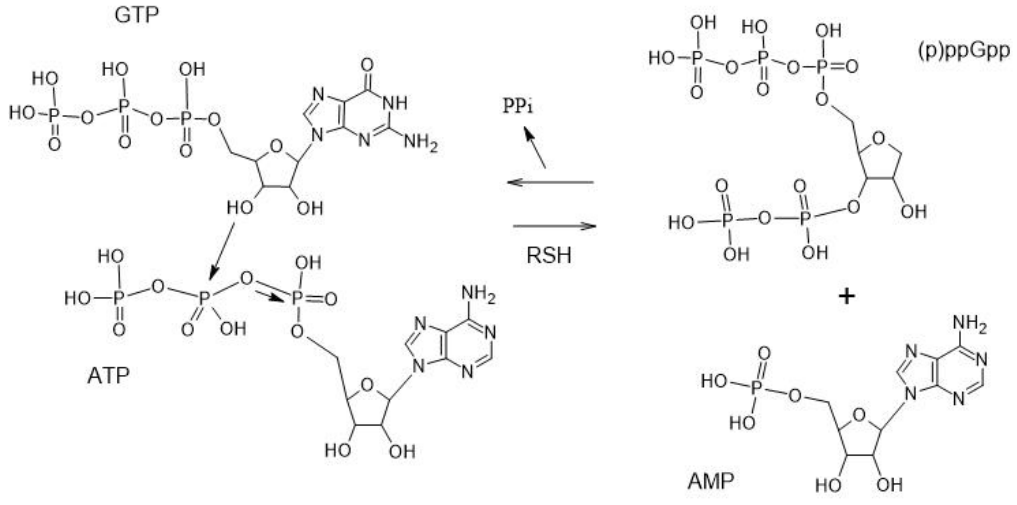


Figure 7. The synthesis of (p)ppGpp is conducted by combining GTP and ATP by RSH (RelA/SpoT Homologs) (ACD/ChemSketch, version 2021.1.2)

In its inactivated state RelA is bound to the ribosome. Protein synthesis is halted when uncharged tRNA is encountered at the A site of the 50S subunit of the ribosome. This results in a reaction in which the ribosome bound RelA is released and activated to begin synthesizing (p)ppGpp. If SpoT is still in the hydrolytic state, it will degrade the alarmone produced by the activated RelA. However, once the conversion is made to synthase activity in SpoT an overproduction of alarmone occurs thus resulting in a stringent response (Erickson et al.2004).

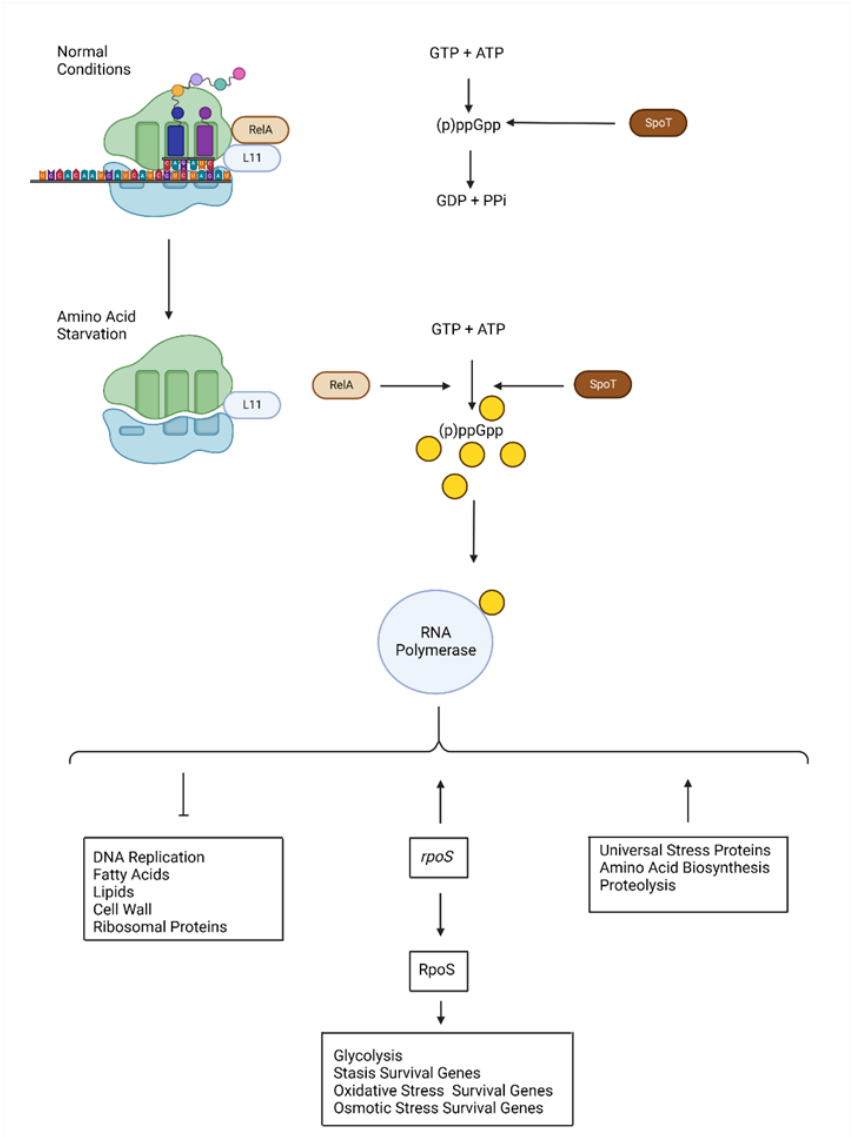


Figure 8. This schematic representation shows how the stringent response in prokaryotes controls various phenotypes

The increased levels of (p)ppGpp results in changes in numerous physiological processes, such as: inhibition of growth and enhancement of amino acid biosynthetic pathways. An article by Khakimova et al. suggests that the stringent response may be required for optimum catalase activity that allows *P. aeruginosa* to tolerate H_2O_2 that is produced during the oxidative burst found in neutrophils, macrophages, dendritic cells, and the soil amoeba *Dictyostelium discoideum* (Cosson and Zulianello 2002; Lima and Lelong 2011; Khakimova and Ahlgren 2013). It is also believed that these two molecules can direct transcription of genes under the control of the alternative sigma factor RpoS (Kim et al. 1998; O' Callaghan et al 2011). The stringent response has been shown to be important for the ability of several different human pathogens to cause disease, for example: *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Bacillus subtilis,* and *Listeria monocytogenes* (Eymann et al. 2002; Friedman and Kolter, 2004; Magnusson et al. 2005; Abu-Zant et al. 2006; Fontan 2008; Takeuchi et al. 2012).

It has been reported that RelA/SpoT in *P. aeruginosa* function like the RelA/SpoT proteins in *E. coli* (Battesti and Bouveret 2009). For SpoT, it has been proposed that the acyl carrier protein (ACP), which is a small protein that acts as a cofactor in fatty acid and lipid metabolism, is a regulating protein that acts as a ligand that binds to the C-terminal (Boes et al 2008). This suggests that there is a link between SpoT and lipid metabolism which also indicates that there is a connection between this enzyme and perturbations in the cellular envelope.

Studies in *Pseudomonas fluorescens* CHAO have shown a possible link between the Gac/Rsm phosphorelay system and the stringent response. *P. fluorescens* is a distant relative of *P. aeruginosa* but both have metabolic pathways that are conserved. *P. fluorescens* produces secondary metabolites that have antibiotic properties, and this bacterium can cause disease in immunocompromised patients (Kay et al. 2005).

In a study by Sana et al., capillary electrophoresis-based metabolomics profiling was used to show that *gacA* and *retS* mutants had opposing effects on intracellular levels of several central metabolites (Sana et al. 2012). This suggests that the Gac/Rsm pathway regulates both primary and secondary metabolism in strain CHAO. Further characterization revealed that ppGpp was the metabolite that was most strongly regulated by GacA.

In both *L. pneumophila* and *E. coli* ppGpp is a signaling molecule that regulates LetS/LetA and BarA/UrvY; both are homologous to GacS/GacA (Mukhopadhyay et al. 2000; Hammer et al. 2002;). In both organisms, ppGpp has been shown to exert indirect positive effects on the signal transduction pathways initiated by the GacS/GacA homologues. In *relA/spoT* mutants the expression of RsmY and RsmZ was attenuated and, as a consequence, it lowered the expression of traits regulated by these sRNAs, such as RpoS (Kay et al. 2006). A *spoT* mutant of strain CHAO showed increased expression levels of the *rsmZ, rsmY*, and *rpoS* genes compared to the wild type, and suggests that ppGpp activates the Gac/Rsm pathway. This also shows that ppGpp levels have a positive regulatory effect of RsmA proteins in strain CHAO. The Gac/Rsm system in *P. fluorescens* is similar, but not identical, to that found in *P. aeruginosa*.

Several researchers noticed that there is a link between this diverse array of signaling pathways in not only *P. aeruginosa*, but other bacteria such as *Myxococcus xanthus* (Crawford et al. 2000). In fact, Erickson et al. suggested that a *relA* mutant produces less RpoS than the wildtype strain in *P. aeruginosa* (Erickson et al. 2004). RpoS is an alternative sigma factor that has been shown to be clearly involved in the regulation of QS by inducing the transcription of the autoinducer molecules (Potvin et al. 2008). RpoS is also involved in the secretion of extracellular virulence factors such as alginate and exotoxin A. In addition, RpoS-negative strains have been shown to be less resistant to membrane stress, which suggests that there is a

link between SpoT and RpoS (Potvin et al. 2008). However, the actual specifics involved in elucidating how the stringent response and QS interact are still unclear. It has been shown that RpoS negatively affects the transcription of *rhlI*. This suggests that (p)ppGpp could control *rhlI* synthesis and these alarmones play a larger role in the production of virulence factors and biofilm formation than previously thought.

Specific Aims

To study, and further illuminate, the role that the stringent response plays in the biochemical pathways involved in the overproduction of alginate a *relA* deletion mutant will be constructed in the mucoid *P. aeruginosa* PAO1 and the isogenic strain PDO300. Within these strains, LacZ transcriptional fusions of the components of the Gac/Rsm system will be inserted and the β-galactosidase activity will be measured. This will aid in establishing whether the stringent response controls the Gac/Rsm system or vice versa.

CHAPTER 2. MATERIALS AND METHODS

Reagents and Chemicals

Restriction enzymes, Taq DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Polymerase Chain Reaction (PCR) primers are obtained from Integrated DNA Technologies (USA). Luria-Bertani (LB), Pseudomonas Isolation Agar (PIA) culture media were purchased from Difco Laboratories (Detroit, MI). Wizard SV Gel and PCR cleanup was purchased from Promega (Madison, WI). Kanamycin, and all other antibiotics, were purchased from Sigma-Aldrich (St. Loius, MO).

Unless otherwise noted, all reagents, growth media, and test solutions used in this study were made by the author.

Bacterial Strains and Growth Conditions

P. aeruginosa strains PAO1 and PDO300 were grown overnight on PIA. *Escherichia coli* strains were grown on LB agar media and then inoculated into 5mL's of LB broth overnight. When required, tetracycline (10 µg/mL), ampicillin (100 µg/mL), and gentamycin (15 or 150 µg/mL) were added to the media.

Sub-cloning into SM10 E. coli cells

The pEX18$_{GM}$ -*ΔrelA* plasmid was constructed previously by Chris Pritchett, PhD and subcloned into JM109 cells. A schematic representation of pEX18$_{GM}$ -*ΔrelA* can be found in Figure 9. This plasmid was specifically designed for gene replacement in *P. aeruginosa*. For these experiments the plasmid was used to introduce a gene deletion construct to a wild type. It contains multiple cloning sites that have unique restriction sites, *oriT* for conjugation mediated plasmid transfer, and two FRT (Flp recombinase targets) that are site specific recombinases. This plasmid has 3 different selectable markers which help in distinguishing the colonies. The *sacB*

gene converts sucrose to levan, which will accumulate in the periplasm and is toxic to *P. aeruginosa.* The *lacZ* alpha allele is used for blue and white screening. When bacteria harboring this plasmid is plated on media containing X-gal the LacZ gene will cleave the colorless X-gal forming a blue precipitate. The third selectable marker is gentamicin which provides bacterial resistance to gentamicin (Hoang et al. 1998).

To improve efficiency of conjugation the plasmid was moved into chemically competent *E. coli* SM10 λ-pir cells. This strain was lysogenized λ-pir phage and was designed for bi-parental mating. The plasmid pEX18 $_{GM}$-*ΔrelA* was extracted via alkaline sodium dodecyl sulfate (SDS) mini-plasmid prep as described previously (Birnboim and Doly 1979). The extracted plasmid was then introduced into the SM10 cells via heat shock transformation as described previously (Froger and Hall 2007).

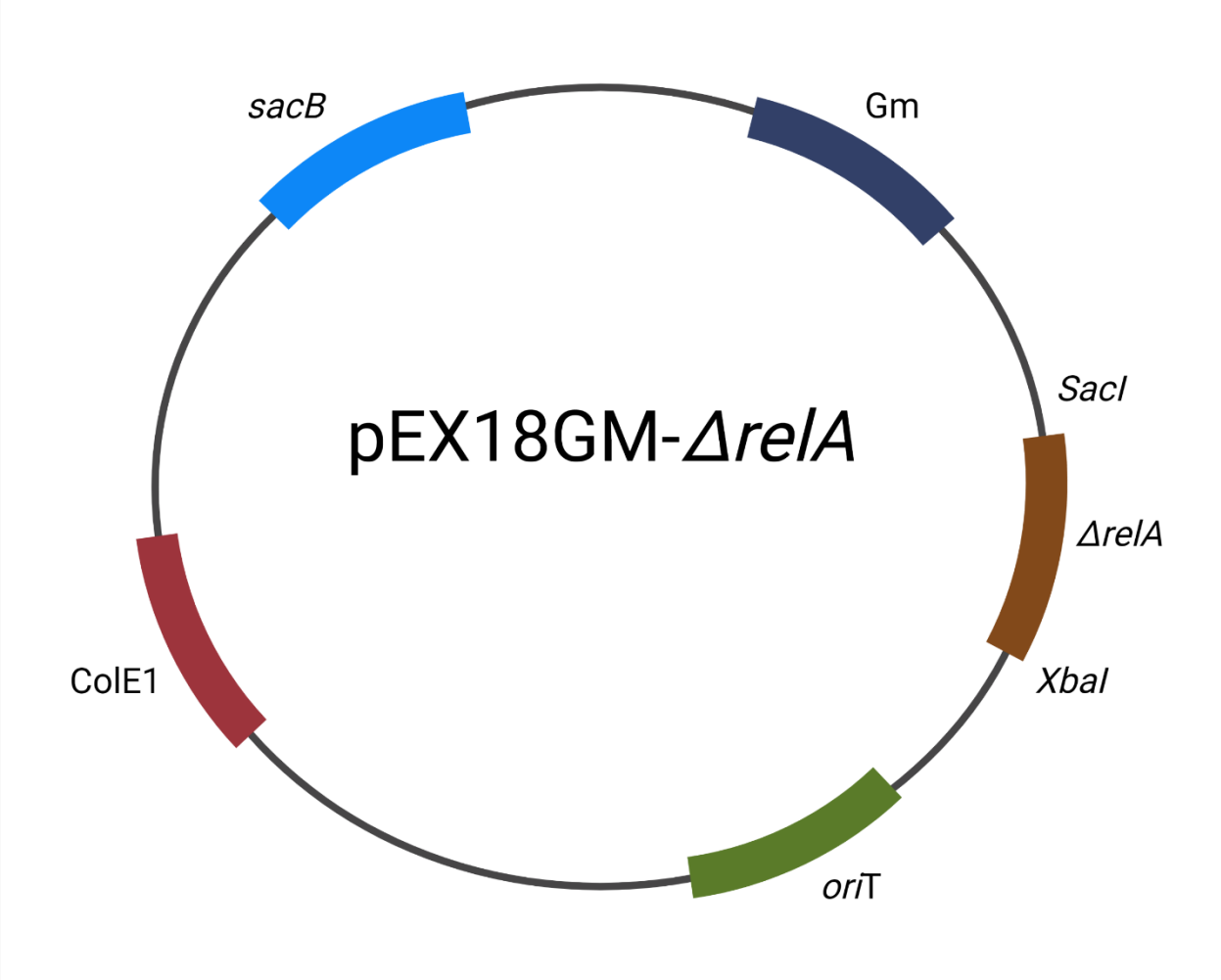


Figure 9. This schematic representation shows the various aspects pEX18GM -*ΔrelA*

Construction of relA Knockout Mutants

Bacterial cultures were grown overnight for 16 hrs, recipient strains were grown at 42 °C and the donor strains were grown at 30°C. The following day the suicide construct pEX18$_{gm}$-*ΔrelA* was conjugated into the wild type PAO1 and PDO300 via bi-parental mating at 30°C. The bacteria were then plated onto PIA-gentamycin plates for antibiotic selection (also to select against the SM10 strain of *E. coli* harboring the plasmid due to the presence of Irgasan). These plates were placed at 37°C and grown overnight. The following day individual colonies were patch plated onto PIA-gentamycin vs. LB-no salt-10% sucrose to screen for merodiploids. If the

plasmid was successfully integrated into the chromosome, then the bacteria should be gentamycin resistant and sucrose sensitive. Bacteria that were potential merodiploids were then screened via colony PCR for confirmation, and grown overnight in 5 mL LB.

The following day the culture was streaked out onto LB-no salt-10% sucrose and grown overnight at 30°C. This is to check whether the merodiploid was resolved and the plasmid containing the *sacB* gene was excised from the genome and degraded. At this point the bacteria were either wild type or mutant. To confirm that the plasmid was excised colonies were patch plated onto PIA-gentamycin versus PIA. The bacteria that grew on PIA and were inhibited from growth on the PIA-gentamycin plates were screened via PCR using internal detection primers. A representation of the *relA* gene with Forward and Reverse check primers can be seen in Figure 10.

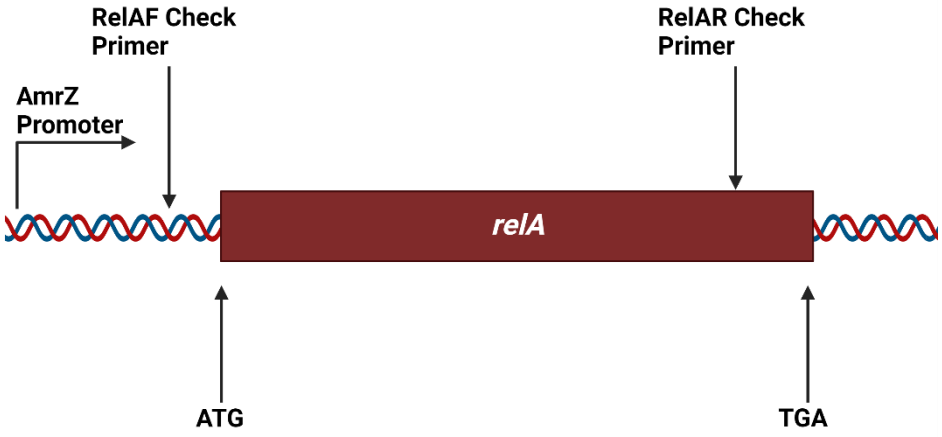


Figure 10. Location of promoter region, start and stop codons, and Forward and Reverse check primers in relation to the relA gene in *Pseudomonas aeruginosa*

Growth Curve of Knockout Mutants

Bacteria were streaked onto a fresh PIA plate for isolated colonies and grown overnight. The following day a single colony was used to inoculate five milliliters of LB broth and grown

overnight. This seed culture was used to inoculate a fresh 100 mL broth in an Erlenmeyer flask. Upon inoculation the optical density (OD) was measured at 600 nm for time 0. After this the OD_{600} was measured at 4 hours and then every 2 hours until 12 hours had elapsed.

Colony forming units (CFU)/mL were performed to ensure there were no growth defects attributed to the deletion of *relA.* At the same timepoints listed above a sample was removed from the broth culture, serially diluted and plated. These plates were grown overnight at 37°C. The following day the colonies counted, and CFU/mL's were calculated.

Integration of Transcriptional Fusion

To begin testing the effects of the stringent response on the Gac/Rsm Two Component System transcriptional fusions were integrated into the chromosome of the *relA* mutants. The *gacA, rsmA, rsmZ,* and *rsmY*-mini-CTX-*lacZ* fusions were constructed previously by Christopher Pritchett, Ph.D. The basic schematic representation of mini-CTX-*lacZ* plasmid can be found in Figure 11. The *E. coli* SM10 cells harboring the mini-CTX-*lacZ* fusions were grown the day before on LB-tetracycline and the *relA* mutants were grown on PIA plates overnight at 30 and 42°C, respectively. The following day bi-parental mating was set up on LB plates overnight at 37°C. Next, the mating mixture was streaked onto LB-no salt-Irgasan-tetracycline overnight at 37°C. This allows the plasmid to be integrated into the chromosome. The following day a bi-parental mating was set up on LB with PAO1/PDO300-*ΔrelA*::mini-CTX-*lacZ* (*gacA, rsmA, rsmZ,* and *rsmY*) with SM10-pFLP2. This will excise the tetracycline cassette from the chromosome. This mating was allowed to proceed overnight at 30°C. Next, the mating mixture was streaked out for isolated colonies onto Vogel-Bonner minimal media (VBMM)-carbenicillin and allowed to grow at 37°C. Carbenicillin is a broad spectrum, semi-synthetic penicillin antibiotic that *P. aeruginosa* is naturally resistant to.

Colonies that successfully grew on VBMM-carb were picked onto LB vs LB-no salt-tetracycline for selection of the strain that had the tetracycline cassette removed. The colonies that were tetracycline sensitive were screened via PCR.

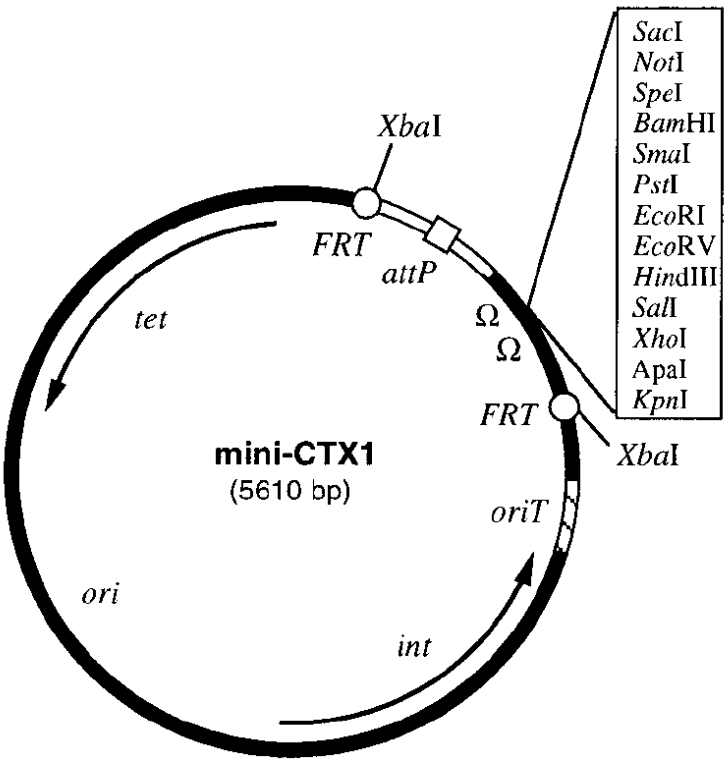


Figure 11. This figure shows the plasmid map of mini-CTX-*lacZ* (Hoang et al. 1998)

β-galactosidase Assays

β-Galactosidase activities were quantified using the Miller Method (Miller, 1972). Strains were grown overnight on PIA at 37°C. The following day a single colony was used to inoculate a 5 mL broth of LB. This culture was grown for 8, 16, and 24 hours. After this time had passed the cells were pelleted and resuspended in 1 mL Z-buffer (Acetamide 5 g/L, Potassium monohydrogenphosphate 3g/L, Potassium dihydrogenphosphate 3g/L, Potassium tetrathionate 1g/L, Magnesium sulfate heptahydrate 0.05g/L)+ β-mercaptoethanol (2.7 µL BME/1 mL Z-Buffer). The OD_{600} of this suspension was taken and recorded. To lyse the cells 100 µL

chloroform and 50 µL 0.1% SDS was added to the previous suspension. This was vortexed for 15 seconds and the reaction continued for 10 minutes. After this time, 100 µL of the supernatant from the previous reaction was added to 700 µL Z-buffer + BME. To begin the β-galactosidase assay 200 µL ortho-nitrophenyl-β-galactoside (ONPG) was added and a timer was started. Upon completion of the reaction, when the tube turned a dark yellow, 500 µL Na_2CO_3 was added; this raises the pH of the solution and stops the reaction from proceeding. After the reaction was completed the OD_{420} and OD_{550} were measured to help quantify the amount of β-galactosidase and cell debris, respectively. From this Equation 1 was used to calculate the Miller Units. The chemical reaction that occurs during the assay is shown in Figure 12.

$$1\ Miller\ Unit = 1000 * \frac{[OD_{420} - (1.75 * OD_{550})]}{(OD_{600} * Volume * time)}$$

Equation 1. Miller Unit calculation for β-galactosidase

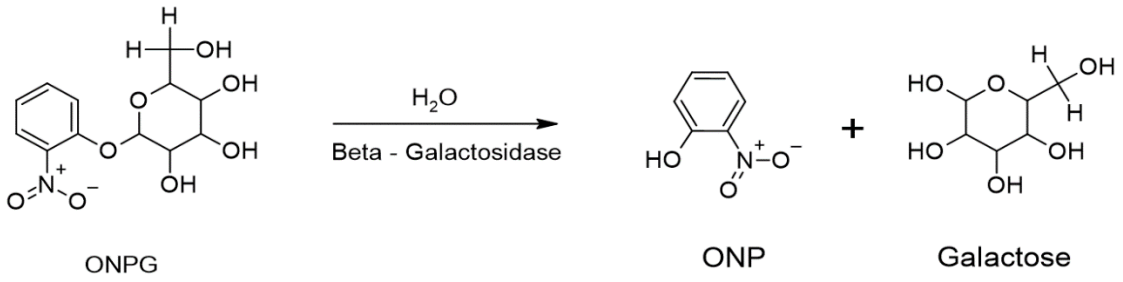


Figure 13. This figure shows the chemical reaction involved in the hydrolysis of ONPG by β-galactosidase (ACD/ChemSketch, version 2021.1.2)

Alginate Assays

After construction of the PDO300-*ΔrelA* mutant it was observed that there was a decrease in alginate production. To quantify this, an alginate assay was performed to measure the amount

being produced by the wild-type vs the mutant. The *P. aeruginosa* PDO300 and PDO300-*ΔrelA* strains were grown for 48 hours at 37°C on PIA plates. Alginate was separated from the cells by washing the culture with 20 mLs of 0.85% NaCl. The cells were centrifuged and the supernatant containing the alginate was moved to a new tube. Three milliliters of sulfuric acid/borate solution were aliquoted into glass tubes. Three hundred and fifty microliters of the supernatant containing the alginate was layered on top of the sulfuric acid/borate solution (Borate solution: 100.9 mg/mL KOH, 247.4 mg/mL Boric Acid; Sulfuric Acid/ Borate Solution: 0.25mL/L Borate Solution, 9.75 mL/L Sulfuric Acid) and vortexed for a second, the solution was put back on ice. Next, 100 µL of 0.1% carbazole in ethanol was mixed in and put in a 55°C water bath for 30 minutes. The change in color from brown to blue was measured at OD_{530} and the weight of alginate produced per cell was calculated by using Equation 2.

$$\frac{OD_{530}\ X\ 1000\ X\ volume\ NaCl\ used\ (mL)}{wet\ cell\ weight\ (mg)}$$

Equation 2. This figure shows the equation used to calculate the Alginate Assay

Colony Morphology

To observe colony morphology, cultures were grown overnight at 37°C in 5 mL LB broth with shaking at 250 R.P.M. The following day the cultures were diluted to a turbidity of 0.005 at OD600 with ddH2O. Two microliters of the diluted cultures were spotted onto Congo-red plates (10 g/L Tryptic soy broth, 40 µg/mL Congo-red, and 20 µg/mL Coomassie Brilliant Blue). Plates were incubated at room temperature for 6 days.

Biofilm Formation Assay

This assay is based on the ability of bacteria to form biofilms on polyvinylchloride plastic (PVC), a material used to make catheter lines and endotracheal tubes. The protocol was modified to accommodate for polystyrene; a polymer that has important uses in the medical field not

limited to housing medical test kits and/or devices, along with being a part of diagnostic components.

Biofilm formation was assayed by the ability of the bacterial cells to adhere to the wells of 96-well microtiter dishes as indicated by a previously reported protocol (O'Toole and George 2011). Bacterial strains were grown overnight in LB broth. The following day the overnight cultures were diluted 1:100 into a fresh M63 minimal media supplemented with magnesium sulfate, glucose, and casamino acids. One hundred microliters of the dilution were added per well in a 96 well dish. The plates were then incubated for 16 hours at 37°C. After this time the media was removed, and the plates rinsed with water. One hundred twenty-five microliters of 1% crystal violet were added to each well and the plates were incubated at room temperature for 10-15 minutes. After this they were rinsed thoroughly and repeatedly with water and then scored for the formation of a biofilm.

The quantification of biofilm formation was performed as described previously (O' Toole et al. 2011). Ninety-five percent ethanol (EtoH) was added to each crystal violet- stained microtiter dish well (2 X 200 µL 95% EtOH). The EtOH was transferred to a 1.5 mL Eppendorf tube, the volume brought to 1 mL with ddH_2O, and the absorbance measured at 540 nm in a spectrophotometer.

Biofilm formation on polystyrene tubes were assayed by diluting an overnight culture 1:100 into 3 mLs of fresh M63 media. Each tube incubated for 24 hrs at 37°C, then the tubes were washed with ddH2O and stained with 1% crystal violet as described above. The resulting stained biofilm formation was photographed and used for comparison alongside the quantitative biofilm assay.

Swimming Motility Assays

The swimming motility in bacteria is dependent only on active flagella and as such a swimming motility assay is a way to test for the presence of functional flagella (Ha et al. 2014). Because the PDO300-*ΔrelA* showed a decrease in alginate production, and as described earlier the phenotypic switch involves flagellate bacteria going to a stationary mucoid production type, a swimming assay was performed to determine if the PDO300-*ΔrelA* showed signs of functional flagella production.

To further screen other phenotypes in the PAO1 vs. PAO1-*ΔrelA* the swimming assay was performed as well. Bacteria were stab inoculated with a needle into the LB-agar plates (0.3% agar), this concentration has been used in previous studies (Ha et al. 2014). The plates were incubated at 30°C for 24 hrs and photographed. The migration zones were directly measured. The experiment was performed in triplicate and the results were expressed as mean ± standard error of the mean.

Twitching Motility Assays

To test for Type IV pili, twitching assays were performed. This assay uses concentrations of agar that creates an environment that is too viscous for flagellar motility (Darzins 1993; Semmler et al. 1999). For this experiment, the bacteria strains were stab inoculated into the bottom the LB-agar plates (1.0% agar). The plates were incubated at 30°C for 72 hrs. Because this assay involves inoculating the bottom of the plate, all agar was removed, and the zones were measured after staining with 0.5% crystal violet. The experiment was performed in triplicate and the results were expressed as mean ± standard error of the mean.

Transmission Electron Microscopy (TEM)

Samples were prepared as described previously (Giltner et al. 2010; Stacey 2013). Cultures were streaked for isolation and grown on PIA plates overnight. Five µl of ddH2O was added to a Formvar/Carbon film coated, 200 Mesh, Cu, Transmission Electron Microscopy (TEM) grid. A single, isolated colony was picked with a sterile toothpick. The colony/toothpick was put in the drop of water for 5 minutes. After the transfer of bacteria to the TEM grid, 5 µl of 1% uranyl acetate (UA) was added to the drop of water and let set for 45 seconds. Then a sterile piece of chromatography paper was placed on the opposite side of the grid, touching the drop of water. This wicked away the excess water/UA, leaving the bacteria on the TEM grid.

The TEM grids with bacteria affixed were stored in a carrier and taken to the Microscopy Core Facility (MCF) at the James H. Quillen College of Medicine. Transmission Electron Microscopy was performed with a Philips Tecnai 10 transmission electron microscope with an acceleration of 80 kV by Dr. Sean D. Stacey with Judy Whittimore aiding with image processing.

Statistical Analysis

Unless otherwise noted, statistical analysis and graphing were carried out using Microsoft Excel. The significance of difference between the mean values of two measured parameters was assessed by One-Way ANOVA.

Statistical calculations for growth curves were performed by permutation tests with the compare growth curve function of the statmod R package.

CHAPTER 3. RESULTS

Construction of relA mutants in PAO1 and PDO300

As stated earlier the pEX18$_{GM}$-*ΔrelA* plasmid was constructed previously. This plasmid was subcloned into competent *E. coli* SM10 cells for bi-parental conjugation with PAO1 and PDO300. After heat shock transformation was performed a plasmid mini prep and restriction digest was performed to confirm the successful integration of the plasmid. The primers used to create the *ΔrelA* gene segment were designed to contain the restriction sites for SacI and XbaI. A schematic representation of how the deletion mutants were created can be found in Figure 13. After the plasmid prep a double restriction digest was performed and the results can be seen in Figure 14 A lane 2. Next, a bi-parental mating was set up. After proper screening a colony was found to grow on the sucrose plate and not the antibiotic selectable plate. This indicated successful homologous recombination occurred and either the wild type allele or the mutant allele was switched out. To identify the deletion mutant colony PCR was run using a special set of primers designed to sit 150 base pairs upstream and downstream of the *relA* gene; labeled as F3 and R3 in Figure 13. This reduces the extension time and allows PCR to run much quicker. This also results in a band that will be present at a different size than what was shown in the restriction digest. A successful homologous recombination of the mutant allele should be 300 base pairs whereas the wild type allele will be closer to 3000 base pairs. Negative and positive controls were used to ensure the reaction ran correctly and the results were not due to contamination. As a negative control a PCR tube was loaded with the reaction mixture as normal but in place of the genomic template the same volume of distilled H_2O was used. This reaction was used to ensure that any bands seen in the other lanes are not due to DNA contamination of any of the reagents. As seen in Figure 14, the lanes indicated for negative controls were blank,

which is what is to be expected. Two different sets of positive controls were used. The first was wild type genomic template; PAO1 and PDO300 for each reaction, respectively. This was used to determine where the band for the wild type *relA* should be located on the gel and to ensure that the primers worked correctly. The second positive control used was the pEX18-*ΔrelA* plasmid itself. This, again, was used to determine the location of the *ΔrelA* band on the gel and to ensure that the primers worked with the plasmid. As seen in Figure 14, the lanes for the positive control showed proper amplification and most importantly in the lanes indicated for the mutant showed a band located at the correct location. This indicates that successful homologous recombination occurred and that the wild type allele was switched out for the deletion mutant allele.

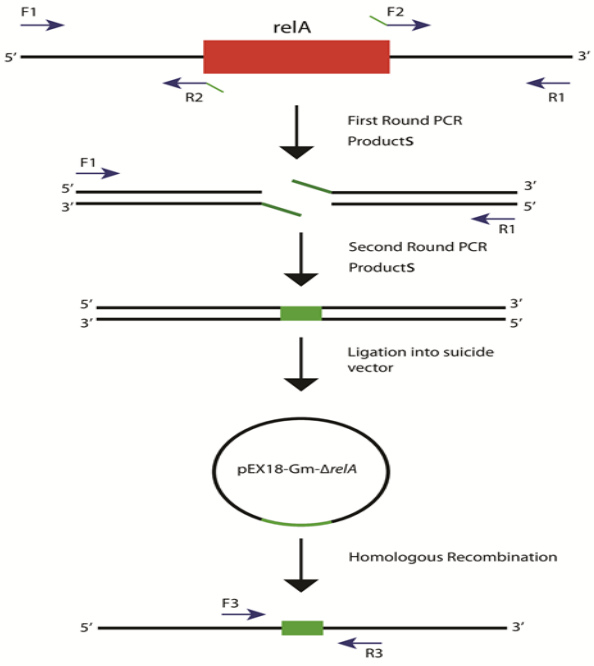


Figure 13. This schematic representation shows how the deletion of *relA* in PAO1 and PDO300 was performed

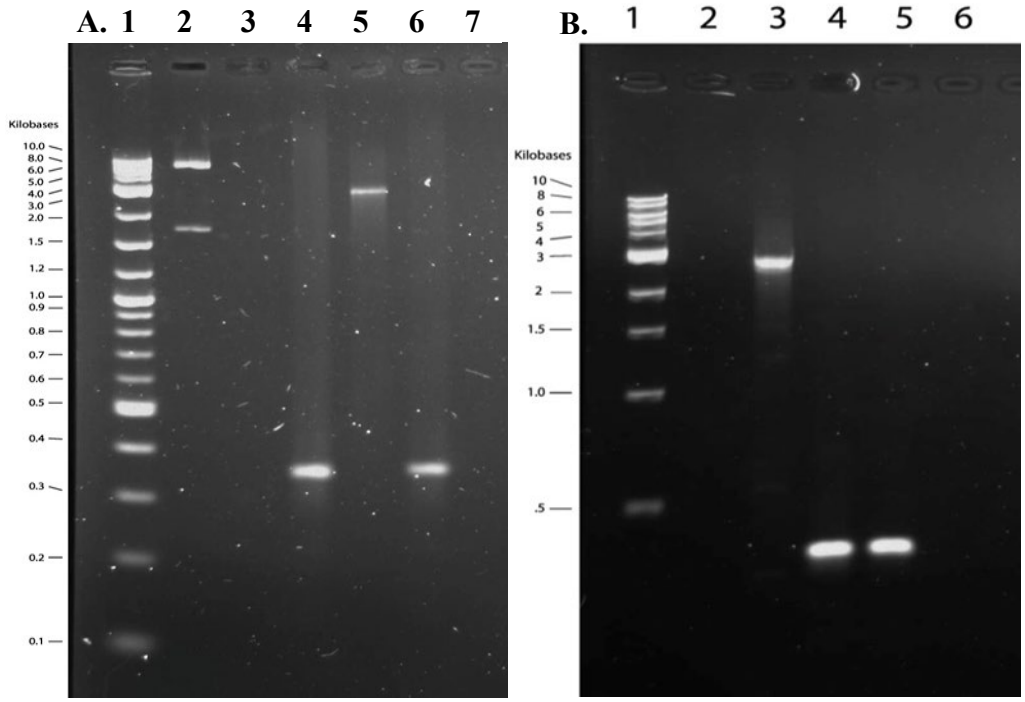


Figure 14. PCR results after successful homologous recombination. A. PDO300- Lane 1: Molecular Weight Marker, Lane 2: Restriction Digest of pEX18-Gm-*ΔrelA*, Lane 3: PCR (-) control: no DNA, Lane 4: PCR (+) control: pEX18Gm-*ΔrelA* using F3 and R3 primers, Lane 5: PCR (+) control: w.t. PDO300 using F3 and R3 primers, Lane 6: PDO300-*ΔrelA* mutant using F3 and R3 primers B. PAO1- Lane 1: Molecular Weight Marker Lane 2: PCR (-) control: no DNA, Lane 3: PCR (+) control: PAO1 using F3 and R3 primers, Lane 4:PCR (+) control: pEX18Gm-*ΔrelA* using F3 and R3 primers, Lane 5: PAO1-*ΔrelA* using F3 and R3 primers

Growth Curve Analysis

The effects of deleting or disrupting the *relA* gene in other bacteria have shown mixed results. The effects of the alarmone on physiology are complex and appear to differ greatly depending on the organism. Therefore, the effect of a *relA* deletion in the ability of *P. aeruginosa* strains PAO1 and PDO300 to grow in liquid media was tested. During exponential phase, the PAO1-*ΔrelA* had a similar growth rate as the wild type (Figure 15). Interestingly, during stationary phase there was no difference in growth between the mutant and the wild type. The same phenotype was observed in the PDO300-*ΔrelA* and the PDO300 wild type (Figure 16).

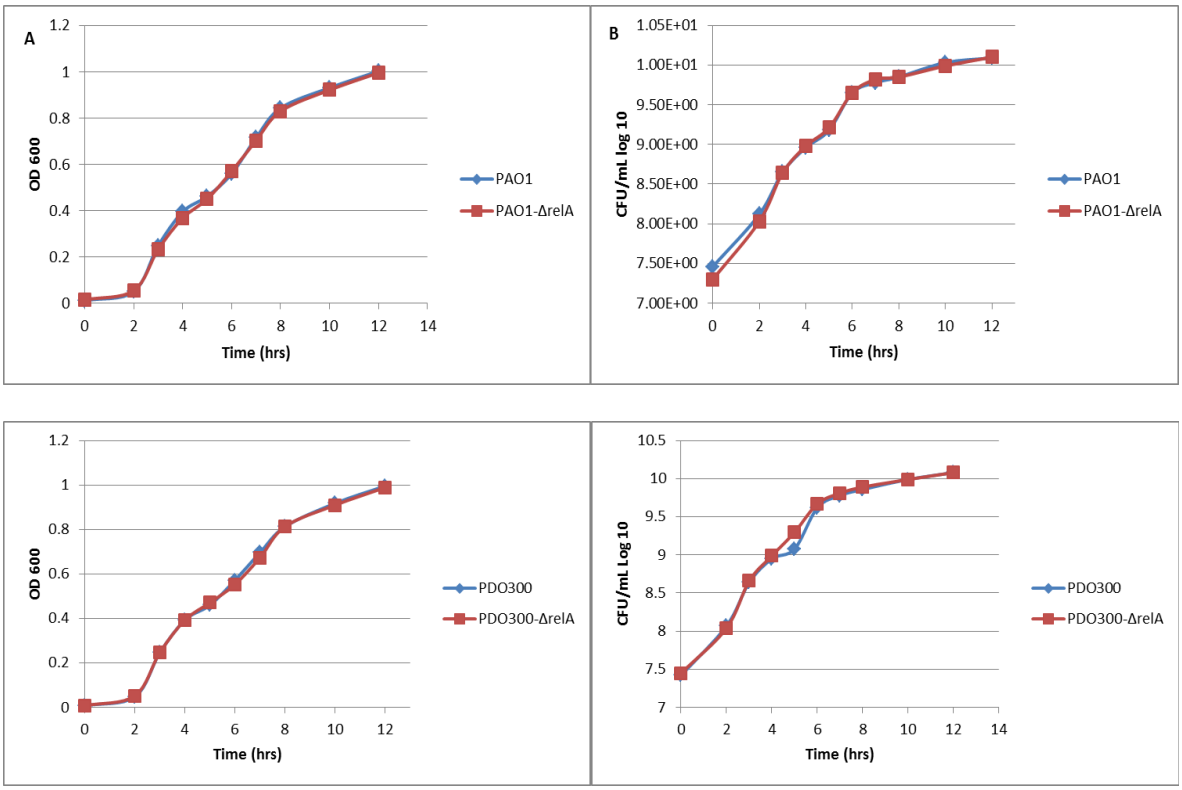


Figure 15. Growth Curves in LB broth A. PAO1 vs PAO1-*ΔrelA* OD600 B. PAO1 vs PAO1-*ΔrelA* CFU/mL C. PDO300 vs PDO300-*ΔrelA* OD600 D. PDO300 vs PDO300-*ΔrelA*

CGGC Permutation Test

To test for statistical significance of the growth curves a CGGC Permutation test was run. This is a statistical test that calculates all pairwise comparisons between two or more groups of growth curves. This set of permutation tests are based on the logic that if the *relA* deletion in the two respective wild-types, PAO1 and PDO300, has no effect on the growth curves then shuffling the data to recalculate the test will not bare a difference as the similar values will be obtained for both groups (Baldwin et al. 2007).

The CGCC Permutation test was completed by the web portal made by the Walter-Eliza Hall Institute of Medical Research which performed the calculations via the compare Growth curves function of the statmod R package (Elso et al. 2004). Ten thousand permutations were

performed between the different growth curves listed in the previous section: OD_{600} of PAO1 vs PAO1-*ΔrelA* and PDO300 vs PDO300-*ΔrelA*, and CFU/mL of the same strains.

In each test run, the data reported no significant statistical differences between wild-types and mutants at $p<0.05$.

Transcriptional Fusion Integration

The mini-CTX-*lacZ* vectors used in this study were originally developed by Hoang et al. (1998) and were designed to provide a means for transcriptional analyses of genes. This vector contains elements to allow for the integration of genetic elements in a single copy at a predetermined location within the chromosome with the prevention of disrupting any important/required gene sequence. This vector contains elements which insert the non-replicative plasmid into the neutral *attB* site found in *P. aeruginosa* chromosome; the *attB* site allows for single copy insertion. It also contains the presence of FRT sites that allow for the efficient Flp-recombinase-mediated excision of unwanted DNA sequences such as antibiotic resistance and the plasmid backbone; in this study tetracycline was used as the antibiotic selectable marker. This leaves only the gene sequence and the *lacZ* fusion inserted into the *attB* site. A schematic representation of this can be found in Figure 16.

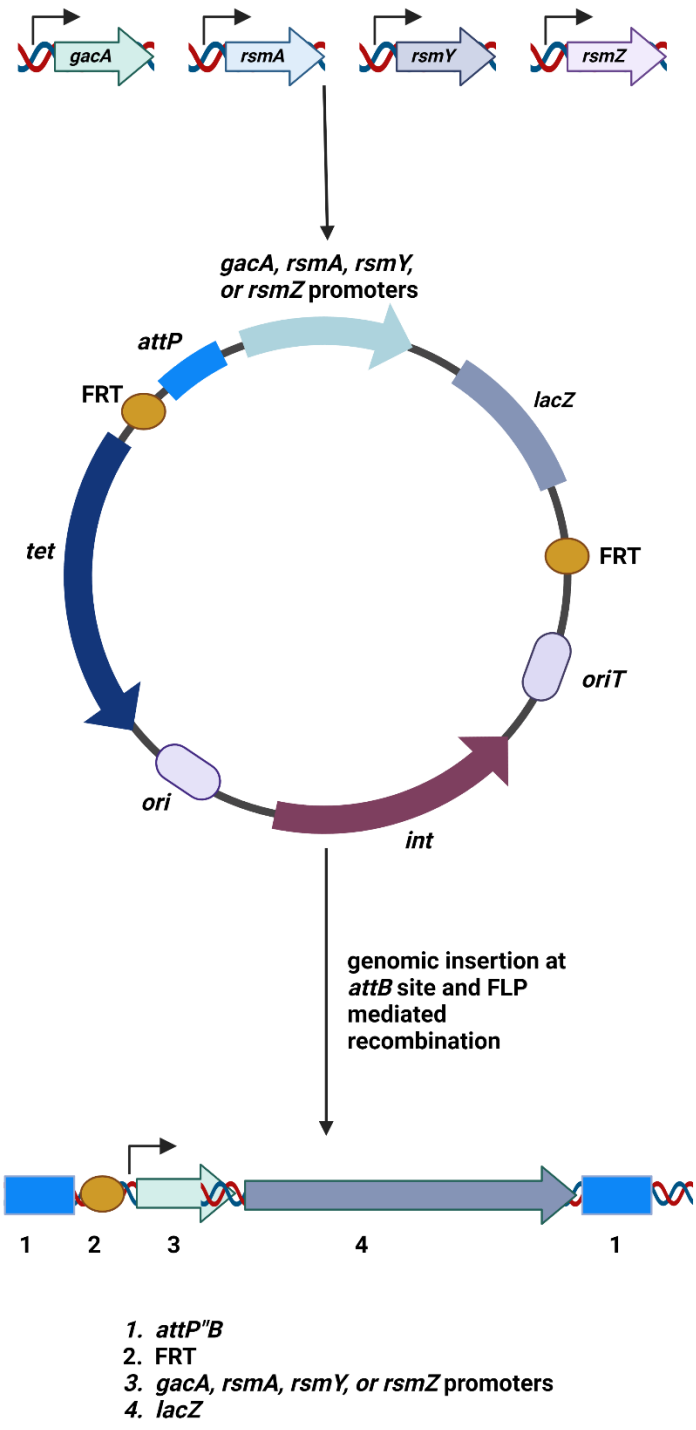


Figure 16. This is a graphical representation showing the *lacZ* transcriptional fusion integration into the *P. aeruginosa* wild type and *ΔrelA* strains

The multiple cloning site (MCS) of the mini-CTX-*lacZ* vector allows for the integration of a gene sequence without interrupting the promoterless reporter *lacZ*. To this end the promoter regions from the following genes were inserted by Chris Pritchett, Ph.D.: *gacA, rsmA, rsmY*, and *rsmZ*.

These segments were then integrated into the chromosome of both the wildtype and *ΔrelA* mutants of PAO1 and PDO300. This was done so the effects of the stringent response on the Gac/Rsm two component regulatory system could be measured via β-galactosidase assay. The bacteria harboring the transcriptional fusions were streaked for isolation on $VBMM_{carb300}$ plates. After screening the fusion strains on LB vs. LB-no salt-tetracycline PCR was performed to confirm the successful integration of gene-*lacZ* transcriptional fusion.

β-galactosidase Assays

The GacA/GacS two component system is a part of a global regulatory pathway. A pathway consisting of LadS/RetS/GacS/GacA/RsmA proteins. Each component of this pathway acts a check on the other (Figure 6). To study the effect of the stringent response on this system *lacZ* transcriptional fusions were created and integrated into the wild types and mutants as described above. The results of this will be addressed in three subsequent sections: one for PAO1 vs PAO1-*ΔrelA*, PDO300 vs PDO300-*ΔrelA*, and PAO1 vs PDO300 / PDO300-*ΔrelA.*

The PAO1 vs PAO1-ΔrelA β-galactosidase Assays do not show significance. Figure 17 shows that in the wildtype PAO1 GacA transcription is around 275 Miller Units, and the mutant is very similar. Which is the same for both RsmY and RsmZ. For RsmA, the average Miller units for the wildtype are a bit lower than the mutant but the individual values are within range of each other.

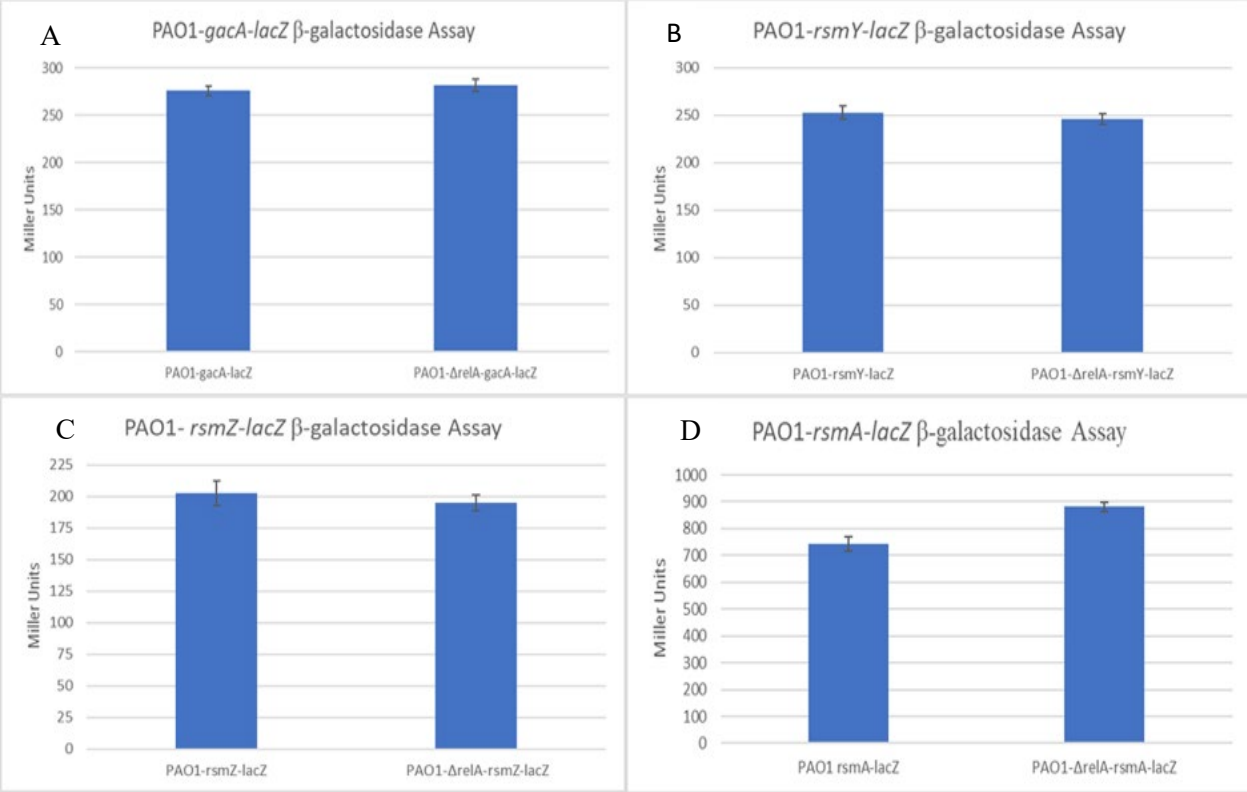


Figure 17. PAO1 vs PAO1-*ΔrelA* β-galactosidase Assay A. *gacA-lacZ* B. *rsmY-lacZ* C. *rsmZ-lacZ* D. *rsmA-lacZ*

The PDO300 vs PDO300-ΔrelA β-galactosidase Assays show significance. Figure 18 shows that the wild-type, PDO300, has higher Miller Units in *gacA, rsmY,* and *rsmz* when compared to the *ΔrelA* mutant. Another difference is that *rsmA* in the mutated strain is higher than the wild type. All results are significant at $p<0.05$.

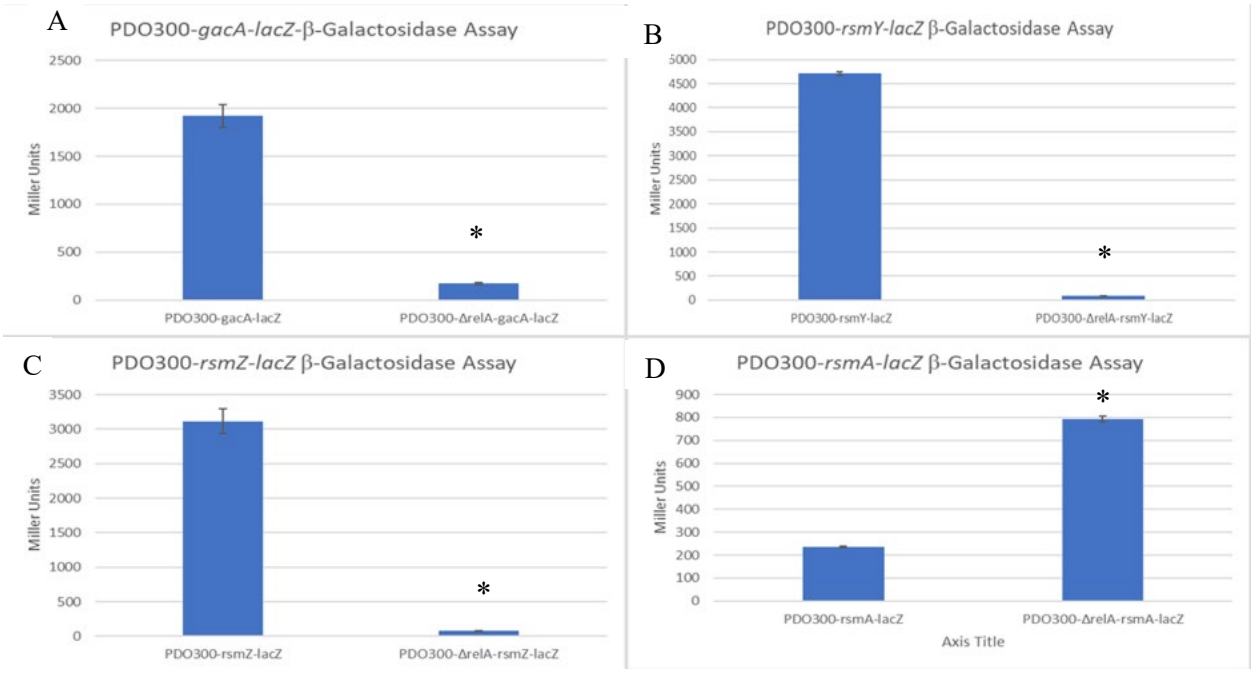


Figure 18. PDO300 vs PDO300-*ΔrelA* β-galactosidase Assay A. *gacA-lacZ* B. *rsmY-lacZ* C. *rsmZ-lacZ* D. *rsmA-lacZ*

The comparisons between PAO1 vs PDO300 / PDO300-ΔrelA β-galactosidase Assays show significance. Figure 20 shows the data from the PAO1 β-galactosidase Assay compared to both the PDO300 and PDO300-*ΔrelA.* For each transcriptional fusion PDO300 and PDO300-*ΔrelA* were tested for significance using one way ANOVA. What is shown is that upon deletion of the *relA* gene in PDO300 the Miller Units for *gacA*, *rsmY,* and *rsmZ* dropped to levels close to those seen in PAO1. Whereas the Miller Units in PDO300-*ΔrelA* increased to levels comparable to PAO1.

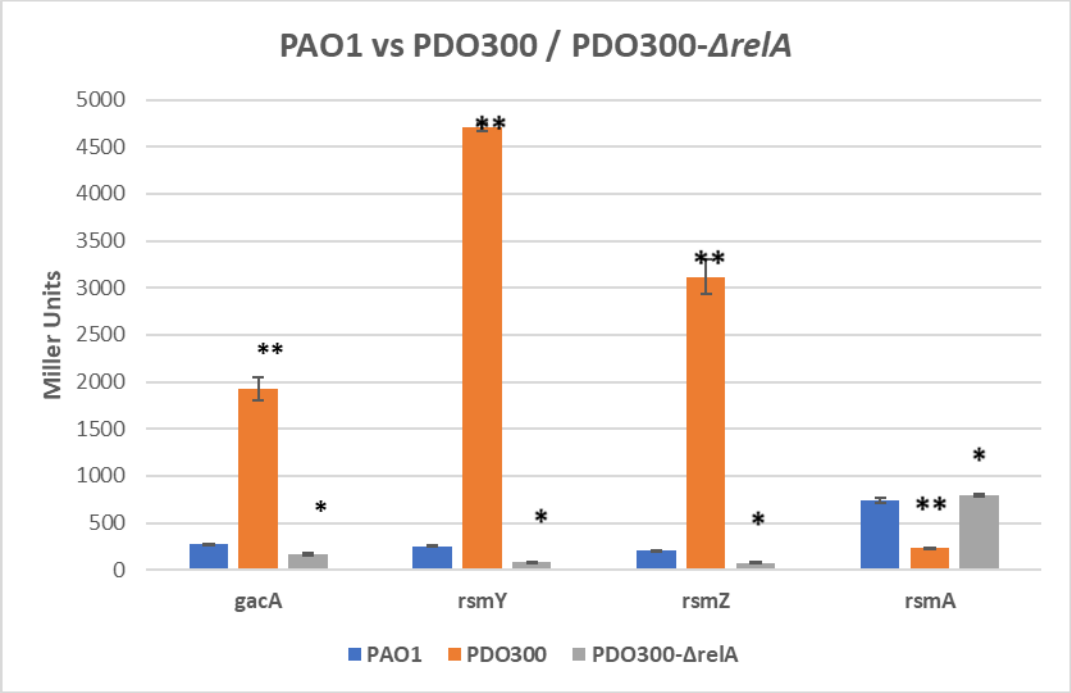


Figure 19. Data from the PAO1, PDO300, and PDO300-*ΔrelA* were tested for significance. For each transcriptional fusion PAO1 vs PDO300 and PAO1 vs PDO300-*ΔrelA* were compared. Two asterisks represent significance at 0.01 and one asterisk represents significance at 0.05.

Alginate Assay

Initially, after the construction of the *relA* mutant in PDO300 it was noticed that the mutant had a decrease in the mucoid phenotype. To determine if this was a result in a decrease in alginate production a carbazole assay to quantify the amount of alginate produced per cell. The modulation of alginate levels in PDO300 and PDO300-*ΔrelA* were determined at 48 hrs. This can be seen in Figure 20. The deletion of *relA* in PDO300 resulted in a near 6-fold decrease in alginate production and was statistically significant ($P < 0.05$).

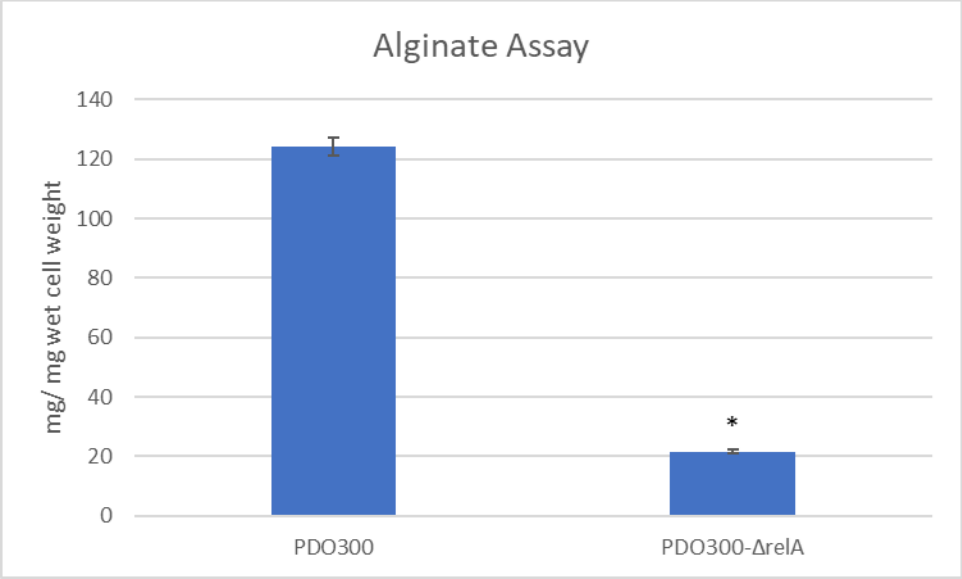


Figure 20. Alginate production in PDO300 and PDO300-*ΔrelA* after 48 hrs growth

Swimming Motility Assay

To test for the presence of functional flagella in both the PDO300-*ΔrelA* and PAO1-*ΔrelA* mutants a swimming assay was performed. The basis of this experiment relies on stab inoculating the bacterial strains into LB-agar plates that have a decreased agar concentration 0.3%. This allows swimming via flagella only. The plates were incubated at 24 hrs.

What is shown in Figure 21 is that PAO1-*ΔrelA* had a slight increase in swimming motility. But PDO300-*ΔrelA* had a significant increase in swimming motility. Both results were statistically significant ($P < 0.05$).

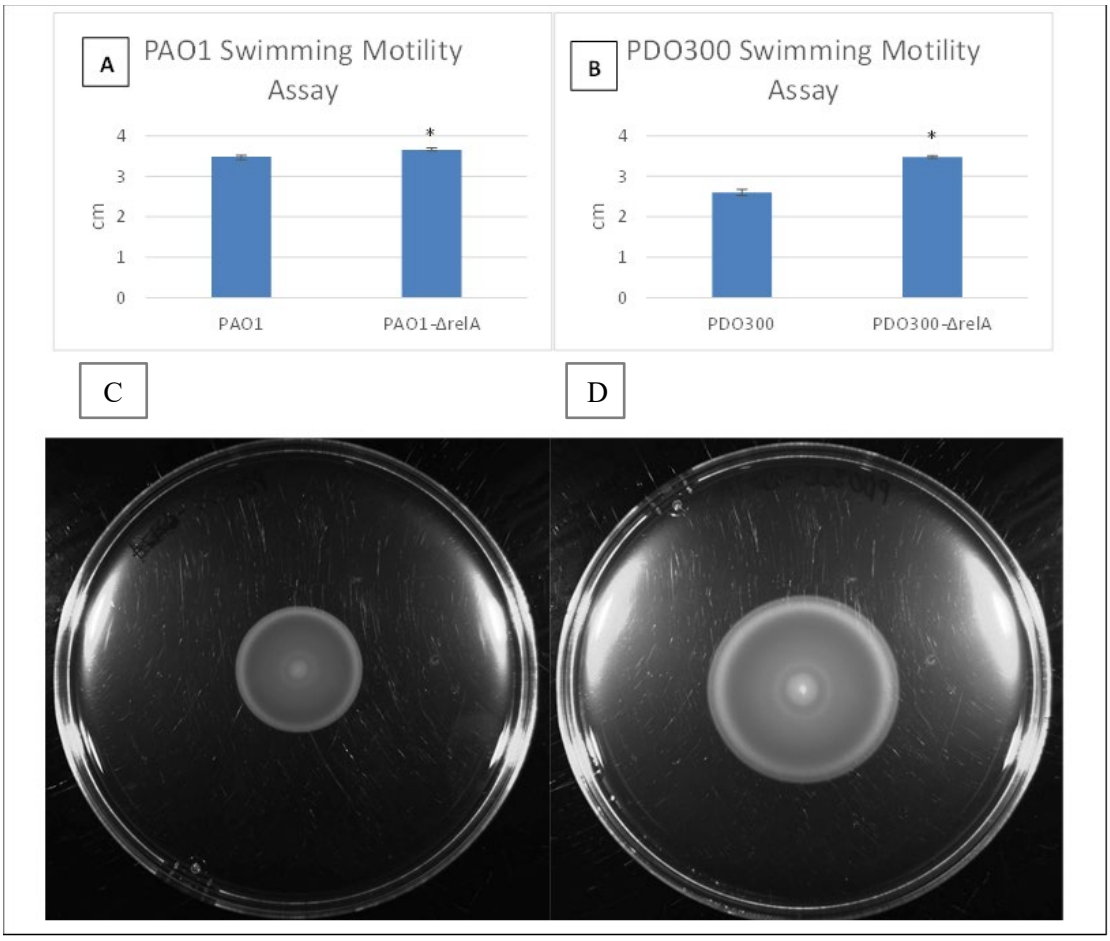
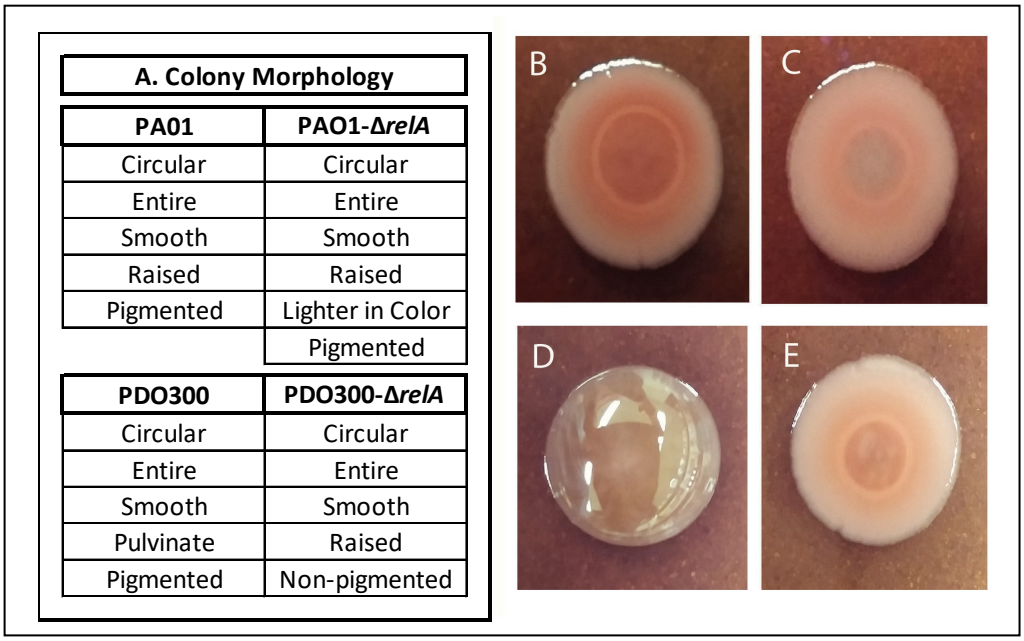


Figure 21. Swimming Motility Assay in PAO1, PAO1-*ΔrelA*, PDO300, and PDO300-*ΔrelA* A. PAO1 vs PAO1-*ΔrelA* B. PDO300 vs PDO300-*ΔrelA* C. PDO300 D. PDO300-*ΔrelA*

Colony Morphology

Cultures were grown on Congo-Red plates at room temperature for 6 days. At the end of which images of representative colonies were taken. These can be seen in Figure 22.

PAO1-*ΔrelA* showed to be a lighter color than the wild-type but had the same basic colony morphologies. The biggest difference seen was in the PDO300-*ΔrelA* vs PDO300. The mutant strain showed a change in surface and elevation morphologies. The PDO300-*ΔrelA* mutant showed a raised elevation vs the pulvinate, or curved convexly/swelled, morphology in the wild type. Another difference is that the PDO300-*ΔrelA* appears less pigmented than PDO300. All these differences are outlined in Figure 22.



A. Colony Morphology

PA01	PAO1-Δ*relA*
Circular	Circular
Entire	Entire
Smooth	Smooth
Raised	Raised
Pigmented	Lighter in Color
	Pigmented

PDO300	PDO300-Δ*relA*
Circular	Circular
Entire	Entire
Smooth	Smooth
Pulvinate	Raised
Pigmented	Non-pigmented

Figure 22. Colony Morphology A. Chart describing the colony morphologies in the tested strain B. PAO1 C. PAO1-*ΔrelA* D. PDO300 E. PDO300-*ΔrelA*

Biofilm Formation Assays

The Quantitative Biofilm Assays show significance. The Biofilm Formation Assay was run in two parts. The first was a quantitative test that measures the ability of bacteria, *P. aeruginosa*, to form biofilms on PVC plastic. This is important as PVC plastic is the same material used to make catheter and endotracheal tubes. This test uses OD_{540} as a measure of the crystal violet for the staining of biofilm on the tubes. As can be seen in Figure 23, both PAO1-*ΔrelA* and PDO300-*ΔrelA* showed significant decreases in biofilm formation as compared to

their respective wild-types. PAO1-*ΔrelA* showed an approximate 2.5-fold decrease, whereas PDO300-*ΔrelA* showed an approximate 2.1-fold decrease. Both the mutant strains showed a statistically significant decrease at $P < 0.05$.

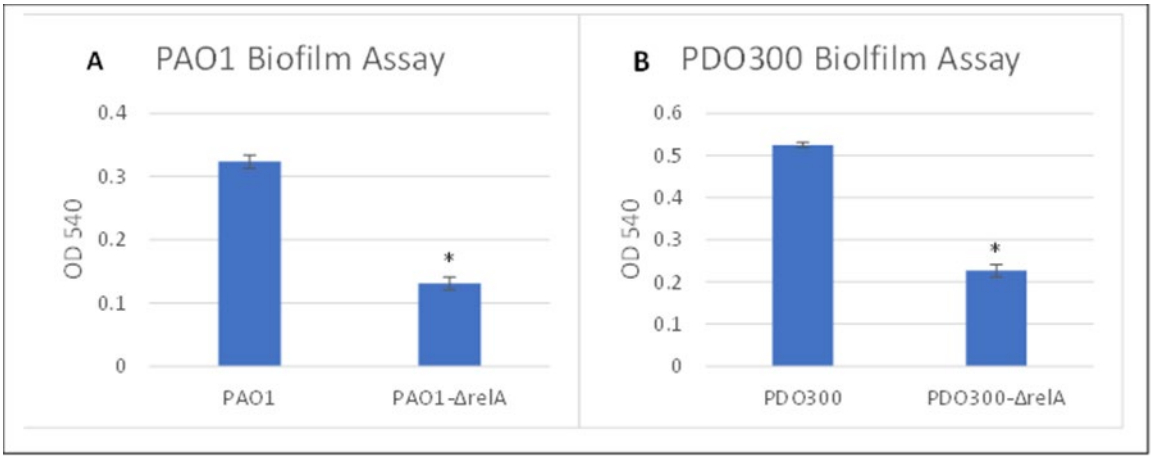


Figure 23. Quantitative Biofilm Assay on PVC Plastic A. PAO1 vs PAO1-*ΔrelA* B. PDO300 vs PDO300-*ΔrelA*

The Visual Representative Biofilm Assays show difference in biofilm formation. The second part of the experiment showed the ability of PDO300 and PDO300-*ΔrelA* to form biofilms on polystyrene tubes; a material used to house medical test kits, and devices, along with being diagnostic components. This was not a quantitative assay as it is only a picture of the tubes. Figure 24 shows a decrease in the crystal violet binding to the polystyrene tubes.

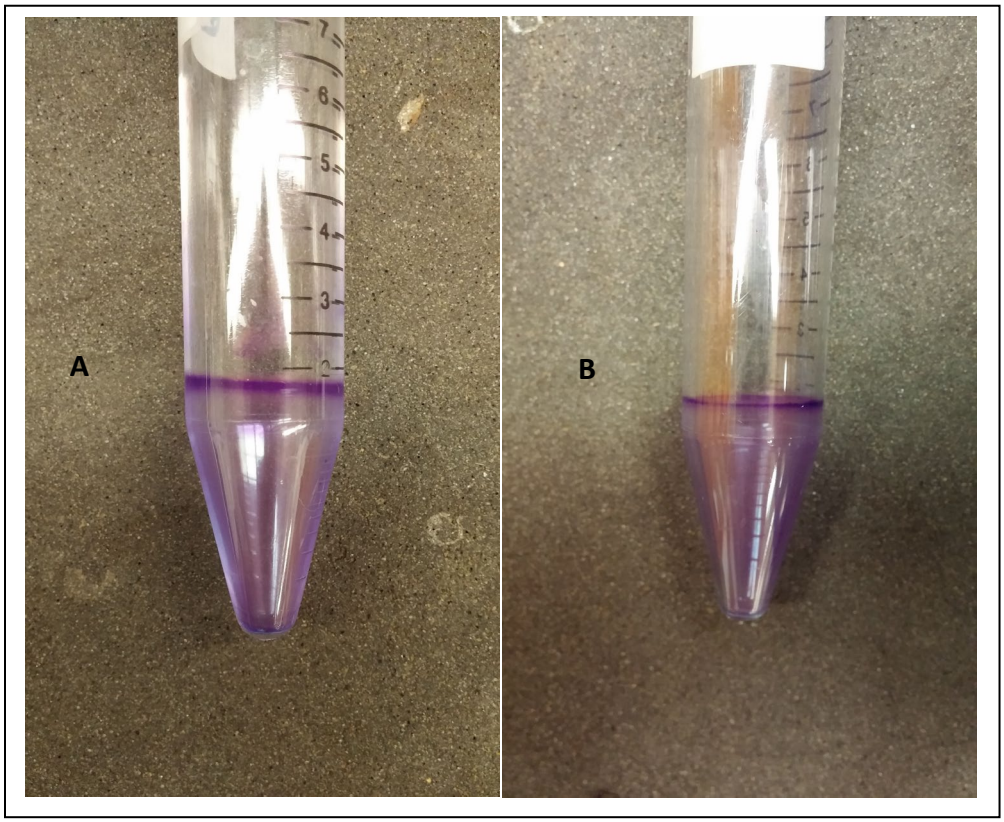


Figure 24. Biofilm Formation on Polystyrene A. PDO300 B. PDO300-*ΔrelA*

Twitching Motility Assay

This test was used to test for type IV pili, a key component for attachment to surfaces in the initial colonization of *P. aeruginosa*. This test used an agar concentration between the swimming motility and the normal growth plates. Growing the cultures for 72 hrs should activate the stringent response. What can be seen in Figure 25 is that PAO1-*ΔrelA* shows an approximate 1.6-fold decrease in twitching motility. This is statistically significant at $P < 0.005$.

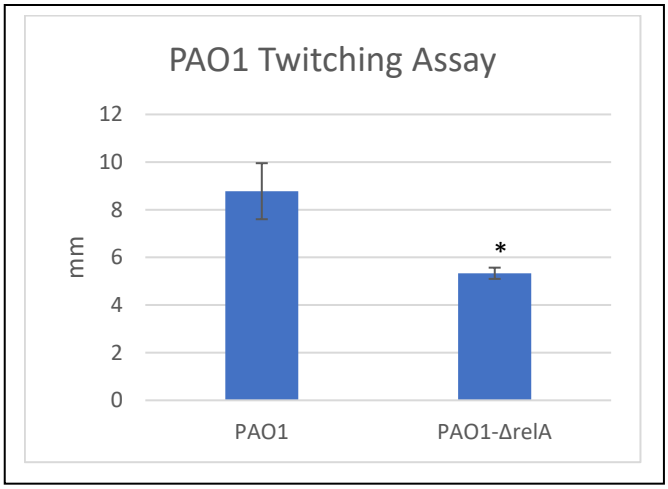


Figure 25. Twitching Motility Assay in PAO1 vs PAO1-*ΔrelA*

Figure 26 shows that the mutated strain of PDO300 had a 9-fold increase in twitching motility. The representative images of this are shown as well. PDO300-*ΔrelA* had a statistically significant increase at $P < 0.05$.

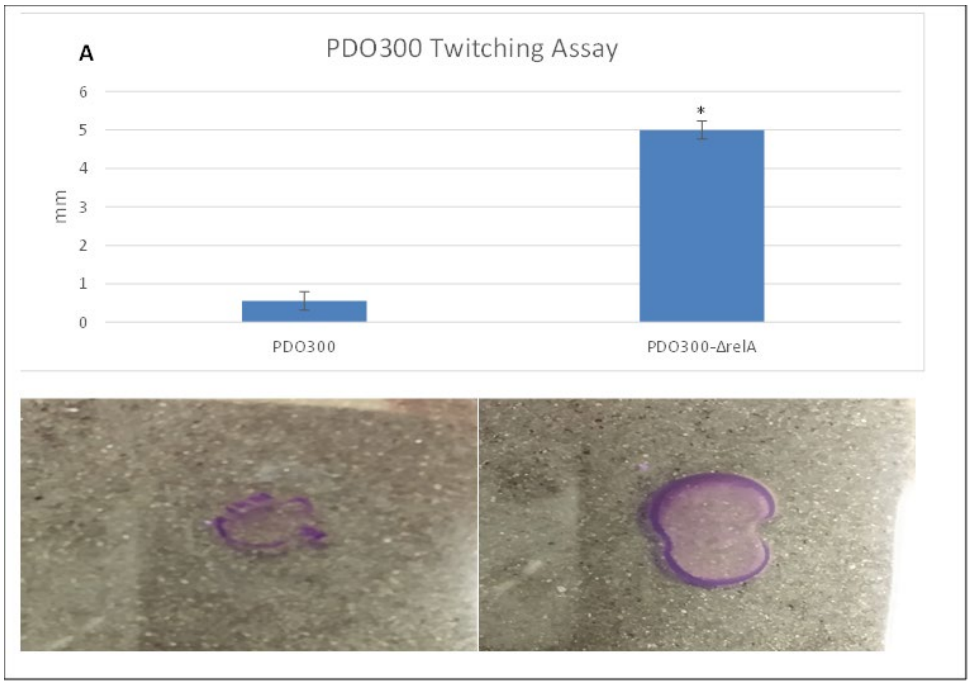


Figure 26. PDO300 Twitching Motility Assay A. Quantitative Analysis of PDO300 vs. PDO300-*ΔrelA* B. PDO300 C. PDO300-*ΔrelA*

Transmission Electron Microscopy (TEM)

RelA negatively regulates motility in mucoid background. Because the *relA* mutant in the mucoid background showed lowered alginate production and increased motility, transmission electron microscopy was performed. Colonies were isolated on PIA plates and placed on a 400-Cu grid, then stained with 2% uranyl acetate. Cell size was noted as the only difference in the non-mucoid background. In the mucoid background, the *relA* mutant possessed a single-polar flagellum as can be seen in Figure 27.

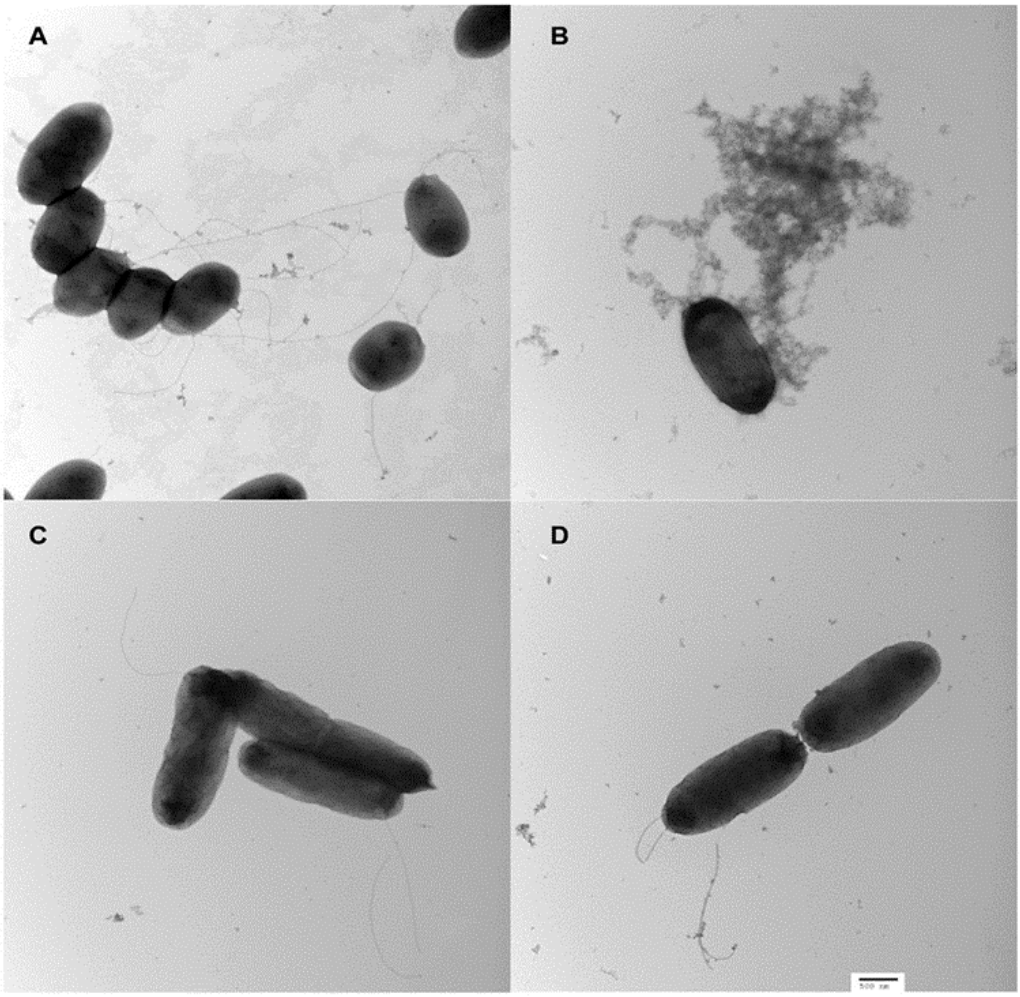


Figure 27. RelA negatively regulates motility in mucoid background. A. PAO1 B. PDO300 C. PAO1-*ΔrelA* D. PDO300-*ΔrelA*. All images are a direct magnification of 17000x at 80kV.

CHAPTER 4. DISCUSSION

Pseudomonas aeruginosa is a Gram-negative bacterium that can cause a wide range of life-threatening infections, both acute and chronic. It is a leading cause of morbidity and mortality in cystic fibrosis patients. According to the 2019 and 2022 CDC Special Reports, the rate of multi-drug resistant nosocomial infections of *P. aeruginosa* increased 32% during the COVID-19 pandemic. This was after years of decline (CDC 2019; Karaba et al. 2020; CDC Special Report 2022). Recent statistics show that *P. aeruginosa* is one of the six antimicrobial resistant bacteria that costs the United States more than $4.6 billion annually (Nelson et al. 2021; Weiner-Lastinger et al. 2022).

Antibiotic resistance is an intrinsic and natural trait for these bacteria. *P. aeruginosa* utilizes many different molecular mechanisms and phenotypes that are required for survival during pathogenesis and antimicrobial treatments. During initial stages of colonization, a vast array of virulence mechanisms helps these bacteria to survive. After infection, bacteria are exposed to oxidative stress due to inflammatory responses and then treatment with antibiotics (Furukawa et al. 2006; Turner et al. 2014). These stressors induce the expression of a different subsect of genes that enable these bacteria to adapt and ultimately switch to persistent and resistant phenotypes. At the same, *P. aeruginosa* becomes less virulent. A common factor during this phenotypic switch is the overproduction of exopolysaccharide alginate. This event often indicates a downturn in prognosis for cystic fibrosis patients (MacDougall et al. 2005; Poole 2011; Gellatly and Hancock 2013).

Thus, finding a mechanism or gene to either reverse or stop this phenotypic switch from occurring may be beneficial to future treatments in patients. However, there are numerous regulatory systems responsible for virulence and adaptation. Quorum sensing plays a central role

as it acts to control the social behaviour of *P. aeruginosa* via many different signaling pathways (LaSarre and Federle 2013). The progress of acute to chronic infection is influenced by quorum sensing dependent gene expression. These genes are critically involved in virulence factor production, motility-sessility switch, biofilm development, and adjusting the pathways for stress responses.

Pathogenesis and infection introduce bacteria to a wide range of environmental stressors and the production of virulence factors is often metabolically costly (Whiteley et al. 1999; Diggle et al. 2002; Wagner et al. 2003; Garcia-Contreras et al. 2014). The stress of response to the depletion of phosphate and iron was found to be intrinsically linked (Slater et al. 2008). Numerous studies have shown that the uptake and acquisition of phosphate and iron is important for survival of *P. aeruginosa*. The system of genes responsible for scavenging these elements are upregulated as soon as the bacteria interact with the human respiratory epithelial cells (Frisk et al. 2004; Chugani and Greenberg 2007). Hypoxia, or oxygen depletion, is a stress factor during pathogenesis and is induced via various factors such as chronic inflammation, microbial populations, and biofilm formation (Hassett 1996; Worlitzsch et al. 2002; Yoon et al. 2002; Alvarex-Ortega and Harwood 2007; Hassett et al. 2009).

It is also important to note that different modes of *P. aeruginosa* motility are associated with virulent traits (Winstantley et al. 2016). The phenotypic switch from a motile to a sessile cell with lowered virulency is advantageous to the survivability of *P. aeruginosa*; allowing it to evade stresses, immune responses, antibiotic treatments, and other bacteria. This allows the bacteria hide within a biofilm and since motile cells are easily detected by host immune cells *P. aeruginosa* are protected from any adverse environmental stressors. (Leid 2009; Amiel et al. 2010; Olsen 2015).

The exopolysaccharides Psl, Pel, and alginate play an important role in biofilm formation. Psl is a key element in the early stages of biofilm formation. It acts as a means for migration via type 4 pili and twitching, cell to cell interaction, and cell-surface adhesion (Overhage et al. 2005; Wang et al. 2013; Zhao et al. 2013). Pel is also important for initiating and maintaining cell to cell interactions (Colvin et al. 2011). The overproduction of alginate is the characteristic phenotype of mucoid and sessile *P. aeruginosa*. It is important in biofilm maturation, stability, and protection (Hay et al. 2009a; Hay et al. 2013; Strempel et al. 2013).

The Gac/Rsm pathway plays an integral role in both motility-sessility and acute-chronic infection transitions. GacA/GacS is a two-component regulatory system that generates two non-coding RNAs, known as RsmY and RsmZ, under stress conditions. These counteract and bind a RNA binding protein, RsmA. This protein binds to *psl* mRNA, inhibiting Psl biosynthesis. (Irie et al. 2010).

The stringent response is method bacteria use to survive amino acid starvation events. The ribosome associated RelA synthesizes the alarmones, (p)ppGpp (known collectively as guanosine pentaphosphate and guanosine tetraphosphate), which are central triggers for both persistence and stringent response (Potrykus and Cashel 2008; Wu et al. 2010; Amato2014).

At the time of the current study, 2012 to 2015, various research articles showed a link between the stringent response and acute-chronic phenotypic switch. But most were in other bacteria, notably in *E. coli* (Kuroda et al. 2001; Fung et al. 2010; Amato et al. 2013). Fung et al. showed that using *E. coli* as a model can be informative for *P. aeruginosa* because it has several homologous pathways (Fung et al. 2010).

The experiments conducted in this study suggest that the stringent response acts as a regulator of the acute-chronic phenotypic switch. Whether that is immediate or intermediate

means is left for future studies. What has been shown is that when *relA* is deleted in a mucoid strain, in this case PDO300, a reversion to a motile, non-mucoid type occurs. Beta-galactosidase assays with the transcriptional fusions for with the promoter regions of the components of the Gac/Rsm pathway (*gacA*, *rsmY*, *rsmZ,* and *rsmA*) were inserted into both the wild type of PDO300 and the knockout strain, PDO300-*ΔrelA*. These showed that knocking out a key component of the stringent response has a significant decrease in GacA, RsmY, and RsmZ transcription which in turn has a significant increase in RsmA transcription; as RsmY and RsmZ are not available to bind to the RNA-binding protein, RsmA.

The growth curves of both mutant and wild type show no significant differences, therefore suggesting that any change in molecular activity is not due to growth defects over 24 hrs. Another notable feature is the overall morphology of the mutant colonies were different from the wild type. PDO300-*ΔrelA* mutants were non-mucoid and was less pigmented; seemingly reverting to the PAO1 phenotype.

Based on the observations of the mutant being non-mucoid, an alginate assay was conducted. This assay showed that PDO300-*ΔrelA* had a significant decrease in alginate against the wild type, PDO300. Quantitative biofilm assays showed that the mutant PDO300-*ΔrelA* had a significant decrease in biofilm formation. Twitching motility assays showed a significant increase in twitching motility mechanisms in the mutant. More studies will need to be conducted to determine if the decrease in biofilm formation is due to both alginate and Pel production being diminished, or one of the two. Swimming motility assays showed that PDO300-*ΔrelA* was more motile than the wild type, PDO300. Suggesting the formation of functional flagella when *relA* is removed.

When comparing PAO1 vs PAO1-*ΔrelA*, it is shown that swimming motility is slightly increased in the mutant. However, biofilm formation and twitching motility decreased significantly.

One possible explanation for these results is found in sigma factors and possibly cyclic di-GMP. It has been shown previously that the stringent response and production of (p)ppGpp is crucial for regulation in numerous virulence factor production (Erickson et al. 2004; Viducic et al. 2006; Boes et al. 2008; Nguyen et al. 2011; Vogt et al. 2011; Sampathkumar et al. 2016). Also, the transcription of *fleQ* depends on the housekeeping sigma factor RpoD (σ^{70}) which is repressed once the transcriptional regulator Vfr is overexpressed (Dasgupta et al. 2002; Romling et al. 2013).

Vfr is a transcriptional regulator protein (virulence factor regulator) that has been shown to regulate the production of type IV pili, exotoxin A, and the *las* quorum sensing system, in which *las,* along with *rhl,* controls the expression of multiple virulence factors (Albus et al. 1997; Beatson et al. 2002; Schuster et al. 2003; Wagner et al. 2003). The alarmone indirectly mediates global changes by releasing RpoD from the RNA polymerase. This acts to shift the use of RpoS (σ^{S}) and the increase in this sigma factor's expression will control QS and, subsequently, biofilm formation (Jishage et al. 2002; Durfee et al. 2008).

Cyclic-3'5'-diguanylic acid, cyclic di-GMP, is a small molecule but plays a key role post-transcriptional regulation of biofilm formation. Experimental data showed that MucR synthesizes cyclic di-GMP near the alginate biosynthesis and secretion protein complex (Hay et al. 2009b; Wang et al. 2015). Cyclic di-GMP binds to FleQ and will repress flagella biosynthesis while at the same time de-repressing *pel* and *psl* genes (Baraquet et al. 2012). When RsmA is bound by RsmY and RsmZ, cyclic di-GMP levels are increased (Nadal-Jimenez et al. 2012).

Figure 28 shows an outline of the interaction between RsmYZ/RsmA complex elicits cyclic-di-GMP indirectly.

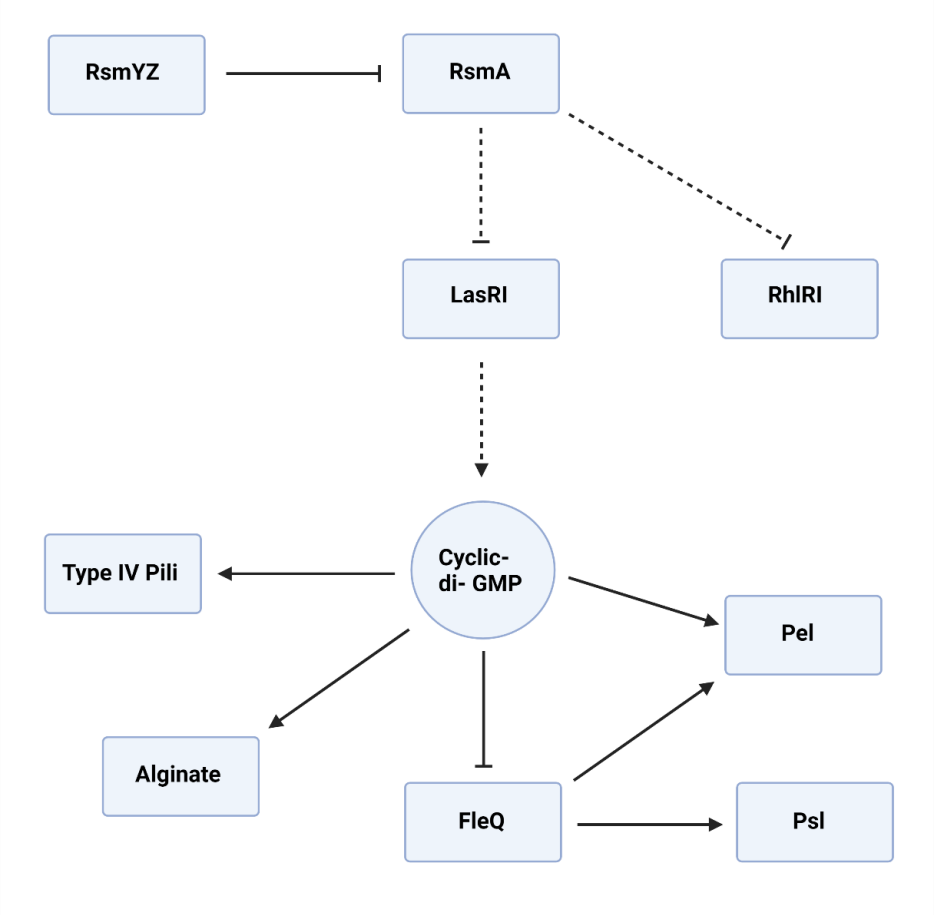


Figure 28. Outline of how the RsmYZ/RsmA complex elicits phenotypic switch in *P. aeruginosa*

To clarify, it is possible that that repressing (p)ppGpp in the *ΔrelA* mutants leads to an increase in RpoD binding to RNA polymerase. Since (p)ppGpp overproduction has shown a significant increase in RpoS expression a decrease in the alarmone in *ΔrelA* mutants may have a negative effect on RpoS binding to the RNA polymerase (Gentry et al. 1993; van Delden et al. 2001). This decrease in RpoS also has a negative effect on QS transcription, notably *vfr*, *las/rhl*, and *gacA/gacS*, which decreases biofilm formation (alginate, Pel, and Psl) (Whiteley et al. 2000). And increases FleQ transcription leading to motile cells via flagella production. The deletion of

(p)ppGpp synthases affects the cyclic di-GMP metabolism as several genes are under the control of RpoS; shown in *E. coli* and *Pseudomonas putida* (Lange et al. 1995; Weber et al. 2006; Matilla et al. 2011). Figure 29 shows the proposed method of how the stringent response can elicit a phenotypic switch as outlined above.

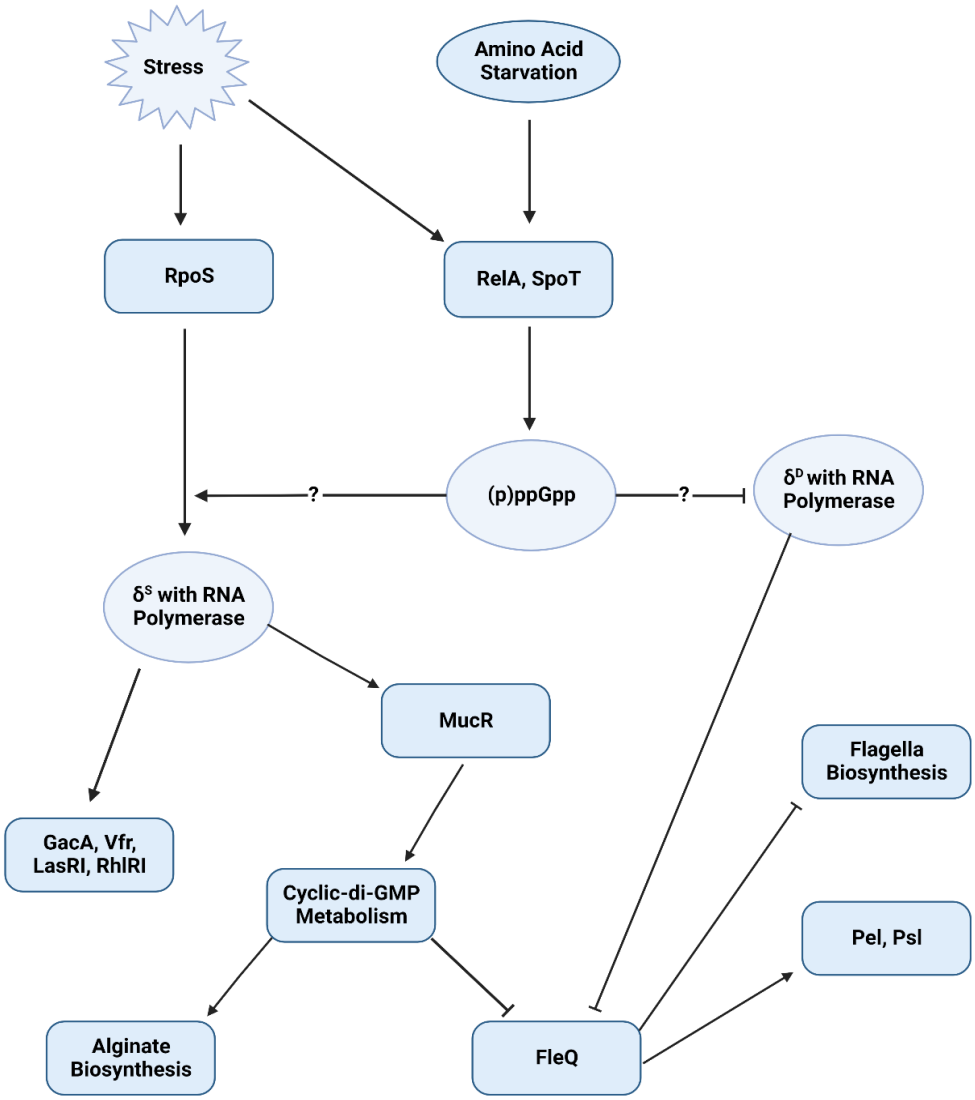


Figure 29. The possible interactions between (p)ppGpp synthesis and σ^S binding with RNA Polymerase and σ^D releasing. This in turn can cause an upregulation of biofilm formation genes while down regulating flagella biosynthesis.

FUTURE EXPERIMENTS

It is important to note that more work will need to be done to establish a direct link as described in the previous chapter. Hard evidence will be needed to fill in the gaps of data presented here. First, to satisfy Molecular Koch's Postulates, the *relA* gene will need to be re-inserted into PAO1-*ΔrelA* and PDO300-*ΔrelA* mutants. Then the tests described in this thesis will need to be conducted in the PAO1-*ΔrelA::relA* and PDO300-*ΔrelA::relA* strains. This is done to either confirm or refute that the phenotypic changes seen in the mutants were caused by the deletion of the *relA* gene.

Next, to establish that (p)ppGpp affects RpoS production, Green Fluorescent Protein tagging will need to be done on the *rpoS* gene and subsequently the protein itself. This will need to be done in the wild types, *ΔrelA* mutants, and the *ΔrelA::relA* constructs. This will show whether a decrease in alarmone synthesis results in a decrease in RpoS. Albeit it will be an indirect link if one is present. The same type of test will need to be conducted with GacA.

Immunocytochemistry utilizes antibodies that bind to specific antigens on cells to allow for easy screening. In this case, it would be used to screen for Psl and Pel production in the wild types, *ΔrelA* mutants, and the *ΔrelA::relA* constructs.

As is always the case, these experiments will only yield more questions. And that in of itself is the nature of science. Always searching for explanation backed by evidence.

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APPENDICES

Appendix A: Primers, Plasmids, and Bacterial Strains

Plasmids Used in this Study.

Plasmids	Description	Source
pRK2013	helper plasmid for mobilization	Figurski, D.H. et al. 1979
pEX18$_{GM}$	allelic exchange vector	Hoang et al. 1998
Δ*relA* pEX18$_{GM}$	allelic exchange for *relA* allele	This Study
rsmA-lacZ mini-CTX	transcriptional fusion	Dr. Christopher Pritchett
rsmY-lacZ mini-CTX	transcriptional fusion	Dr. Christopher Pritchett
rsmZ-lacZ mini-CTX	transcriptional fusion	Dr. Christopher Pritchett
gacA-lacZ mini-CTX	transcriptional fusion	Dr. Christopher Pritchett

Bacterial Strains used in this Study.

P. aeruginosa strains		
PAO1	wild type	
PDO300	*mucA*-22 mutation	Mathee, K. et al. 1997
PAO1- Δ*relA*	*relA* nonpolar deletion	This study
PDO300- Δ*relA*	*relA* nonpolar deletion	This study
PDO300-*rsmA-lacZ*	*rsmA* transcriptional fusion strain	This Study
PDO300-*rsmY-lacZ*	*rsmY* transcriptional fusion strain	This Study
PDO300-*rsmZ-lacZ*	*rsmZ* transcriptional fusion strain	This Study
PDO300-*gacA-lacZ*	*gacA* transcriptional fusion strain	This Study
PDO-Δ*relA-rsmA-lacZ*	*rsmA* transcriptional fusion strain	This study

PDO-Δ*relA-rsmY-lacZ*	*rsmY* transcriptional fusion strain	This study
PDO-Δ*relA-rsmZ-lacZ*	*rsmZ* transcriptional fusion strain	This study
PDO-Δ*relA-gacA-lacZ*	*gacA* transcriptional fusion strain	This study
PAO1-*rsmA-lacZ*	*rsmA* transcriptional fusion strain	This Study
PAO1-*rsmY-lacZ*	*rsmY* transcriptional fusion strain	This Study
PAO1-*rsmZ-lacZ*	*rsmZ* transcriptional fusion strain	This Study
PAO1-*gacA-lacZ*	*gacA*t transcriptional fusion strain	This Study
PAO1-Δ*relA-rsmA-lacZ*	*rsmA* transcriptional fusion strain	This study
PAO1-Δ*relA-rsmY-lacZ*	*rsmY* transcriptional fusion strain	This study
PAO1-Δ*relA-rsmZ-lacZ*	*rsmZ* transcriptional fusion strain	This study
PAO1-Δ*relA-gacA-lacZ*	*gacA* transcriptional fusion strain	This study

Primer Sequences used in this study.

Primers

rsmATFF	GCG CGG CCG CGT CGA CAT GAT CGT GCA GAA C
rsmYTFF	GCG CGC GGC CGC GTG TTG CGT TCG TTG GTC AC
rsmZTFF	GCG CGC GGC CGC CCT TAG ACC CAC TGA AGA CC
gacAintF	CCA GGG TGC TTG CGC TTT AC
lacZRforTF	GAT GTG CTG CAA GGC GAT TAA G
relARcheck	GCC GAA TTC GGG ATC GCG C
relAFcheck	GCA GGG CTA TCG TCT GAA GC

Appendix B: PCR Conditions

Figure 30. Optimal conditions for PCR

Ingredients	Final Concentration
10X Thermopol Reaction Buffer	1X
10 mM dNTPs	0.3 mM
10 µM Forward Primer	0.4 µM
10 µM Reverse Primer	0.4 µM
Template DNA	50 ng/mL
Taq DNA polymerase	0.625 units
Double distilled H_2O	To 25 µL

Figure 31. PCR Cycling Conditions to amplify *relA* in *P. aeruginosa*

Steps	*relA* gene PCR Parameters
Denaturation	95 °C- 5 minutes
Amplification	95°C – 30 seconds
	59°C- 30 seconds
	75°C- 1 minute
Number of cycles	30
Final Extension	72°C- 10 minutes

Figure 32. PCR cycling conditions to amplify *lacZ* transcriptional fusions

Steps	Transcriptional Fusion PCR Parameters
Denaturation	95 °C- 5 minutes
Amplification	95°C – 30 seconds
	64°C- 30 seconds
	72°C- 30 seconds
Number of cycles	30
Final Extension	72°C- 10 minutes

VITA

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