MOLECULAR MECHANISMS OF CYTOTOXICITY REGULATION *IN PSEUDOMONAS AERUGINOSA* BY THE MAGNESIUM TRANSPORTER MGTE

by

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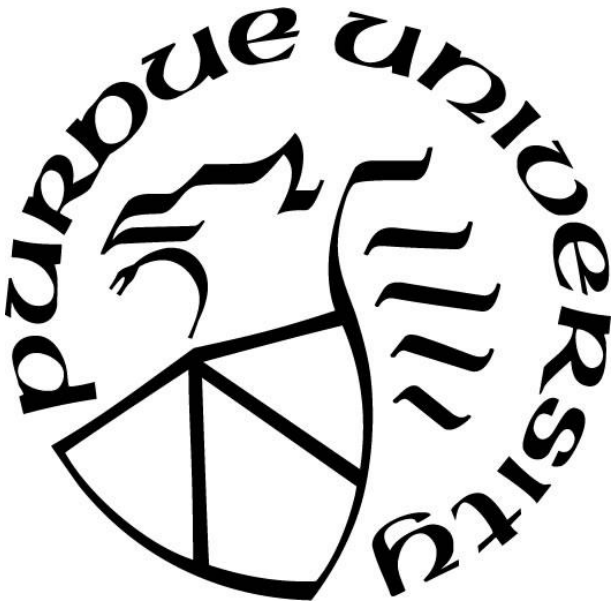

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Dedicated to my Wife Pallabi and my Parents

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LIST OF ABBREVIATIONS

T3SS	Type III Secretion System
QS	Quorum Sensing
LPS	Lipopolysaccharide
TCS	Two Component System
CF	Cystic Fibrosis
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ONPG	*Ortho*-nitrophenyl-β-D-galactopyranoside
CPRG	Chlorophenol red–β-d-galactopyranoside
SDDM	Sodium Dodecyl Maltoside
Δ	Deletion mutation
Tn	Transposon mutation

ABSTRACT

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Title: Molecular Mechanisms of Cytotoxicity Regulation in *Pseudomonas aeruginosa* by the Magnesium Transporter MgtE
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The Gram-negative bacterium *Pseudomonas aeruginosa* causes numerous acute and chronic opportunistic infections in humans. One of its most formidable weapons is a type III secretion system (T3SS), a multi-protein molecular syringe that injects powerful toxins directly into host cells. The toxins lead to cell dysfunction and, ultimately, cell death. Identification of regulatory pathways that control T3SS gene expression may lead to the discovery of novel therapeutics to treat *P. aeruginosa* infections. In a previous study, it was found that expression of the magnesium transporter gene *mgtE* inhibits T3SS gene transcription. MgtE-dependent inhibition appeared to interfere with the synthesis or function of the master T3SS transcriptional activator ExsA, although the exact mechanism was unclear. In this work, we demonstrate that *mgtE* expression acts through the GacAS two-component system to activate transcription of the small regulatory RNAs RsmY and RsmZ. This event ultimately leads to inhibition of *exsA* translation. Moreover, our data reveal that MgtE acts solely through this pathway to regulate T3SS gene transcription. Our study reveals an important mechanism that may allow *P. aeruginosa* to fine-tune T3SS activity in response to certain environmental stimuli.

In addition, a previous study has shown that the *P. aeruginosa* gene *algR* abrogates *mgtE* mediated regulation of cytotoxicity. AlgR has pleiotropic effects in *P. aeruginosa*,

including regulation of synthesis of the exopolysaccharide alginate. In the second part of my thesis, I show that *algR* and *mgtE* genetically crosstalk to inhibit ExsA driven T3SS gene transcription. This genetic interaction between *algR* and *mgtE* seems to be specifically directed towards regulation of T3SS gene expression rather than having an indiscriminate effect on multiple virulence attributes in *P. aeruginosa*. Additionally, we have further demonstrated that AlgR inhibits *mgtE* transcription. These studies suggest the presence of a T3SS inhibitor that is inhibited by both AlgR and MgtE. Future work will involve transcriptomic and proteomic analysis to identify such an inhibitor.

Taken together, this study provides important insight into the molecular mechanisms of *mgtE* expression and function in *P. aeruginosa*. We have established that *mgtE* has pleiotropic effects on cytotoxicity in *P. aeruginosa*. Thus, given the role that cytotoxicity regulation plays in shaping *P. aeruginosa* pathogenesis and associated clinical outcomes, *mgtE* might be an interesting drug target, though extensive future studies are required to validate this proposition. Nevertheless, this research, provides clues for identification of novel therapeutic targets in *P. aeruginosa*. Hence this work, in the long run, serve to ameliorate the morbidity and mortality in patients infected with *P. aeruginosa*.

CHAPTER 1. INTRODUCTION

This chapter in part, has been published in references (1) and (2). Required permissions have been obtained.

Pseudomonas aeruginosa is a Gram-negative pathogen implicated in a multitude of acute and chronic infections (3). Like other members of the bacterial genus *Pseudomonas*, they are ubiquitous in soil and water ecosystems (4). Their genetic flexibility, versatility in optimizing the usage of a vast array of organic and inorganic compounds, and innate capability to survive under diverse, often trying, environmental conditions are the key factors behind their successful inhabitation of most environmental niches found on our planet. These characteristics also enable them to become notorious opportunistic pathogens. From the point of view of mankind, this genus is threatening indeed due to its inherent and acquired antibiotic resistance (5). It is not surprising therefore, that it has been increasingly implicated in nosocomial infections and deaths (5). This, and its involvement in the permanent lung infections in cystic fibrosis patients (described later), which severely increase morbidity and mortality, warrants the study of its virulence mechanisms and their regulation.

1.1 Taxonomy

P. aeruginosa is part of the bacterial family *Pseudomonadaceae,* which is also comprised of *Azotobacter*, *Azomonas*, *Cellvibrio*, and *Azorhizophilus*, among others. *Pseudomonadaceae* are commonly characterized as chemoorganotrophic, aerobic, and incapable of photosynthesis. These are also able to survive under a myriad of nutritional

environments (6). While the common methods of taxonomical organization of the *Pseudomonas* genus include cell morphology, pigment types, nutritional behaviors, and genetic organization (7), the widespread phenotypic characteristics mostly studied are carbon utilization patterns, antibiotic resistance, and antibiotic and amino acid synthesis (8). Over 30 species exist in the genus *Pseudomonas* (7), and 13 species (and numerous strains thereof) are sequenced and annotated, *P. aeruginosa* amongst them. While the benefits of chemotaxonomic studies have been widely published (9), it is generally accepted that gene sequencing studies hold the greatest promise for solving *Pseudomonas* taxonomic issues (7).

1.2 Cellular Characteristics

P. aeruginosa is Gram-negative, catalase and oxidase positive. These microbes are slightly curved or linear rod-shaped cells, having a maximum length of approximately 4 μm. They are often motile via polar flagella, although lateral flagella are occasionally found, usually associated with swarming (6, 10). The flagellar number is decided by FleN, a putative ATP–GTP binding protein (6). Pili have been reported and are particularly studied for their role in pathogenesis in *P. aeruginosa* (11); nevertheless, other typical nonpathogenic *Pseudomonas* species like *P. fluorescens* have also been cited to possess pili (12). Intriguingly, low G + C content of pilin genes, in relation to the higher average chromosomal G + C, indicates horizontal acquisition of pilin genes by *Pseudomonas* species (13). *Pseudomonas* species produce a number of colored pigments. For instance, under limiting iron conditions, they produce iron chelator molecules that fluoresce under UV light (14). Other pigments are non-fluorescent and participate in other processes. *P.*

aeruginosa commonly produces the pigments pyocyanin, pyoveridin and pyorubin. Intracellularly, *Pseudomonas* species form inclusion bodies of various substances. For instance, under nitrogen-deficient conditions, *Pseudomonas* accumulates poly-β-hydroxybutyrate (PHB) granules. When growing on gluconate or alkanes, they are found to assemble poly-hydroxyalkanoates.

1.3 Physiology and Metabolism

As mentioned, *P. aeruginosa* exhibits great metabolic plasticity (6), and extensive analysis has been performed to elucidate *Pseudomonas* metabolism. The TCA cycle has been reported in *P. aeruginosa* and in all other species of *Pseudomonas*. The Entner–Doudoroff glycolytic pathway often prevails over the Embden–Meyerhof pathway due to the lack of 6-phosphofructokinase in many species. The Pentose Phosphate pathway, the glyoxylate shunt, and other important metabolic pathways are also widespread in this genus. Catabolite repression is also reported in *Pseudomonas* (15, 16), which allows *Pseudomonas* to utilize a medley of available carbon sources in a preferential and orderly manner. Organic and amino acids are preferred to glucose (17), followed by mannitol and histidine. Catabolite repression is facilitated by Crc and other signal transduction proteins (18, 19). Intriguingly, in some cases, like during repression of phenol metabolism in *P. putida*, cells mediate catabolite repression by discerning the redox state of respiratory chains.

P. aeruginosa has a propensity to use amino acids as carbon and nitrogen sources. Using specific membrane permeases, amino acids available in the environment are translocated

into the cell cytoplasm (20). The ability of using amino acids as nutrients poses a great advantage to *Pseudomonas* because amino acids can be readily assimilated into the cell biomass with minimal processing. Alternatively, amino acids can be further metabolized to enter central metabolism. Among amino acids, arginine metabolism, especially, is key in *Pseudomonas* biochemistry, with a number of catabolic pathways identified for breaking down this substrate (21, 22). In *P. aeruginosa*, numerous arginine-responsive genes are controlled by the arginine regulatory protein ArgR (23).

Though typically considered a strict aerobe, *P. aeruginosa* can perform fermentations and/or anaerobic respiration as a facultative anaerobe. Specifically, arginine (24) and pyruvate fermentation (24) pathways have been reported. Additionally, it utilizes nitrogenous substances, like nitrate, as terminal electron acceptors for anaerobic respiration (25).

1.4 Habitat

P. aeruginosa is ubiquitous in the environment, which is attributed to its metabolic versatility (26). It can be found in soil and water environments, as well as plant and animal tissues and many other niches. *P. aeruginosa* cells have even been found in hospital saline solutions (27), Antarctic cyanobacterial mats, and in water from plumbing fixtures (28). It grows over a temperature range of 4°C–42°C, and pH 4–8 is considered favorable. Though culture-dependent studies indicate that *P. aeruginosa* might be an important soil microorganism (29), culture-independent methods, such as metagenomics, suggest that *Pseudomonas* species actually might be relatively scarce in the soil environment.

Nevertheless, when growing in soil, *Pseudomonas* does so in conjunction with other bacteria, like *Streptomyces,* that supply monomeric carbon compounds (6). *Pseudomonas* is considered extremely suited to thriving in the rhizosphere (30), the territory impacted by vegetation. Importantly, *Pseudomonas* outcompetes other microorganisms to optimize their hold on the rhizosphere. While some species, like *P. syringae,* are epiphytic in nature and effectively colonize plant leaves and other plant surfaces to cause disease (31), and others benefit the plant by producing phytohormones (32), *P. aeruginosa* is a saprophyte or pathogen, found to be scavenging organic matter (33).

1.5 Control of Gene Expression

P. aeruginosa has one of the largest genomes among sequenced bacteria, containing over 6 Mbp, and over 5,000 genes. Expression of these numerous genes is controlled by a complex network of transcriptional and post-transcriptional regulatory mechanisms (34). Several of these regulatory mechanisms are discussed later.

1.5.1 Quorum Sensing

P. aeruginosa uses three overlapping quorum sensing (QS) systems to regulate gene expression. QS facilitates coordinated gene expression in response to environmental signals and bacterial population density. Thus, QS is used to coordinate population-wide gene expression for pathogenesis, metabolism, and many other processes (35). Two of these systems, Las and Rhl, produce acylhomoserine lactone (AHL) autoinducers, while the third involves the quinolone molecule PQS (35). QS controls expression of type II

secretion (T2SS), elastase, pyocyanin, and pyoverdine in *P. aeruginosa*, among other factors (35).

1.5.2 Two-Component Systems

There are about 130 genes involved with two-component systems (TCSs) in *P. aeruginosa* (36). TCS are commonly found also in other species belonging to this genus. These regulatory systems mediate a variety of functions for the microbes (Table 1.1).

Table 1.1: Some Important Two-Component Systems in *P. aeruginosa* and Their Functions (1)

Sensor Histidine Kinase	**Response Regulator**	**Function**
PilS	PilR	Pilus gene expression
NtrB	NtrC	Nitrogen metabolism
PhoR	PhoB	Phosphate assimilation
FleS	FleR	Motility and adhesion properties
GacS	GacA	Pathogenesis
PfeS	PfeR	Iron Uptake

1.5.3 Sigma Factors

A large number of sigma factors have been reported in *Pseudomonas* species, and these sigma factors are critical for optimal RNA polymerase-mediated gene transcription activity. Some important ones are described below.

1.5.3.1 σ^{70}

This sigma factor is important for housekeeping gene transcription (37). The consensus sequence found in σ^{70}-dependent promoters in *P. aeruginosa* has similarity to that found in *Escherichia coli*.

1.5.3.2 Extracytoplasmic Function Sigma Factors

This subgroup of σ^{70} family factors is important for control of extracytoplasmic stress (38). An important extracytoplasmic function (ECF) sigma factor is AlgU, which regulates biosynthesis of the secreted polysaccharide alginate (39). It is also considered to regulate heat shock sigma factor σ^{32}, and hence is implicated to be a global gene regulator (40). Another ECF sigma factor, PvdS, regulates pyoverdine siderophore biosynthesis. A sequence at the −35 position, known as the "IS box," is critical for PvdS-dependent promoter functioning (41). Interestingly, orthologues of the *P. aeruginosa* PvdS have been reported in *P. putida* (strain KT2440) and *P. fluorescens* (42).

1.5.3.3 σ^{54}

This sigma factor is encoded by *rpoN* and is distinct from the σ^{70} family. Some of the major functions of RpoN include regulation of glutamine synthetase and urease in *P. aeruginosa*, as well as some virulence factors. The number and function of σ^{54}-dependent genes varies greatly among various *Pseudomonas* species (43, 44). For example, it is involved in the regulation of the phytotoxin coronatine in *P. syringae.*

1.5.4 Other Transcriptional Regulators

There are quite a few families of transcriptional regulators found in *P. aeruginosa,* including members of the AsnC, GntR, LacI, LuxR, and MarR families (1). For catabolism of aromatic hydrocarbons, various species use σ^{54}-dependent regulator families such as XylR, TouR, and DmpR (45).

1.5.5 Post-Transcriptional Regulation

Post-transcription regulation has also been reported in *P. aeruginosa*. For example, the TCS GacAS and the membrane bound proteins RetS and LadS regulate transcription of two small noncoding regulatory RNAs: RsmY and RsmZ (3) (Fig. 1.1). These RNAs control the intracellular concentration of free RsmA, a prominent member of the CsrA family of RNA-binding proteins. RsmA controls translation of certain transcripts by binding to conserved 5′-ANGGAN-3′ motifs present within stem-loop secondary structures of target mRNAs, such as those for type III secretion system (T3SS) genes. Such binding sites often overlap or are proximal to the ribosome binding site of these target mRNAs (46, 47), and thus RsmA binding blocks ribosome recognition and translation initiation. RsmA also post-transcriptionally stimulates gene expression through effects on mRNA stability and secondary structure (48, 49). RsmY and RsmZ each have 4–6 RsmA binding sites. Thus, presence of these regulatory RNAs controls the free concentration of RsmA and its ability to bind target mRNAs (3). Post-transcriptional regulation is reported in other *Pseudomonas* species as well. As an example, in *P. fluorescens*, the *hcnABC* operon (involved in expression of hydrogen cyanide synthase expression) is under control of the small noncoding RNAs RsmA and RsmE (50, 51).

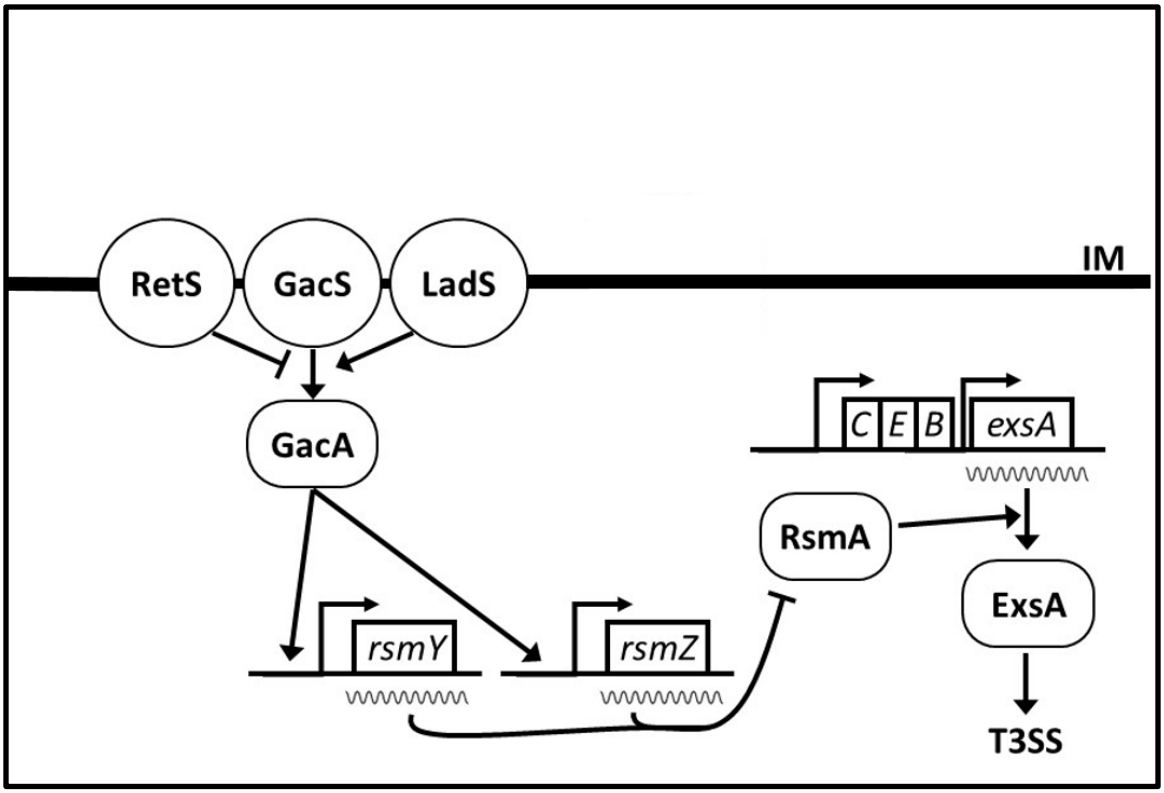

Figure 1.1. Post transcriptional regulation by small RNAs RsmY and RsmZ. The inner membrane (IM) associated sensor kinase GacS, phosphorylates and activates its cognate response regulator GacA, which directly binds to and activates transcription of regulatory small RNAs RsmY and RsmZ. Increased *rsmY*/*rsmZ* levels, in turn, sequester RsmA, preventing it from enhancing translation of *exsA*, thus inhibiting T3SS. RetS forms a heterodimer with GacS and prevents it from activating GacA, while LadS activates GacA phosphorylation by an unknown mechanism (see text for details).

1.6 Pathogenesis of *P. aeruginosa*

P. aeruginosa causes infection in a wide range of different host organisms, including humans. The importance of *P. aeruginosa* as an opportunistic pathogen stems from three major concerns: high incidence of infection, high morbidity and mortality, and recalcitrance to immune or antibiotic clearance (52). *P. aeruginosa* is the leading pathogen causing acute respiratory infections in mechanically ventilated and immunocompromised individuals and chronic infections in the airways of individuals

with cystic fibrosis (CF) (53, 54). *P. aeruginosa* has also been implicated in other opportunistic diseases such as those of the urinary tract, eye, skin, and burn wounds (55).

1.6.1 General Pathogenicity Attributes

During infection, *P. aeruginosa* is thought to be acquired from the environment, including, importantly, the hospital. Bacterial cell surface virulence factors, such as flagella, pili, and lipopolysaccharide (LPS), aid in initial adhesion to the host epithelial layer (52). This is often followed by injection of effector molecules (ExoU, ExoS, ExoT, ExoY) into the host epithelium through the T3SS. These effectors facilitate host cell cytoskeleton rearrangement, escape from host cell phagocytic engulfment, and cytotoxicity leading to tissue necrosis (55). Other secreted virulence factors involved in host cell cytotoxicity and tissue destruction include elastase, phospholipase C, and pigments such as pyoverdine and pyocyanin (56, 57). Production of this arsenal of virulence factors is tightly regulated by an intricate network of QS, TCSs, stress response systems, and other factors (55, 58, 59).

1.6.1.1 Cell Surface Virulence Factors

As the name suggests, these factors are localized on the cell surface. They mediate bacterial motility and the early stages of infection, such as initial adhesion to the host cell surface.

1.6.1.1.1 Flagella

In addition to providing motility for *P. aeruginosa*, flagella bind to asialoGM1, a normal constituent of the host epithelial cell membrane (60). They are also known to prompt an NFκB mediated inflammatory reaction, such as production of interleukin IL-8, by interacting with TLR2 and TLR5 (61). Because flagella are immunogenic, they often are downregulated during infection, especially in chronic infections like those in the CF airways (see below). Immunogenicity of flagellar proteins has been exploited by researchers to develop immunological products targeted towards them (62).

1.6.1.1.2 Pili

Pili are also critical for *P. aeruginosa* virulence (63), particularly for adherence to the host cell surface by binding to asialoGM1 (64, 65). They are also involved in twitching motility, which is dependent upon retractile movement of the pili. This activity leads to *spreading*, rather than *swimming*, of the bacterial population on the host tissue surface. This kind of locomotion is especially prevalent in respiratory tract infections (66). Because of the importance of pili for virulence, immunological targeting of pili is a major area of research (67-69).

1.6.1.1.3 LPS

LPS is crucial in *P. aeruginosa* pathogenesis (69). LPS assists *P. aeruginosa* in binding to asialoGM1 (65) and elicits pro-inflammatory cascades through interaction of lipid A with TLR4/CD14 (70).

1.6.1.2 Type III Secretion System

T3SS is a complex molecular syringe that is used by *P. aeruginosa* to inject four effector toxins (ExoU, ExoS, ExoT, and ExoY) into the host cell cytosol. Intriguingly, it has been reported that mutants that lack effectors, but still express the T3SS structure are still virulent (71), indicating that the T3SS needle complex by itself might be important in *P. aeruginosa* pathogenesis. PcrV is an important structural protein making up the tip of the T3SS needle (72), and immunotherapeutics targeted at PcrV have achieved some success (73).

1.6.1.2.1 ExoU

ExoU is the most potent of the *P. aeruginosa* T3SS effectors (71, 74) and it is the predominant cytotoxin injected by the T3SS (74, 75). It destroys host cell membranes through its phospholipase/lysophospholipase activities (76, 77). Anti-ExoU immunotherapy has been generally unsatisfactory (52), but some success has been achieved with phospholipase A2 inhibitors in vitro (78). As the mechanism of ExoU activity becomes clearer, newer therapeutic strategies will emerge.

1.6.1.2.2 ExoS

The ExoS cytotoxin (71) disrupts the host cell cytoskeleton through two different activities (52). The C-terminus, which requires a 14–3–3 cofactor protein, contains ADP-ribosyltransferase activity (74, 79) and the N-terminal domain acts as a Rho GTPase activating protein (GAP). ExoS provokes inflammatory responses through TLR2 and TLR4 (80).

1.6.1.2.3 ExoT

ExoT is regarded a minor effector (74). This toxin contains domains similar to ExoS, although the ADP-ribosyltransferase domain in ExoT affects a more restricted subset of host cellular proteins, including Crk (81). ExoT induces cytoskeletal rearrangements, leading to inhibition of *P. aeruginosa* internalization and stunted wound healing (82). Some studies have reported that ExoT production decreases ExoU-mediated cell cytotoxicity (71).

1.6.1.2.4 ExoY

ExoY functions as an adenylate cyclase (55), and upon being injected into the host cell cytoplasm, increases intracellular cAMP levels (83). Increased host intracellular cAMP leads to increased tissue porosity, especially in lung infections (84).

1.6.1.3 Other Secreted Virulence Elements

P. aeruginosa secretes a number of other factors that contribute to disease progression. These factors, such as secreted pigments, enzymes, and proteases, destroy host tissues, increase tissue porosity, and induce host inflammatory reaction, among other functions (Table 1.2) (52).

Table 1.2: Secreted Virulence Factors in *Pseudomonas aeruginosa* and Their Functions (1)

Factor	Description/Function
Pyocyanin	Blue pigment; induces IL-8 expression and apoptosis of neutrophils; causes oxidative damage in host cells
Pyoverdine	Siderophore for iron chelation and uptake; virulence regulator controlling secretion of exotoxin A and itself
Alkaline protease	Cleaves fibrin in host tissue; prevalent in corneal infections and in lung disease
Protease IV	Important in keratitis caused by *P. aeruginosa*; also implicated in airway infections due to destruction of lung surfactant proteins A, B, and D
Elastase	Metalloproteinase secreted into the extracellular space; destroys tight junctions leading to porosity of airway epithelial tissue; induces inflammation by recruiting neutrophils and eliciting IL-8 response; also reported to disrupt lung surfactant proteins A and D
Phospholipase C	Secreted into the extracellular space; disrupts host cell membrane phospholipids, especially in acute lung disease; participates in surfactant destruction, inflammation induction, and inhibition of neutrophil oxidative burst
Exotoxin A	Functions as an ADP-ribosyltransferase that blocks elongation factor 2, leading to impaired protein synthesis and host cell death

1.6.1.4 Biofilm formation

By definition, ‘biofilms’ are ‘matrix enclosed bacterial populations adherent to each other and/or to surfaces or interfaces’ (85). Thus, a surface attached bacterial sedentary biofilm lifestyle is markedly different from a ‘planktonic’ or free-swimming one. There are four distinct stages of biofilm formation in *P. aeruginosa*. The first stage involves initial attachment to a biotic (eg. host epithelium) or abiotic surface. The second phase is characterized by microcolony formation, wherein microbes cluster with each other and the sedentary surface. In stage three, the microcolonies progress in size and complexity to develop into the mature three-dimensional biofilm structure, with water channels and an exopolysaccharide matrix. This is followed by the final stage, biofilm dispersion, wherein some bacteria detach from the biofilm, switch to a planktonic mode of growth and leave, either to remain planktonic or to form a biofilm elsewhere (also known as ‘biofilm cycling’). Changes in gene expression occur at each stage as the bacteria transit from the planktonic phase and slowly adapt to the biofilm lifestyle.

Though biofilms are ubiquitously found in various natural environments (86), in the clinical setting, biofilm formation is severely problematic as it renders survival advantage to the pathogen. Typically, biofilms are associated with higher recalcitrance to clearance by the host immune system and to antimicrobial therapy (87). There are three main mechanisms to account for this. One, the biofilm matrix (composed mainly of exopolysaccharides and extracellular DNA and protein) provides a mechanical barrier to host immune system based cells and chemicals and other foreign antimicrobials such as antibiotics from reaching the bacterial population. Secondly, due to altered gene regulation in biofilm-associated bacteria, stress response genes become transcriptionally

activated, further resulting in high antibiotic resistance. Additionally, oxygen and nutrient deprivation in the center of the biofilm render the bacteria non-growing and thus impervious to antibiotics.

P. aeruginosa biofilms generally display lower expression of acute virulence factors like the T3SS and diminished cytotoxicity, but importantly, increased production of factors, like exopolysaccharide, that enhance persistence (88). Indeed, *P. aeruginosa* biofilms are widely implicated in chronic infections like that in a diabetic foot wound, on prosthetic devices and, as a prototype example, in the airways of CF patients.

1.6.2 *P. aeruginosa* Pathogenesis in the CF Lung Environment

CF is a congenital genetic disease, marked by improper chloride secretion across cell membranes (89). Particularly in the airway epithelium, this defect leads to unusually excessive amounts of mucus building up (89), which provides an excellent niche for microbes to proliferate. Though there are multiple pathogens that colonize the CF lung, *P. aeruginosa* predominates from adolescence though adulthood (90).

P. aeruginosa causes a persistent, lifelong infection in the airways of individuals with CF (90). These chronic infections initiate as *P. aeruginosa* adjusts to the CF airway environment and transitions to a biofilm lifestyle (91). As mentioned previously, this biofilm infection in CF lungs is strikingly dissimilar to acute *P. aeruginosa* infections. T3SS and expression of toxins like elastase are diminished. Virulence features that trigger host immunity, like flagella and pili, are downregulated once the pathogen has established

adherent contact with the host respiratory epithelium. On the other hand, biofilm bacteria produce vast amounts of the exopolysaccharide alginate, leading to a highly mucoid phenotype (92).

P. aeruginosa biofilms in the context of CF airways hold serious clinical implications (93). Firstly, CF biofilms are nearly impossible to eradicate due to elevated antibiotic resistance (94). Secondly, despite intense host immune infiltration, bacteria are protected from destruction within the biofilm. Moreover, inflammation leads to damage of surrounding host tissues (92). Thus, *P. aeruginosa* persists for the life of the individual, and it is recognized as the leading cause of morbidity and mortality in CF patients (95). Thus, understanding the molecular cascades that enable *P. aeruginosa* to form biofilms has been the focus of much research.

1.7 Relevant background studies

Because the *P. aeruginosa* biofilm lifestyle is associated with much poorer clinical outcomes (96), it is pivotal to elucidate the genes and biochemical pathways that help the bacteria either 1) switch to the biofilm form from the acute (high T3SS) lifestyle, or 2) maintain the biofilm lifestyle. The key to the identification of such genes, is to investigate the pathogen in conditions which best simulate those where *P. aeruginosa* indeed forms antibiotic resistant biofilms (like that in the CF airway environment).

1.7.1 A co-culture biofilm system to simulate the CF lung environment

A major hindrance to studying microbial pathogenesis in the CF lung is the lack of suitable animal models possessing the CF phenotype and sustaining chronic microbial infection (97, 98). To overcome this, a novel tissue culture based model system was developed (87, 91), wherein *P. aeruginosa* forms biofilms directly on an immortalized human CFBE cell monolayer in culture. Such a co-culture model is more physiologically relevant than biofilms grown on abiotic surfaces such as plastic, because of their distinctive response to stimuli like antibiotics (91). In other words, this model, to a considerable extent, simulates the actual *P. aeruginosa* pathogenesis going on in the CF airway environment.

1.7.2 MgtE modulates virulence in *P. aeruginosa*

Using the co-culture model described above, it has been shown that the *P. aeruginosa* inner membrane magnesium transporter MgtE is a virulence modulator (91). Specifically, biofilm formed by a Δ*mgtE* isogenic deletion strain exhibits higher cytotoxic effects towards cultured airway cells compared to the wild type *P. aeruginosa* strain. Furthermore, complementation of the mutant with a plasmid expressing *mgtE* results in restoration of the wild type phenotype. Genetic analyses revealed that *mgtE* inhibits cytotoxicity in *P. aeruginosa* by decreasing transcription the T3SS. Additionally, *mgtE* was found to inhibit T3SS expression also in liquid (non-biofilm, free swimming lifestyle) culture (99). Because cytotoxicity inhibition is a prominent feature of the biofilm lifestyle, these results suggest that MgtE might be a key signaling molecule facilitating the transition of the bacteria between the acute and chronic phases of growth. Intriguingly, MgtE's toxin modulating function appear to be separate from its transport functions (99).

1.7.3 MgtE is a putative antibiotic-stress response regulator

Recent studies showed that the antibiotic tobramycin, which is extensively used clinically to treat *P. aeruginosa* airway infection in CF patients (100), enhanced MgtE transcription in co-culture biofilms (91). It was additionally found that multiple antibiotics (9 out of 12 tested) induced *mgtE* expression in *P. aeruginosa* (2). This indicates that MgtE responds to antibiotic stress, possibly in connection with its role as a virulence modulator. Since the CF lung environment challenges *P. aeruginosa* with a wide range of stresses, including antibiotic stress, MgtE might be an important molecule necessary for *P. aeruginosa* growing predominantly in its biofilm lifestyle to survive in the CF lung.

1.7.4 AlgR inhibits MgtE's activity

AlgR is pleiotropic response regulator in *P. aeruginosa*, known to affect T3SS transcription (101) as well as production of the biofilm exopolysaccharide alginate (102), among other things. Through a genetic screen of putative virulence modulators, mutation of *algR* disrupted *mgtE*'s activity (2). Specifically, a Δ*mgtE*/Δ*algR* double isogenic mutant led to decreased toxicity (Fig.1.2), instead of the expected toxicity increase.

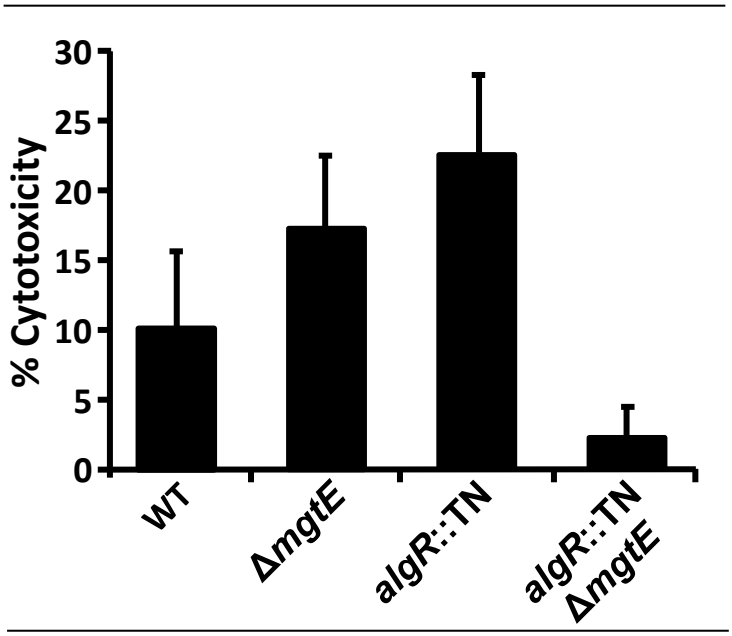

Figure 1.2. AlgR disrupts the cytotoxicity phenotype of *mgtE*.
The Δ*mgtE* and *algR*::Tn strains both display increased cytotoxicity, compared to WT, when grown as biofilms on cultured CFBE cells. However, mutation of both in the same strain leads to decreased cytotoxicity. Figure courtesy Carly V. Redelman (2).

1.8 Vital Questions

These previous studies have provided valuable insight into a novel mechanism by which *P. aeruginosa* regulates virulence during biofilm formation in chronic infections. However, these studies failed to elucidate the exact molecular mechanism by which *mgtE* affected T3SS. Furthermore, it was unclear how MgtE and AlgR cooperated to alter toxicity. Addressing these vital questions will provide novel understanding of how *P. aeruginosa* responds to environmental cues to appropriately regulate transitions between planktonic and biofilm lifestyles.

1.9 Study Aims

The overall goal of my research is to discern the molecular mechanisms of *mgtE*-mediated regulation of cytotoxicity in *P. aeruginosa*. This was accomplished by elucidating the pathway through which *mgtE* inhibits the T3SS, as well as investigating the molecular basis of the genetic interaction between *algR* and *mgtE*, whereby these two genes crosstalk to exert effects on the T3SS. Though the role of *mgtE* in T3SS regulation in *P. aeruginosa* was first discovered using *P. aeruginosa* biofilms growing in vitro on CFBE cells (91), in this study, the mechanistic characterization of *mgtE*'s effect on the T3SS has been done using planktonic cultures of *P. aeruginosa* instead of biofilm models of the bacteria. This is because of two reasons: 1) *mgtE* mediated regulation of T3SS gene expression is conserved between both the biofilm and the planktonic modes of growth in *P. aeruginosa* (99) and 2) ease of experimentation using planktonic *P. aeruginosa* cultures as opposed to its biofilms. The studies done shed more light on the virulence regulatory networks in *P. aeruginosa*, and bacterial pathogenesis in general, which going forward, would help in the development of novel therapeutic targets against *P. aeruginosa*.

CHAPTER 2. SOLUBILIZATION AND SELECTIVE PROTEOMIC ANALYSIS OF MGTE IN PSEUDOMONAS AERUGINOSA

2.1 Introduction

As described in Chapter 1, the T3SS is a molecular syringe-like apparatus that is employed by *Pseudomonas aeruginosa* to inject toxins into host cells to facilitate cytotoxicity. 40 gene products, which include proteins that form the injectisome, chaperones and effector toxins, constitute the T3SS in *P. aeruginosa*. The proteins which control the central or 'intrinsic' regulation of the T3SS are also among these 40 genes. The whole of the T3SS regulon (i.e. these 40 proteins) is transcribed from 10 operons, and it is dependent upon free levels of the master T3SS transcription factor ExsA, which regulates its own transcription as well (55). Under conditions of low or no T3SS, another protein, ExsD, sequesters ExsA and prevents it from binding to its target promoters. Under these conditions, 2 other regulatory proteins ExsE and ExsC remain bound to each other. Under conditions that upregulate the T3SS in *P. aeruginosa* (such as host cell contact, serum, or low Ca^{2+}), ExsE is transported out of the cell, thus liberating ExsC. This free ExsC then binds to and sequesters ExsD away from ExsA, hence increasing free levels of ExsA in the cell. ExsA in turn, binds to all the T3SS promoters (including the operon from which it is transcribed) and turns on T3SS gene expression (55) (Fig. 2.1). The proteins ExsE, ExsC, ExsD and ExsA constitute the 'intrinsic' regulatory machinery of the *P. aeruginosa* T3SS.

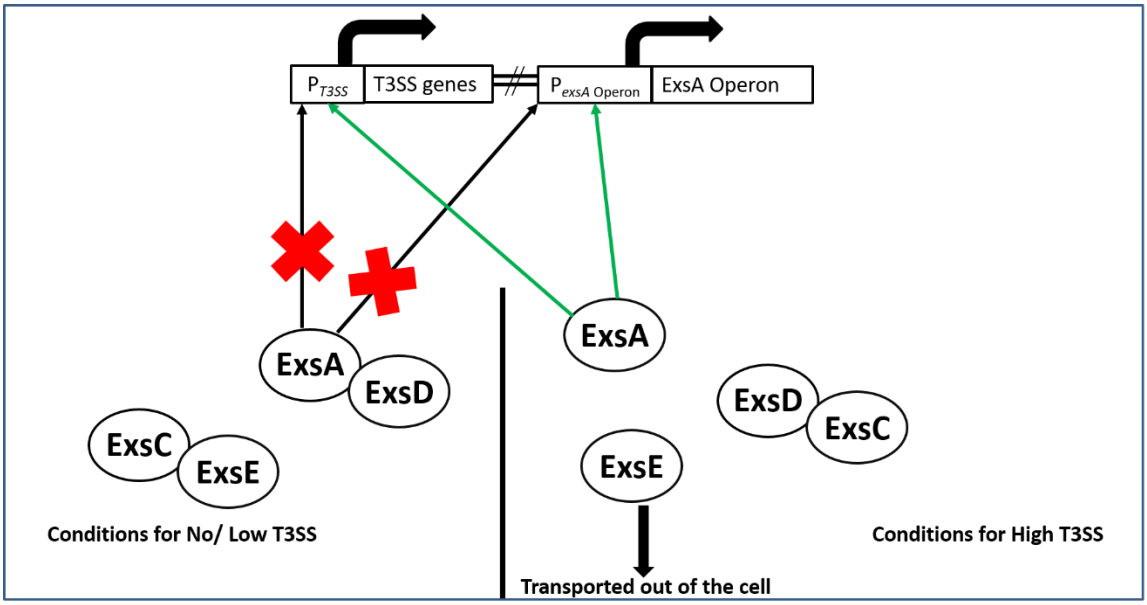

Figure 2.1. Schematic representation of the 'intrinsic' regulation of the *P. aeruginosa* T3SS and autoregulation of ExsA. See text for details.

Previous studies have shown that MgtE, an inner membrane magnesium transporter (99), inhibits ExsA-driven T3SS gene transcription in *P. aeruginosa* (91, 99). Further, *mgtE* was found to bypass the intrinsic regulatory steps governed by *exsE*, *exsC* and *exsD* and act at the level of *exsA*, but the exact mechanism was unknown. Considering the protein/protein interactions involved in the intrinsic regulatory cascade, we hypothesized that MgtE might also be sequestering ExsA directly (like ExsD), thus preventing it from facilitating T3SS gene transcription. In this chapter, I describe my exploration of this hypothesis.

2.2 Materials and Methods

Bacterial strains, plasmids and culture conditions. The bacterial strains used in this study are wild type (WT) *P. aeruginosa* strain PA14 and isogenic deletion mutants in *mgtE* (Δ*mgtE*) and *exsA* (Δ*exsA*). All of these strains have been reported earlier (99). Strains were transformed with plasmid p*mgtE* (PA14 *mgtE* with N-terminal 6x histidine tag cloned into

vector pMQ72) (99) or its empty backbone vector pMQ72 (103) by electroporation. Plasmids were maintained in *Escherichia coli* S17 (99) cultured on LB agar plates or LB containing 10 µg/mL gentamicin. Transformed *P. aeruginosa* was cultured in LB agar plates with 60 µg/ml gentamicin, and the presence of the respective plasmids was confirmed by performing PCR with primers p729 (5' CAGACCGCTTCTGCGTTCTG 3') and p730 (5' GCAACTCTCTACTGTTTCTCC 3') (2). For biochemical analysis, overnight cultures of respective strains with the respective plasmids were subcultured (1:1000 dilution) in LB containing 60 µg/ml gentamicin and 2mM EGTA (2) and grown to OD_{600}=1.0.

Preparation of whole cell lysate and Western blotting. Whole cell lysates were prepared as previously described (3). Briefly, 1.25 ml of cells from the respective cultures (grown to OD_{600}=1.0) were pelleted by centrifugation at 10,000 rpm. The pellet was thereafter washed with PBS (phosphate buffered saline), resuspended in 250 µl SDS-PAGE sample buffer, sonicated for 10s and finally boiled at 95^0C for 5mins. The whole cell proteome samples from different strains were analyzed by SDS-PAGE, followed by Western blotting using either anti-ExsA (3) antibody (1:1000) (kind gift from Dr. Timothy L. Yahr, University of Iowa) or anti-Histidine antibody (1:1500) (Qiagen) as the primary antibody. Appropriate fluorescent tagged (Alexa Fluor 690 or 780, Thermofisher or Jackson Immunologicals) secondary antibodies (1:10000) (donkey anti-rabbit and donkey anti-mouse for anti-ExsA and anti-His primary antibodies respectively) were utilized and fluorescence was measured by Image Studio (LI-COR Biosciences, Lincoln, NE) as described previously (104).

Cell lysis, solubilization of inner membrane proteins, protein purification, Western blotting and mass spectroscopic analysis. *P. aeruginosa* cultures were grown to an optical density of OD_{600}=1.0. For crosslinking of proteins, formaldehyde was added to the respective cultures at 0.6% final concentration and incubated for 20mins at 37^0C, as described previously for crosslinking inner membrane and cytosolic proteins in Gram-negative bacteria (105). This was followed by harvesting of cells by centrifugation at 10,000 rpm and washing them with Tris-HCl buffer (20mM Tris-HCl, 0.5M Sucrose, pH 8.0). Subsequently, spheroplasts were prepared by incubating the harvested cells in lysozyme (2mg/ ml in 0.1M EDTA, pH 7.5) for 30mins at 4^0C. Spheroplasts thus obtained were harvested by centrifugation at 2700xg for 30mins, resuspended in a lysis buffer composed of 20mM Tris-HCl, pH 8.0 containing the protease inhibitor PMSF at 0.1mM concentration and lysed by either 4 1min pulses of sonication (105, 106) (30% power, alternating 10s on and off) on ice, by using a French pressure cell (107) (courtesy department of Biochemistry, IUSM), or by bead beating (107) (courtesy Dr. Richard Gregory). Unlysed cells and cellular debris were eliminated by centrifugation for 10mins at 10,000xg in a microcentrifuge. To separate cytosolic proteins from those in the inner membrane, ultracentrifugation (100,000xg) (Beckman, rotor type 70Ti) was performed for 30mins. To solubilize proteins from the inner membrane, the pellet containing the membrane fraction from the previous step was resuspended in the lysis buffers containing different concentrations (1%, 2%, 3%, 4%) of the detergent sodium dodecyl maltoside (SDDM) (105) followed by stirring for 1hr at 4^0C. Since 2% SDDM was the most effective in solubilizing MgtE out of the inner membrane (see Results), this concentration was used in subsequent experiments. Following solubilization, the insoluble fraction was separated

from the solubilized membrane proteins by ultracentrifugation (100,000xg) for 30mins, as above. Solubilized MgtE (containing 6x histidine tag) was purified as described previously (104, 105) with a few modifications. The solubilized membrane fraction was treated with anti-His antibody at a final concentration of 5µg/ml and incubated for 1hr at 4^0C with constant stirring. Magnetic Protein G beads were then added to the samples followed by rotation for 2hrs at 4^0C. The beads were then magnetically separated and washed thrice with the lysis buffer containing 0.05% SDDM. SDS-PAGE sample buffer (SDS 20g/L, Tris-base 9.7g/L, b-mercaptoethanol 7.5mL/L, glycerol 100mL/L, bromophenol blue 100mG/L, pH 6.8) was added to the beads thus obtained, followed by heating for 10mins at 70^0C, protein separation by SDS-PAGE and subsequent mass spectroscopic analysis or Western blotting for ExsA or MgtE-6xHis (as described above). For mass spectroscopy, the immunoprecipitates resolved by SDS-PAGE were stained with Imperial Stain (104) (ThermoFisher). Several acrylamide gel regions were excised (courtesy Dr. Michael Edler, Baucum lab, IUPUI), and mass spectroscopy was performed as described previously (104) by Dr. Lisa Jones (Department of Chemical Biology, IUPUI) and analyzed using the annotated genome of *P. aeruginosa* strain PA14 (courtesy Dr. AJ Baucum).

2.3 Results and Discussion

Standardization of the anti-His and anti-ExsA Western blots. Various blocking buffer compositions, incubation times, primary and secondary antibody conditions were tested to standardize the anti-His (Fig 2.2A) and anti-ExsA (Fig 2.2B) immunoblots in our laboratory. The best blocking appeared to be in 5% non-fat dry milk in buffer composed of 150 mM NaCl, 50 mM Tris-HCl pH 7.5 and 0.5% (v/v) Triton X-100 with overnight incubation at 4^0C. The ideal treatments for the anti-His and anti-ExsA Western blots

seemed to be 1:1500 and 1:1000 primary antibody dilutions in blocking buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.5% (v/v) Triton X-100, 5% non-fat dry milk), respectively.

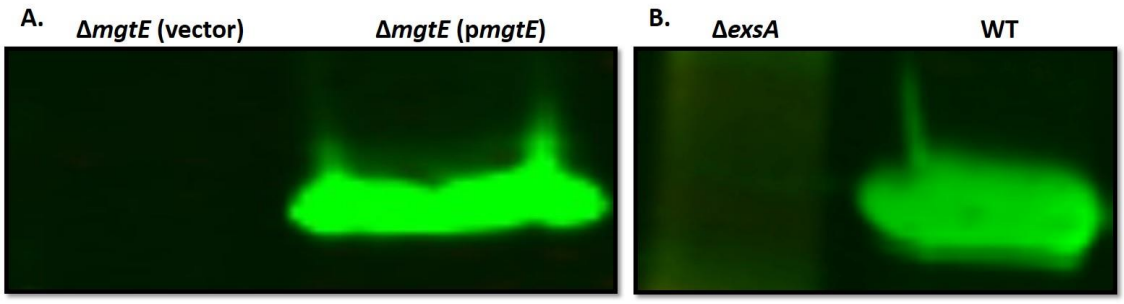

Figure 2.2. Anti-MgtE-6xHis (A) and anti-ExsA (B) immunoblots.
As described in the Materials and Methods, anti-His primary antibody was used to identify MgtE-6xHis (55kDa) (A) and anti-ExsA primary antibody was used to detect ExsA (31.6 kDa) (B). Donkey anti-mouse and donkey anti-rabbit secondary antibodies were used in (A) and (B) respectively.

MgtE expression does not lead to localization of ExsA to the inner membrane. Despite being an inner membrane protein in *P. aeruginosa* (99), MgtE has been reported to have cytosolic domains (108). Thus, we hypothesized that MgtE, by its cytoplasmic domains, directly binds to and sequesters ExsA, thereby preventing it from binding to its target promoters and activating T3SS gene transcription. To test this hypothesis**,** we lysed the cells, separated the membrane fraction from the cytosolic proteins and performed anti-ExsA immunoblot on the membrane lysate to see if ExsA co-localized to the inner membrane under elevated levels of *mgtE* expression (from a plasmid), keeping a Δ*mgtE* mutant as the negative control. As evident, ExsA did not localize to the membrane fraction even high MgtE expression (Fig. 2.3).

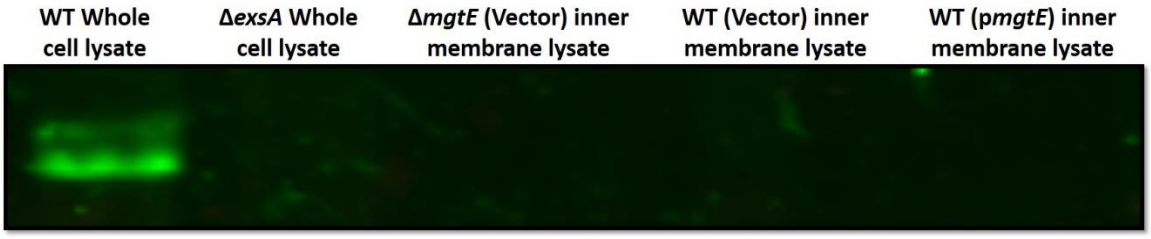

Figure 2.3. Anti-ExsA immunoblot to test for membrane localization of ExsA under *mgtE* overexpression.
Δ*mgtE* strain with empty vector (Vector) and wild type (WT) strains with either vector or p*mgtE* (*mgtE*-6xHis cloned into vector) were cultured, membrane fractions were separated from cytosolic proteins (see Materials and Methods), resuspended in SDS- PAGE sample buffer and immunoblotted for ExsA. Whole cell lysates from the WT and Δ*exsA* strains were used as positive and negative controls respectively.

A major caveat in this experiment is the absence of crosslinking. Also, there is a possibility that MgtE does not tether ExsA directly, but indirectly affects ExsA by binding to some other protein.

Standardization of the detergent concentration for MgtE solubilization out of the inner membrane. We wanted to quantitatively identify any protein(s) that potentially co-immunoprecipitated with MgtE. Thus, we first solubilized MgtE out of the inner membrane using 4 different concentrations of the detergent Sodium Dodecyl Maltoside (SDDM). As shown in Figure 2.4, solubilization using 2% SDDM yielded the best results.

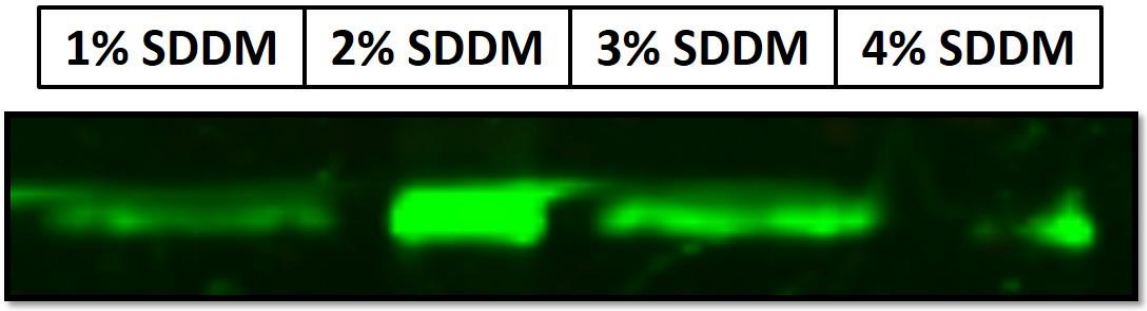

Figure 2.4. Standardization of MgtE solubilization from the inner membrane. MgtE-6xHis was solubilized out of the inner membrane using 4 different concentrations of the detergent SDDM and the solubilized MgtE-6xHis in the supernatant was quantitatively detected using anti-His Western blot.

We then purified MgtE from membrane preparations using an anti-His antibody (as described in the Materials and Methods) and Protein A beads, unconjugated it from the beads by heating, and analyzed the samples by SDS-PAGE to see if any protein(s) co-precipitated with MgtE, keeping the Δ*mgtE* mutant strain as negative control. As seen from Figure 2.5, there are multiple bands that we found in the lanes corresponding to the immunoprecipitated (with MgtE-6xHis) lysate under high expression of MgtE (p*mgtE*) with either formalin crosslinking and non-crosslinking, as compared to that in the Δ*mgtE* mutant. We cut these bands out and analyzed by mass spectroscopy. This analysis indicated that these bands were truncated forms of MgtE. There were a few other proteins that were identified in our study, but the corresponding spectral counts were negligible as compared to those for MgtE.

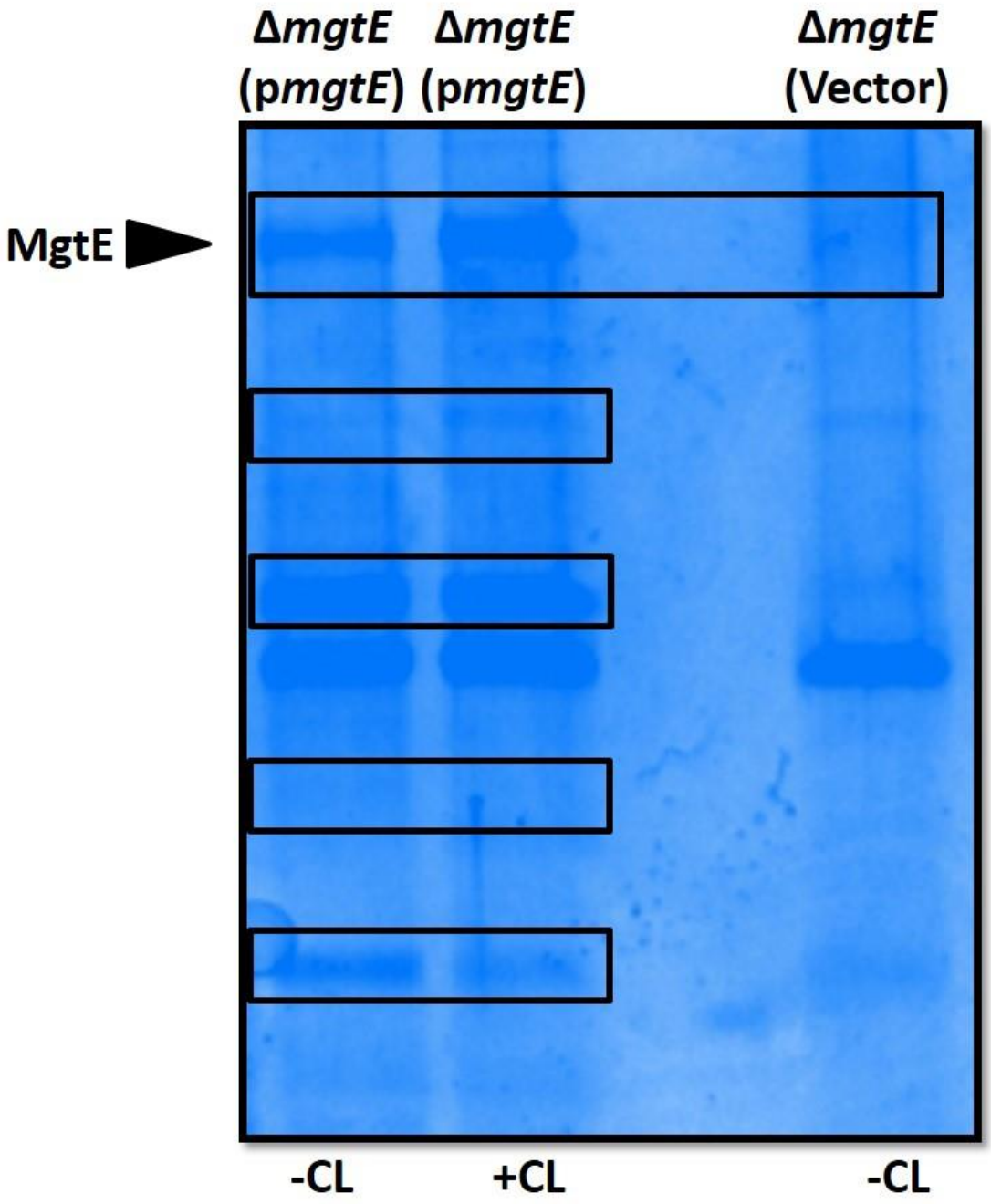

Figure 2.5. SDS-PAGE analysis for selective proteomics of MgtE.
Δ*mgtE* strain with p*mgtE* (*mgtE*-6xHis cloned into vector) was cultured, membrane fractions were separated from cytosolic proteins (see Materials and Methods), solubilized using 2% SDDM and MgtE-6xHis was purified using Protein A beads and the respective immunoprecipitates were analyzed by SDS-PAGE using Imperial stain. –CL and +CL indicates absence and presence of crosslinking by formalin. The 55kDa MgtE protein and the bands excised and sent for mass spectroscopic analysis are highlighted in boxes. The Δ*mgtE* strain with empty vector (Vector) was used as the negative control.

These results suggest that MgtE does not directly bind to ExsA. We recognize two caveats. First, the protein-protein interactions we were looking for might be transient and thus could have been cleaved during the process of MgtE solubilization and purification. Though we used formalin crosslinking, it might not be the most appropriate reagent for our purpose. Second, we analyzed only the prominent bands in the immunoprecipitated eluate, rather than the whole eluate. Thus, proteins that were immunoprecipitated in very low amounts, such that they did not show up as prominent bands on the SDS-PAGE, were not analyzed at all. However, considering the low likelihood of finding a binding interaction, versus the efforts required to optimize additional co-immunoprecipitation experiments, we decided to take a more genetic approach to uncover the mechanisms behind MgtE-mediated regulation of T3SS, as detailed in Chapters 3 and 4.

CHAPTER 3. THE *PSEUDOMONAS AERUGINOSA* MAGNESIUM TRANSPORTER MGTE INHIBITS TYPE III SECRETION SYSTEM GENE EXPRESSION BY STIMULATING *RSMYZ* TRANSCRIPTION

3.1 Introduction

The Gram-negative bacterium *Pseudomonas aeruginosa* is implicated in a wide range of opportunistic infections in humans (109, 110). A major virulence factor used by *P. aeruginosa* to initiate acute infections is a type III secretion system (T3SS) (81, 111). This macromolecular apparatus spans the bacterial cell envelope and acts like a syringe, injecting several toxins directly into host cells (112). This leads to actin cytoskeleton rearrangement, host cell rounding, and cell death (112, 113). These actions promote tissue damage and decrease phagocytic clearance (71, 88, 114). In addition to acute infections, *P. aeruginosa* is also able to establish chronic infections through biofilms formation, most notably in the airways of cystic fibrosis (CF) patients (88, 115). During *P. aeruginosa* biofilm formation, altered gene regulation typically leads to a reduction in T3SS gene expression (88, 116-119). Additionally, isolates from chronically colonized CF patients usually contain mutations that decrease T3SS production (88, 120). Thus, during both acute and chronic infections, *P. aeruginosa* appears to tightly regulate T3SS gene expression in response to environmental conditions.

P. aeruginosa T3SS gene expression is controlled by the master transcription factor ExsA, which is responsible for activating transcription of all T3SS genes, including *exsA* itself by autoregulation (55). Under non-inducing conditions, ExsA is bound by the anti-activator protein ExsD and is unable to bind to its target promoters to initiate gene transcription.

Two other proteins important for T3SS regulation, ExsC and ExsE, form a separate complex. Under inducing conditions (contact of *P. aeruginosa* with host cells, the presence of serum, or low Ca^{2+} conditions), ExsE is secreted through the T3SS apparatus, thus permitting ExsC to sequester ExsD. ExsA, released from ExsD, subsequently activates the T3SS regulon. This mechanism has been referred to as 'intrinsic regulation' (55) (Fig. 2.1).

In addition to the ExsDCE network, several other pathways also control *exsA* expression and/or synthesis (55, 88). These pathways work concurrently but distinctly from 'intrinsic regulation' to further control T3SS gene expression and are referred to as 'extrinsic regulation'. One example of extrinsic regulation is the RsmA/RsmY/RsmZ signaling cascade (Fig. 1.1). RsmA is an RNA binding protein belonging to the CsrA family (121). CsrA family members regulate gene expression at the post-transcriptional level by binding to target mRNAs at conserved sequence motifs and impacting their stability and/or translation (121). RsmA appears to control T3SS gene expression by increasing *exsA* translation through an undetermined mechanism (3). This activity depends upon the concentration of free RsmA in the cell and is controlled by two non-coding RNAs, RsmY and RsmZ (3, 122). RsmY and RsmZ function by directly sequestering RsmA from target mRNA (3, 123-125) and are thus negative regulators of ExsA synthesis. Transcription of *rsmYZ* is directly controlled by the GacAS two-component system (TCS) (3, 121, 126, 127). The environmental signals governing RsmY and RsmZ expression are poorly understood but include two additional sensor kinases, LadS and RetS. Both GacS and LadS are able to phosphorylate the GacA response regulator to enhance *rsmY* and *rsmZ* transcription (128, 129). In contrast, RetS inhibits GacA-mediated *rsmY* and *rsmZ*

transcription by forming a heterodimer with GacS and preventing GacA phosphorylation (130).

Previous studies found that the *P. aeruginosa* inner membrane magnesium transporter MgtE inhibits T3SS gene expression (99). Whereas an *mgtE* mutant demonstrates enhanced T3SS gene expression, *mgtE* overexpression inhibits the T3SS (99). The mechanism by which *mgtE* inhibits the T3SS was not elucidated in these prior studies, although the effect of MgtE on T3SS gene expression is distinct from its role as a Mg^{2+} transporter in *P. aeruginosa* (99). In the current study, we show that *mgtE* expression inhibits ExsA translation by increasing *rsmY* and *rsmZ* transcription. We also demonstrate that *mgtE* acts exclusively through the RsmA/RsmY/RsmZ signaling pathway to inhibit ExsA-mediated T3SS gene transcription. Because *mgtE* transcription is significantly upregulated by growth under low Mg^{2+} conditions and in the presence of some antibiotics (2, 106), this pathway may provide a mechanism for *P. aeruginosa* to modulate T3SS gene expression in response to signals encountered during infections.

3.2 Materials and Methods

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 3.1. Plasmids were maintained in *Escherichia coli* S17 (99) cultured on LB agar plates or LB containing 10 µg/mL gentamicin. Plasmids were isolated from *E. coli* using QIAprep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions and electroporated into appropriate *P. aeruginosa* strains. Transformed *P. aeruginosa* was cultured in Vogel Bonner Minimal (VBM) medium (3, 131) agar plates with 60 µg/ml gentamicin, and the presence of the respective plasmids was confirmed by performing PCR

with primers p729 (5' CAGACCGCTTCTGCGTTCTG 3') and p730 (5' GCAACTCTCTACTGTTTCTCC 3') (2). For beta-galactosidase assays, *P. aeruginosa* strains were cultured overnight on VBM agar plates with gentamicin. Cells were sub-cultured the next day to a starting concentration of OD_{600}=0.1 in Trypticase Soy Broth (TSB) supplemented with 100 mM monosodium glutamate and 1% glycerol (3). 2 mM EGTA was added to the media to induce T3SS gene expression (132, 133).

Table 3.1. Bacterial strains used in this study.

Bacterial Strains	**Relevant Characteristics**	**References**
PA103 P_{exsD}-*lacZ*	WT strain, with the ExsA-dependent P_{exsD}-*lacZ* reporter chromosomally integrated at the CTX site	(3, 81, 115, 134)
UY241	Constitutive transcription of *exsCEBA* in PA103 P_{exsD}-*lacZ* background	(135)
PA103 P_{lacUV5}-*exsCEBA*'-'*lacZ*	P_{lacUV5}-driven *exsA* translational reporter integrated at the CTX site	(3)
PA103 P_{lacUV5}-*exsCEB*'-'*lacZ*	P_{lacUV5}-driven *exsB* translational reporter integrated at the CTX site	(3)
PA103 P_{lacUV5}-*exsCE*'-'*lacZ*	P_{lacUV5}-driven *exsE* translational reporter integrated at the CTX site	(3)
PA103 P_{lacUV5}-*exsC*'-'*lacZ*	P_{lacUV5}-driven *exsC* translational reporter integrated at the CTX site	(3)
PA103 CVS Reporter	P_{lacP1}-*lacZ* reporter for cAMP-Vfr Signaling	(136)

Table 3.1 continued

PA103 P_{exsD}-*lacZ* Δ*vfr*	Isogenic deletion of *vfr* in PA103 P_{exsD}-*lacZ* background	(3)
PA103 P_{exsD}-*lacZ* Δ*algZR*	Isogenic deletions of *algZ* and *algR* in PA103 P_{exsD}-*lacZ* background	(3)
PA103 P_{exsD}-*lacZ* Δ*rsmYZ*	Isogenic deletions of *rsmY* and *rsmZ* in PA103 P_{exsD}-*lacZ* background	(3)
PA103 P_{rsmY}-*lacZ*	*rsmY* transcriptional reporter chromosomally integrated at the CTX site	(3)
PA103 P_{rsmZ}-*lacZ*	*rsmZ* transcriptional reporter chromosomally integrated at the CTX site	(3)
PA103 P_{rsmA}-*lacZ*	*rsmA* transcriptional reporter chromosomally integrated at the CTX site	(3, 137)
PA103 P_{rsmY}-*lacZ* Δ*gacA*	Isogenic deletion of *gacA* in PA103 P_{rsmY}-*lacZ* background	(3)
PA103 P_{rsmY}-*lacZ* Δ*gacS*	Isogenic deletion of *gacS* in PA103 P_{rsmY}-*lacZ* background	(3)
PA103 P_{rsmZ}-*lacZ* Δ*gacA*	Isogenic deletion of *gacA* in PA103 P_{rsmZ}-*lacZ* background	(3)
PA103 P_{rsmZ}-*lacZ* Δ*gacS*	Isogenic deletion of *gacS* in PA103 P_{rsmZ}-*lacZ* background	(3)

Beta-galactosidase assays. *P. aeruginosa* was grown to OD_{600}=1.0 and beta-galactosidase activity was measured as reported earlier (3). *Ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) was used as the substrate for beta-galactosidase in all the beta-galactosidase assays involving transcriptional reporters; chlorophenol red–β-d-galactopyranoside (CPRG) was used as the substrate in assays involving translational reporters (3, 138). Plasmid p*mgtE* (99), and its empty backbone vector pMQ72 (103), were used to assess the effect of *mgtE* expression on transcription and translation.

RNA isolation and quantitative real time PCR (qRT-PCR). *P. aeruginosa* strains were cultured as above for beta-galactosidase assays and harvested at OD_{600}=1.0, whereupon the pellet was washed with phosphate buffered saline (PBS). This was followed by RNA isolation using the RNeasy Plus kit (Qiagen) according to the manufacturer's instructions. A few modifications were made to the protocol, as described earlier (2). Briefly, the RNA was subjected to on-column DNase digestion prior to elution. Additionally, after elution, a second DNase digestion was performed, followed by the RNA Cleanup procedure. These digestions result in negligible DNA contamination of the final isolated RNA sample (2). cDNA was synthesized from the RNA using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer's guidelines (2). DNA contamination of the RNA preparations was tested in control reactions by performing cDNA synthesis in the absence of reverse transcriptase. Quantitative Real Time PCR (qRT-PCR) was performed as previously reported (2) using primers *lacZ*RTfor (5' CAACTGTTTACCTTGTGGAG 3') and *lacZ*RTrev (5' TATGAACGGTCTGGTCTTTG 3'), which bind to a central region in the *exsA-lacZ* transcript. Samples were normalized to

the *fbp* transcript using primers PA5110for (5' CCTACCTGTTGGTCTTCGACCCG 3') and PA5110rev (5' GCTGATGTTGTCGTGGGTGAGG 3') (2, 91, 99, 139).

Statistical analyses. At least three independent experiments were performed for each assay. Two-sample Student's T test was used to determine statistical significance ($p<0.05$).

3.3 Results

MgtE inhibits T3SS gene expression at the post-transcriptional level. Previous studies found that *mgtE* expression inhibits T3SS gene transcription (99). We considered three possibilities to account for the inhibitory effect of *mgtE* expression: 1) reduced *exsA* transcription, 2) reduced ExsA synthesis, and/or 3) impaired ExsA function. Because *exsA* regulates its own transcription (by acting at the P_{exsC} promoter to control transcription of the *exsCEBA* operon) (140), it was necessary to uncouple *exsA* transcription from its own control to analyze potential *mgtE* effects on *exsA* transcription. To this end, we used the previously described *P. aeruginosa* UY241 strain (135), in which the ExsA-dependent P_{exsC} promoter has been replaced with a constitutive variant of the P_{lacUV5} promoter (P_{con}). As a control, P_{exsD}-*lacZ* reporter activity (as a marker for ExsA-dependent transcription) was measured in wild type (WT) PA103 and the UY241 strain following growth under non-inducing (high calcium, –EGTA) and inducing (low calcium, +EGTA) conditions for T3SS gene expression (133, 141, 142). Whereas ExsA is sequestered by ExsD in the WT strain under non-inducing conditions and P_{exsD}-*lacZ* reporter activity is low, EGTA stimulation results in the release of ExsA from ExsD and induction of P_{exsD}-*lacZ* reporter activity (Fig. 3.1A) (143). Strain UY241 also demonstrates EGTA-dependent induction of P_{exsD}-*lacZ* reporter activity, but the overall level of activity is reduced due to the lack of

ExsA autoregulation at the P_{exsC} promoter (Fig. 3.1A). We next expressed *mgtE* in the WT (Fig. 3.1B) and UY241 strains (Fig. 3.1C) and measured P_{exsD}-*lacZ* reporter activity. In both the WT and UY241 strains, *mgtE* expression resulted in a significant reduction in P_{exsD}-*lacZ* reporter activity. These data suggest that MgtE inhibits T3SS gene expression at a post-transcriptional level.

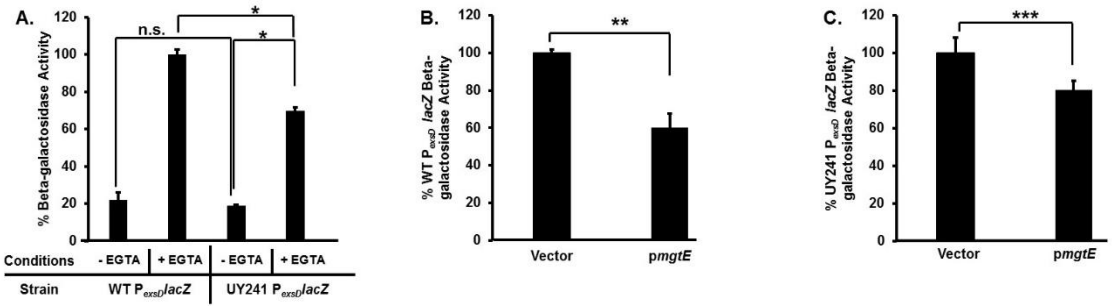

Figure 3.1. *mgtE* inhibits T3SS gene expression at the post-transcriptional level. (A) PA103 P_{exsD}-*lacZ* (WT) and UY241 were assayed either under T3SS non-inducing (–EGTA) or inducing (+EGTA) conditions and assayed for beta-galactosidase activity from the P_{exsD}-*lacZ* reporter construct. Percent activity was calculated considering the P_{exsD}-*lacZ* activity in EGTA-treated WT as 100%. *p<0.0005, n.s.=not significant. (B) PA103 P_{exsD}-*lacZ* (WT) with either vector control or p*mgtE* was assayed under T3SS inducing conditions and beta-galactosidase activity from the P_{exsD}-*lacZ* construct was measured. Percent activity was calculated considering the P_{exsD}-*lacZ* activity in WT with blank vector as 100%. **p<0.05. (C) Strain UY241 with either vector control or *pmgtE* was assayed under T3SS inducing conditions and beta-galactosidase activity from the P_{exsD}-*lacZ* construct was measured. Percent activity was calculated considering the P_{exsD}-*lacZ* activity in UY241 with blank vector as 100%. ***p<0.005.

ExsA translation is repressed by *mgtE* expression. We next tested the hypothesis that *mgtE* expression inhibits ExsA translation. We introduced the *mgtE* expression vector (p*mgtE*) into a panel of WT PA103 strains carrying *lacZ* translational reporters fused to *exsC*, *exsE*, *exsB*, or *exsA* (fused in-frame with *lacZ* at codons 15, 15, 2, and 77, respectively) (3). Each reporter is integrated in single copy on the chromosome at the CTX phage attachment site and transcribed from a constitutive P_{lacUV5} promoter. Whereas plasmid-expressed *mgtE* reduced *exsCEBA*'-'*lacZ* translational reporter activity by almost

3-fold (Fig. 3.2A), *mgtE* had no negative impact on the *exsC*'-'*lacZ* (Fig. 3.2B), *exsCE*'-'*lacZ* (Fig. 3.2C), or *exsCEB*'-'*lacZ* reporter activities (Fig. 3.2D). These data suggest that *mgtE* expression inhibits ExsA translation and that this activity is specific to *exsA* in the *exsCEBA* operon. For reasons that are unclear, the *exsCEB*'-'*lacZ* reporter showed a significant increase upon *mgtE* expression.

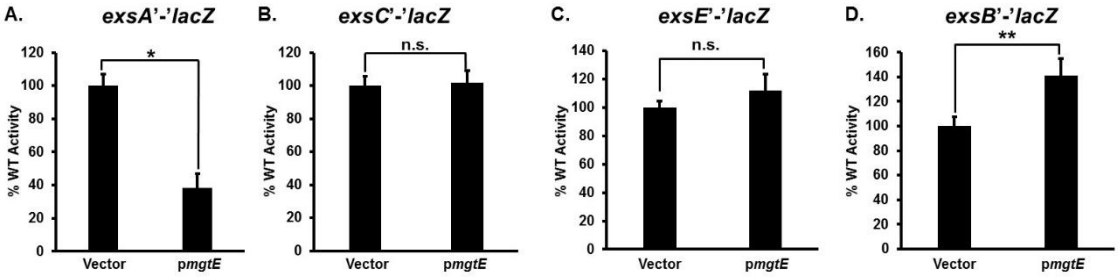

Figure 3.2. *mgtE* expression specifically represses *exsA* translation.
Translational reporter strains of (A) *exsA* (*exsCEBA*'-'*lacZ*), (B) *exsC* (*exsC*'-'*lacZ*), (C) *exsE* (*exsCE*'-'*lacZ*), and (D) *exsB* (*exsCEB*'-'*lacZ*), with either vector control or p*mgtE* were assayed under T3SS inducing (+EGTA) conditions for beta-galactosidase activity. Percent activity was calculated considering the *lacZ* activity from the respective strains with the blank vector as 100%. The reporter constructs were transcribed from a constitutive P_{lacUV5} promoter. *p<0.005, **p<0.05, n.s.=not significant.

The small intergenic region between *exsB* and *exsA* contains a Vfr-dependent promoter (P_{exsA}) (144). Because the P_{exsA} promoter is present in the *exsCEBA*'-'*lacZ* translational reporter, we considered the possibility that *mgtE* inhibits P_{exsA} promoter activity, thus accounting for the observed reduction in *exsCEBA*'-'*lacZ* activity. To investigate this hypothesis, we monitored the effect of *mgtE* expression on a cAMP-Vfr signaling (CVS) reporter (136). The CVS reporter consists of *lacZ* fused to the cAMP- and CRP/Vfr-dependent *lac*P1 promoter from *E. coli*. As shown in Fig. 3.3A, *mgtE* expression had no significant effect on CVS reporter activity. This result is further supported by the finding that *mgtE* expression in a *vfr* mutant still inhibits P_{exsD}-*lacZ* activity (Fig. 3.3B). We infer

from these data that *mgtE* does not alter transcription from the P_{exsA} proximal promoter by acting through *vfr*.

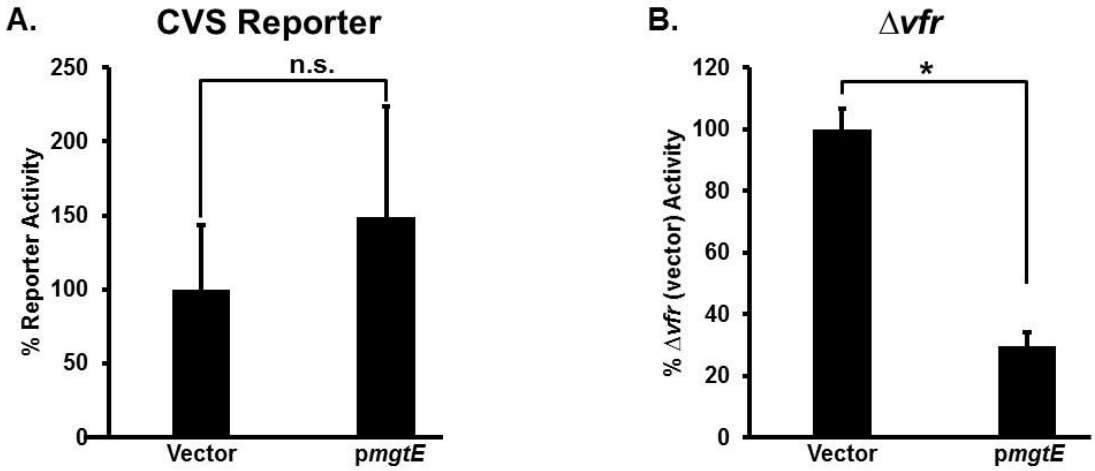

Figure 3.3. The CVS cascade is not required for *mgtE*-mediated inhibition.
(A) *mgtE* expression does not repress CVS reporter activity. PA103 CVS reporter strains with either vector control (Vector) or p*mgtE* were assayed for beta-galactosidase activity from the CVS reporter under T3SS inducing conditions (+EGTA). n.s.=not significant. (B) *mgtE* inhibits T3SS gene transcription in a Δ*vfr* background. Δ*vfr* strains with either vector control (Vector) or p*mgtE* were cultured under T3SS inducing (+EGTA) conditions and assayed for beta-galactosidase activity from the P_{exsD}-*lacZ* reporter construct. Percent activity was calculated considering the P_{exsD}-*lacZ* activity in the Δ*vfr* strain with blank vector as 100%. *p<0.005.

It is also possible that *mgtE* inhibits steady state levels of *exsA* mRNA rather than specifically inhibiting *exsA* translation. To distinguish between these two possibilities, we isolated mRNA from strains carrying the *exsCEBA*'-'*lacZ* translational reporter with either a vector control or p*mgtE* and performed quantitative real time PCR (qRT-PCR) using primer pairs designed for a region central to the *exsA*-*lacZ* transcript. Intriguingly, *mgtE* expression increased *exsA*-*lacZ* transcript levels (Fig. 3.4). This finding suggests that *mgtE* might have positive effects on *exsA* mRNA steady state levels, but more importantly, it strongly suggests that the post-transcriptional inhibition on ExsA synthesis by *mgtE* is due to repression of ExsA translation as opposed to impaired *exsA* mRNA levels.

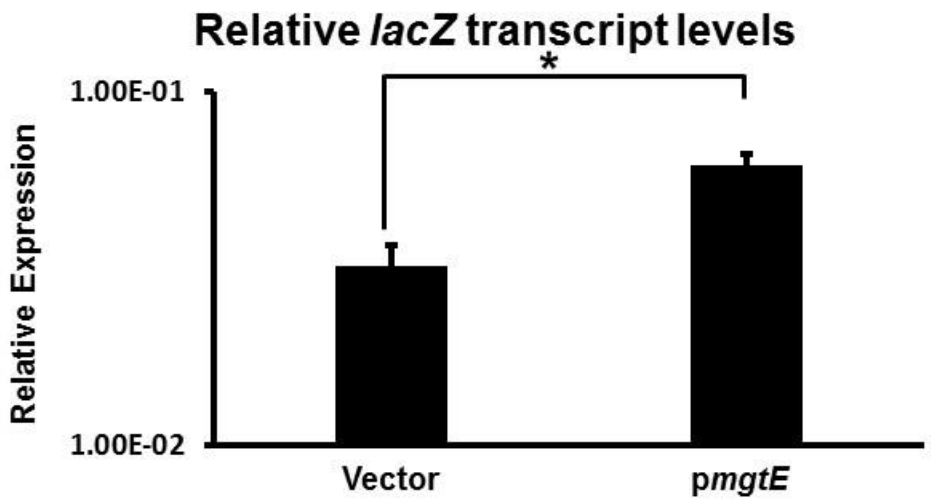

Figure 3.4. *exsA* mRNA remains stable upon *mgtE* expression.
mRNA was isolated from the *exsA*'-*'lacZ* translational reporter strain containing either the vector control or p*mgtE*, and *exsA* transcript stability was analyzed by qRT-PCR. *lacZ* transcript abundance was normalized to that of the control transcript of *fbp*. *p<0.05.

***mgtE* upregulates *rsmY* and *rsmZ* transcription in a GacAS dependent manner.** We next examined possible mechanisms for the *mgtE*-mediated *exsA* inhibition. We reasoned that since *mgtE* is an inner membrane protein (99), its translation repressive effects on *exsA* were likely indirect, possibly by stimulating one of the established signaling pathways that control ExsA translation. For this reason, we investigated whether *mgtE* affected RsmA/RsmY/RsmZ signaling (3). To explore this hypothesis, we measured P_{rsmY}-*lacZ*, P_{rsmZ}-*lacZ*, and P_{rsmA}-*lacZ* transcriptional reporter activity (3) and found that *mgtE* expression significantly upregulates *rsmY* and *rsmZ* transcription by approximately 2.5-fold and 2-fold, respectively (Fig 3.5A-B). Interestingly, *mgtE* expression also stimulated *rsmA* transcription (Fig. 3.5C), but to a smaller degree than measured for *rsmY* and *rsmZ*. Because the GacAS two-component system is essential for *rsmY* and *rsmZ* transcription (17, 18, 23, 24), we hypothesized that the *mgtE* effect requires GacAS. As evident from

Fig. 3.5A-B, *mgtE* expression failed to stimulate P$_{rsmY}$-*lacZ* and P$_{rsmZ}$-*lacZ* reporter activities in the absence of either *gacA* or *gacS*, thus supporting a role for GacAS in *mgtE*-mediated *rsmYZ* regulation.

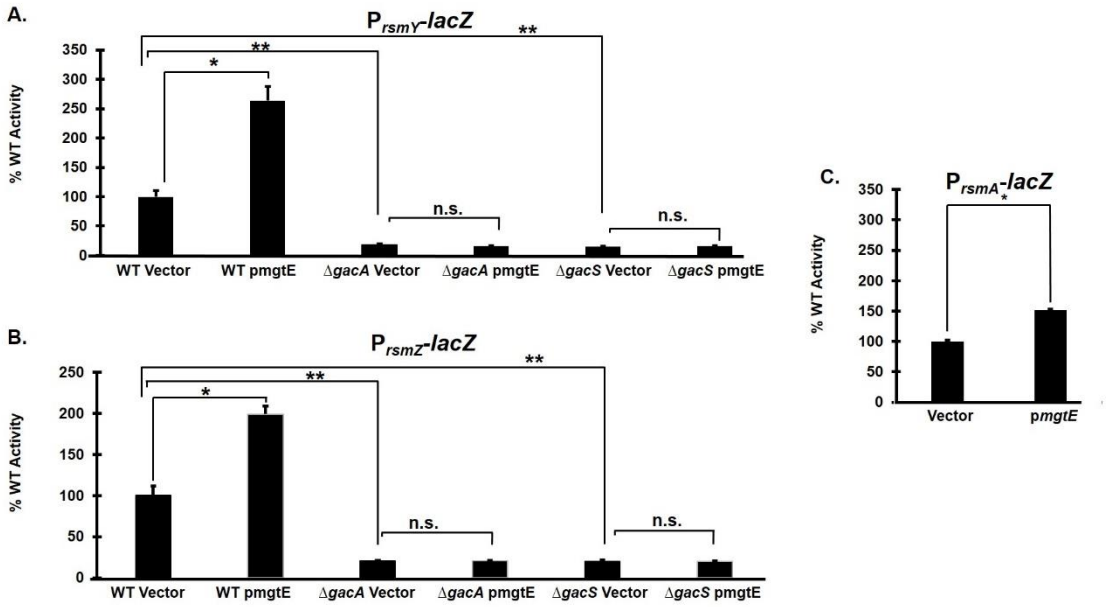

Figure 3.5. *mgtE* expression affects *rsm* signaling by a GacAS-dependent mechanism. Transcriptional reporter strains of (A) *rsmY* (P*rsmY-lacZ*), (B) *rsmZ* (P*rsmZ-lacZ*), and (C) *rsmA* (P*rsmA-lacZ*), in WT, Δ*gacA*, and Δ*gacS* backgrounds with either vector control or p*mgtE* were assayed under T3SS inducing (+EGTA) conditions for beta-galactosidase activity. Percent activity was calculated considering the *lacZ* activity from the respective WT reporter strains with the blank vector as 100%. *p<0.005, **p<0.05.

***mgtE* expression in an *rsmY/rsmZ* mutant fails to inhibit T3SS gene expression.** Since our data suggest that *mgtE* affects RsmA/RsmY/RsmZ signaling to inhibit *exsA* translation, we next tested whether the effect of MgtE functions solely through RsmA/RsmY/RsmZ signaling to inhibit ExsA-dependent transcription by expressing *mgtE* in an *rsmY/rsmZ* double mutant. Consistent with a previous report (55), the *rsmY/rsmZ* mutant demonstrates increased P$_{exsD}$-*lacZ* reporter activity when compared to WT (Fig. 3.6). Whereas *mgtE* expression significantly inhibited P$_{exsD}$-*lacZ* reporter activity in the WT background,

reporter activity was unaffected in the *rsmY/rsmZ* mutant (Fig. 3.6). These data suggest that *mgtE* works solely through the RsmA/RsmY/RsmZ signaling cascade to inhibit *exsA* translation.

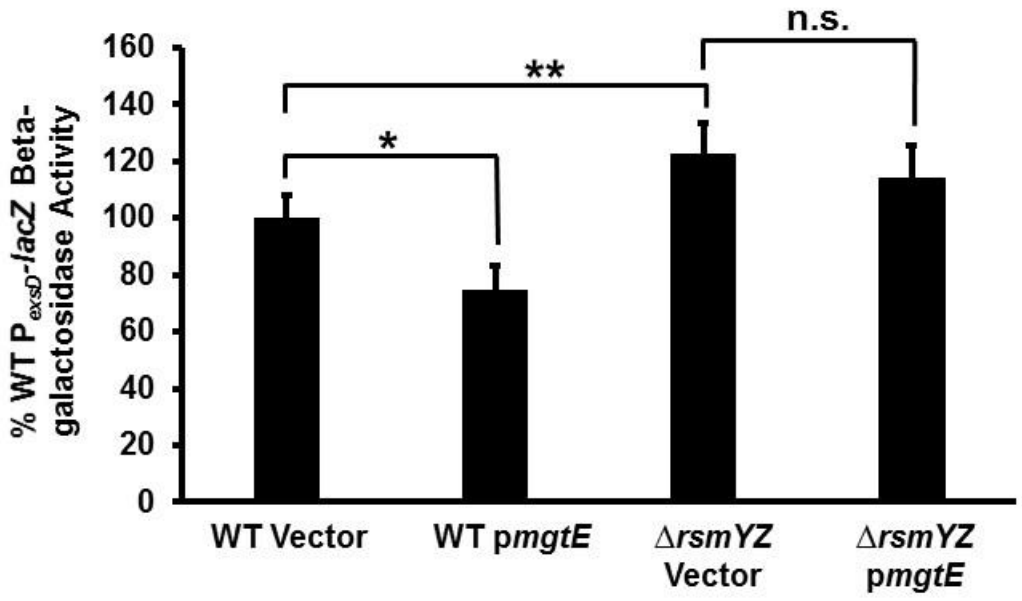

Figure 3.6. RsmYZ are required for MgtE-mediated inhibition of T3SS gene transcription.

PA103 P_{exsD}-*lacZ* and Δ*rsmYZ* P_{exsD}-*lacZ* strains with either vector control or p*mgtE* were assayed under T3SS inducing (+EGTA) conditions for beta-galactosidase activity from the P_{exsD}-*lacZ* reporter construct. Percent activity was calculated considering the P_{exsD}-*lacZ* activity in WT with blank vector as 100%. *p<0.0005, **p<0.005, n.s.=not significant.

3.4 Discussion

The central role of ExsA as the primary regulator of *P. aeruginosa* T3SS makes it an attractive target for therapeutic development (55). Defining signaling networks that control *exsA* expression, synthesis, and activity is critical to realizing that goal. Previous work found that the MgtE magnesium transporter inhibits T3SS gene expression (99). In this study, we demonstrate that MgtE accomplishes this activity by inhibiting *exsA* translation (Fig. 3.7).

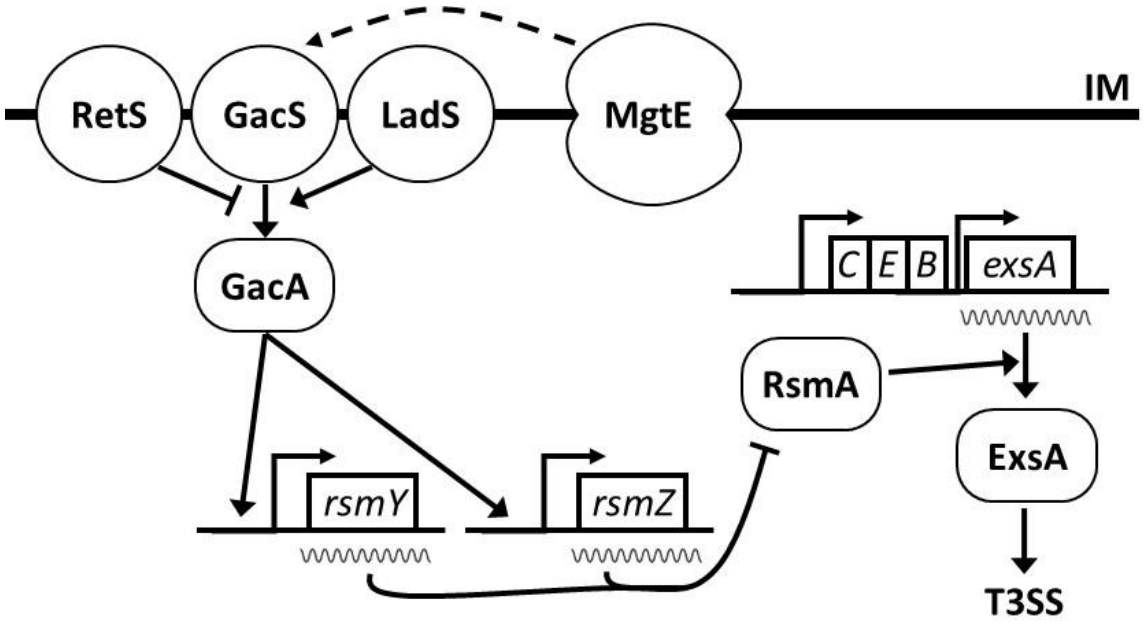

Figure 3.7. A model for *mgtE*-mediated translational repression of *exsA*.
MgtE affects signaling through GacAS, through an unknown mechanism, to stimulate transcription of *rsmY* and *rsmZ*. Increased *rsmY*/*rsmZ* levels, in turn, sequester RsmA, preventing it from enhancing translation of *exsA*, thus inhibiting T3SS.

MgtE-mediated inhibition of *exsA* translation appears to occur through the RsmA/RsmY/RsmZ pathway (Figs. 3.5-3.6). Increased RsmY and RsmZ levels sequester RsmA, resulting in decreased *exsA* translation. Importantly, we found that *mgtE* expression does not decrease *exsA* transcript levels (Fig 3.4), further supporting the conclusion of a specific effect on translation. Additionally, lack of involvement of Vfr in MgtE-mediated T3SS gene transcription inhibition strongly indicates that *exsA* translation inhibition is not the result of decreased transcription activity from the P_{exsA} promoter located on translational fusion constructs (Fig. 3.3). The fact that MgtE does not inhibit other genes in the *exsCEBA* operon (Fig. 3.2) suggests that there may be regulatory sequences specific to *exsA* translational control.

Overexpression of *mgtE* also enhances *rsmA* transcription (Fig. 3.5C), although to a much lesser degree than *rsmY* and *rsmZ* (Fig. 3.5A and B). As was proposed in another study, it is plausible that the net result from increased *rsmA*, *rsmY*, and *rsmZ* transcription is reduced RsmA availability (3). First, RsmA positively regulates *rsmY* and *rsmZ* transcription, which likely plays a role in maintaining homeostasis (3, 145). Second, RsmA binds to its own mRNA to repress translation (146). Therefore, the *mgtE*-dependent increase in *rsmA*, *rsmY*, and *rsmZ* expression could result in reduced RsmA availability and decreased *exsA* translation (see Fig. 3.2).

MgtE lacks helix-turn-helix or other DNA binding motifs (108, 147), which would be needed to directly impact transcription. Therefore, it seems that the positive effect of MgtE on *rsmY* and *rsmZ* transcription is indirect. This is indeed supported by our data that *mgtE* expression fails to affect *rsmY* and *rsmZ* transcription in both a *gacA* and a *gacS* mutant (Fig. 3.5A-B). These results also establish that MgtE influences *rsmY* and *rsmZ* transcription through GacAS. As a membrane protein, MgtE could be involved in direct or indirect binding interactions with GacS, LadS, RetS, or a novel membrane protein that affects signaling through GacS. Because MgtE signals through GacA and GacS, it would be interesting to investigate whether MgtE expression leads to higher phosphorylated states of GacA. It is important to note that RetS was found to regulate biofilm formation in response to magnesium limitation (148), a condition that also enhances *mgtE* transcription (106). Future work will investigate the mechanism by which *mgtE* affects GacAS signaling.

It is noteworthy that *mgtE* transcription is significantly upregulated in low Mg^{2+} and high antibiotic conditions (2, 106), commonly found during host infections like the CF lung environment (149-155). We expressed *mgtE* from a plasmid to simulate the effects of high *mgtE* expression, such as could occur during host infection. Thus, our study describes a mechanism that might allow *P. aeruginosa* to respond to the host environment and optimize T3SS gene expression. Additionally, because MgtE signals through GacAS, our results indicate two environmental signals encountered by *P. aeruginosa* during infection (low Mg^{2+} and high antibiotics during infection) that potentially affect the GacAS signaling pathway. Thus, it is possible that MgtE serves as a sensor, altering T3SS expression in response to changes in the extracellular environment (*i.e.* magnesium levels and antibiotics). Because the effect on *exsA* levels and T3SS gene expression are modest (Figs. 3.1-3.2, 3.6) (99), we suggest that MgtE "fine-tunes" the T3SS response in accordance with the chemical environment, rather than acting as a binary on/off switch.

MgtE is important for the pathogenesis of other microorganisms, such as *Aeromonas hydrophila* and *Campylobacter jejuni* (156, 157). *A. hydrophila*, in particular, has both a T3SS and an RsmA homologue (158, 159). An intriguing avenue of future research will be to investigate whether MgtE homologs in other pathogens inhibit T3SS through a conserved mechanism of action (*i.e.* modulation of RsmA activity). Similar to MgtE in *P. aeruginosa*, the housekeeping Mg^{2+} transporter CorA, found in numerous bacteria, is reported to transport Mg^{2+} and modulate virulence as two distinct functions (160). Future work will investigate whether CorA signaling is similar to that of *P. aeruginosa* MgtE.

Taken together, our current study describes the mechanism by which MgtE inhibits T3SS gene transcription in *P. aeruginosa*. This signaling cascade is one mechanism used by *P. aeruginosa* to respond to Mg^{2+} scarcity and high antibiotic conditions. Additional characterization of upstream events of this signaling cascade (*i.e.* how MgtE affects GacAS signal transduction) would further increase our understanding of the mechanism used by *P. aeruginosa* to orchestrate signaling pathways in response to the host environment.

CHAPTER 4. *MGTE* AND *ALGR* GENETICALLY INTERACT TO AFFECT THE T3SS IN *PSEUDOMONAS AERUGINOSA*

This chapter in part, has been published in reference (2). Required permission has been obtained.

4.1 Introduction

Pseudomonas aeruginosa possesses an intricate network of signaling pathways to regulate cytotoxicity. Typically, cytotoxicity is downregulated in chronic *P. aeruginosa* infections, wherein they form antibiotic resistant biofilms, as in the CF airways (3). The gold standard antibiotic used to treat *P. aeruginosa* biofilm infections in the CF lungs is tobramycin (161), which still fails to completely eradicate the pathogen, because of inherent antibiotic resistance mechanisms and robust transcription of stress response genes (162). In a previous study, to identify the genes in *P. aeruginosa* biofilms which are upregulated upon tobramycin treatment, a co-culture of *P. aeruginosa* biofilms growing on CFBE cells was treated with physiologically relevant levels of tobramycin, followed by microarray analysis (91). One of the major genes in *P. aeruginosa* that was found to be transcriptionally upregulated was the inner membrane Mg^{2+} transporter gene *mgtE*. Additionally, as mentioned in Chapters 2 and 3, *mgtE* was found to inhibit cytotoxicity in *P. aeruginosa*. A follow up study by our lab revealed that, in *P. aeruginosa* biofilms, several other antibiotics (in addition to tobramycin) enhance *mgtE* transcription (2). This indicates that *mgtE* might be involved in stress response pathways in *P. aeruginosa*.

A transposon mutagenesis screen, to elucidate the pathways through which *mgtE* might be signaling, revealed that the *P. aeruginosa* gene *algR* abrogated *mgtE* mediated inhibition

of cytotoxicity (Fig 1.1) (2). This indicates a genetic crosstalk between *algR* and *mgtE*. AlgR has pleiotropic effects in *P. aeruginosa* (163). It is a response regulator involved in production of the biofilm polysaccharide alginate by binding to the *algD* promoter and activating transcription (164). AlgR also activates various stress response cascades. Importantly, *algR* is responsive to several antibiotics, including tobramycin (91), imipenem (165) and numerous cell wall-active antibiotics (166). Additionally, it inhibits cytotoxicity in *P. aeruginosa* by at least two different mechanisms (3).

In this current study, I have shown that *mgtE* may react particularly to antibiotics, among stress conditions. Also, in spite of enhanced transcription under antibiotic pressure, *mgtE* does not confer antibiotic resistance to *P. aeruginosa*. Further, I have attempted to characterize the genetic crosstalk between *mgtE* and *algR* in *P. aeruginosa*. Specifically, I have established that a Δ*mgtE*/Δ*algR* double mutant inhibits cytotoxicity in *P. aeruginosa* (Fig 1.1) due to inhibition of T3SS gene transcription. My data also reveals that the *algR*-*mgtE* genetic interaction is specifically targeted towards regulation of the T3SS in *P. aeruginosa*, rather than indiscriminately affecting other virulence phenotypes. To further elucidate the interaction between *mgtE* and *algR*, I demonstrate that *algR* inhibits *mgtE* transcription, but not vice versa. Taken together, this study sheds more light on the complicated signaling networks that govern biofilm formation, cytotoxicity and stress tolerance in *P. aeruginosa*.

4.2 Materials and Methods

Strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 4.1. Primers used in this study are listed in Table 4.2.

Table 4.1. Strains and plasmids used in this study

Strain or plasmid	**Relevant genotype or description**	**Reference or source**
PA14 WT	*P. aeruginosa* burn wound isolate	(167)
SMC3604	PA14 Δ*mgtE*	(91)
GGA225	PA14 Δ*algR*	This study
GGA226	PA14 : : P_{mgtE}–*lacZ*	This study
GGA231	PA14 Δ*mgtE* Δ*algR*	This study
SC100	PA14 : : P_{algR}–*lacZ*	This study
SC101	SMC3604 : : P_{algR}–*lacZ*	This study
SC102	GGA225 : : P_{mgtE}–*lacZ*	This study
SMC4405	PA14 : : P_{exsD}–*lacZ*	(91)
SMC4406	SMC3604 : : P_{exsD}–*lacZ*	(91)
PA14 P_{exsA}-*lacZ*	PA14 WT having P_{exsA}-*lacZ* reporter chromosomally integrated at the CTX site	This study

Table 4.1 continued

PA14 P_{exsA}-*lacZ* Δ*mgtE* Δ*algR*	Isogenic deletion of *mgtE* and *algR* in PA14 P_{exsA}-*lacZ* background	This study
S17-1	*Escherichia coli* laboratory strain for cloning	(91)
Saccharomyces cerevisiae	InvSc1: *In vivo* cloning; ura3-52/ura3-52	Invitrogen
pMQ30	Allelic replacement vector, yeast cloning	(168)
pMQ70	P_{BAD} expression vector; Cb	(168)
pMQ72	P_{BAD} expression vector; Gm	(168)
pSMC233	*mgtE* deletion; pMQ30 backbone; Gm	(91)
pSMC233-Amp	*mgtE* deletion; pMQ30 backbone; Amp	This study
pSMC291	*mgtE* complementation; pMQ70 backbone	(91)
pSC1	*algR* deletion; pMQ30 backbone; Gm	This study
pMQ30-lacZ	Promoterless *lacZ* in pMQ30; Gm	This study
pGA233	pMQ30-P_{mgtE}–*lacZ*; Gm	This study
pSC2	pMQ30-P_{algR}–*lacZ*; Gm	This study

Table 4.2. Primers used in this study

Primer	**Sequence (5'– 3')**
913for	CCCATGGACTTACCCAGTAG
913rev	CCGTCGACGAGTATTTCGTC
p729	CAGACCGCTTCTGCGTTCTG
p730	GCAACTCTCTACTGTTTCTCC

Table 4.2 continued

30AMPfor	TAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATG A TGAGCACTTTTAAAGT
30AMPrev	AATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTA C CAATGCTTAATCAGTG
AlgRKO#1	TCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGTTCCCTGG ACCTGTCCGACCTGTT
AlgRKO#2	CATGCAGGAAGCCTGAGCTTCACTCGGGTTCGAACTGTTA
AlgRKO#3	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGT A GGTGTTCGAGACGAAAG
AlgRKO#4	TAACAGTTCGAACCCGAGTGAAGCTCAGGCTTCCTGCATG
AlgRKOUP	CTTATCTATGGCATCCAGCC
AlgRKODOWN	CTTGACGAACAGGCCCTTGC
LacZfor	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTAA GATCCAAATGACCATGATTACGGATTC
LacZrev	AGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATTTAT T TTTGACACCAGACCA
MgtEPromLacZfor	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGC C TTTTTCGTCTTCGTCTC
MgtEPromLacZrev	GTTGTAAAACGACGGCCAGTGAATCCGTAATCATGGTCATAG C GCGCTCCACCCCCAGTA

Table 4.2 continued

AlgRPromFor	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGC TTGT TCATGTGCACGTCTTCC
AlgRPromLacRev	GTTGTAAAACGACGGCCAGTGAATCCGTAATCATGGTCA TCGA CAGAGTTTCCGCAAGGC

Genetic manipulations. *Construction of isogenic deletion mutants.* Isogenic deletion mutants were created as described previously elsewhere (91, 168). For deleting the *algR* gene, *algR* flanking regions were amplified by PCR with primer pairs AlgRKO#1/AlgRKO#2 and AlgRKO#3/AlgRKO#4, and the resultant PCR fragments were joined to suicide vector pMQ30 via homologous recombination in *Saccharomyces cerevisiae* (168). This deletion vector, pSC1, was then electroporated into *Escherichia coli* S17-1 cells and plasmid construction was confirmed by restriction digestion. The deletion vector harbored by S17-1 transformants was transferred by conjugation into target *P. aeruginosa* strains. Exconjugants were isolated on selective agar plates (either 50 mg gentamicin/ ml and 30 mg nalidixic acid/ ml or 250 mg carbenicillin/ ml and 30 mg nalidixic acid/ ml). Following the selection, the exconjugants were grown overnight in LB, and spontaneous excision of the vector was selected for by plating on 10% sucrose/LB plates. Mutations were confirmed by PCR, using primer pair AlgRKOUP/AlgRKODOWN, and sequencing. For deletion of *mgtE*, we started with the plasmid pSMC233, which has a gentamicin selective cassette (91). However, because the transposon mutants that we used already have gentamicin resistance, we generated pSMC233-AMP, which is pSMC233 with a carbenicillin gene cassette replacing the gentamicin cassette. This was accomplished by PCR-amplifying the bla gene from pMQ70 using primer pair 30AMPfor/30AMPrev.

The resultant PCR fragment was mixed with BglII-digested pSMC233, and, using the *S. cerevisiae* homologous recombination technique (168), the *bla* gene replaced the *aacC1* gene on pSMC233, thus creating pSMC233-AMP. Deletions of *mgtE* were confirmed via PCR using the primers 913for and 913rev.

Transcriptional fusions with lacZ. The P_{exsD}–*lacZ* reporter constructs were reported earlier (141). We created *algR* and *mgtE* isogenic deletions in strains carrying the P*exsD–lacZ* construct (99) as described above. To create strains with P_{algR}–*lacZ* and P_{mgtE}–*lacZ* transcriptional fusions, we first generated plasmid pMQ30-*lacZ*, which carries a promoterless *lacZ* gene on plasmid pMQ30, which is a suicide vector in *P. aeruginosa*. This was accomplished by amplifying the *lacZ* gene from *E. coli* S17-1 using primers LacZfor and LacZrev. This PCR fragment was recombined with BamHI-digested pMQ30 via homologous recombination in *S. cerevisiae*, as above. Next, the putative promoter regions of *mgtE* and *algR* were PCR amplified using primers MgtEPromLacZfor and MgtEPromLacZrev, or AlgRPromFor and AlgRPromLacRev. These PCR fragments were recombined with BamHI-digested pMQ30-*lacZ* via homologous recombination in *S. cerevisiae*, creating plasmids pGA233 (P_{mgtE}–*lacZ*) and pSC2 (P_{algR}–*lacZ*). These plasmids were then electroporated into *E. coli* S17-1 cells and plasmid constructs were confirmed via restriction digestion. Plasmids were transferred to *P. aeruginosa* strains by conjugation, and exconjugants were identified via selective agar plates (LB with 50 mg gentamicin/ ml and 30 mg nalidixic acid/ ml). These exconjugants contained a single crossover insertion of the plasmid at the *mgtE* or *algR* native sites (strains GGA226 and SC100 in the PA14 background, and strains SC102 and SC101 in the Δ*algR* or Δ*mgtE* backgrounds,

respectively), which positioned the promoterless *lacZ* gene immediately downstream of the *mgtE* or *algR* promoters. The pMQ30 plasmid contains strong transcriptional terminators downstream of the *lacZ* gene (168).

Complementation of strains. The plasmid pSMC291 contains full length *mgtE*, and it has been previously described (99).

Beta-Galactosidase assays. The Miller assay was carried out as we have previously performed (99) with a few modifications. Overnight cultures of bacterial strains were subcultured 1:100 into LB with 2 mM EGTA (to chelate calcium for enhancing T3SS stimulation), and these subcultures were grown until mid-exponential phase (OD_{600}~0.5). Subsequently, the cultures were diluted in appropriate volumes of Z buffer [16.1 g Na_2HPO_4. $7H_2O$/ l, 5.5 g $NaH_2PO_4.4H_2O$/ l, 0.75 g KCl/ l, 0.246 g $MgSO_4.7H_2O$/ l, 2.7 ml beta-mercaptoethanol (pH 7.0)], and then 40 ml chloroform and 10 ml 0.1% SDS were added to each of the aliquots. This solution was vortexed for 10 s and incubated at 30^0C for 5 min. A total of 200 ml (4 mg/ml) ONPG was added for 30 min at 30^0C, and then 0.5 ml 1 M Na_2CO_3 was added to stop the reaction. Expression of *lacZ* was determined by the standard Miller unit equation (Miller Units=1000*(OD_{600}/ (time in mins*volume of culture*OD_{600})) wherein the data were normalized to the OD_{600} value of the bacterial culture, as we have previously calculated (99). Chlorophenol red–β-d-galactopyranoside (CPRG) was used as the substrate in assays involving the P_{exsA}-*lacZ* transcriptional reporter (144). Data are representative of three independent experiments. For the beta-galactosidase

assay of the P_{mgtE}–*lacZ* and P_{algR}–*lacZ* strains, the same procedure as above was followed, except that EGTA was omitted.

For testing of stresses on the P_{mgtE}–*lacZ* construct, we subcultured WT *P. aeruginosa* from overnight cultures into 1.5 ml fresh LB in 24-well plates. Plates were incubated for 12 h, statically, and then bacteria were subjected to the indicated stresses for 30 min. The M63 salts consisted of 15 g KH2PO4/ l, 35 g K2HPO4/ l, 10 g (NH4)2SO4/ l in water. Beta-galactosidase assays from co-culture biofilms were performed as previously described (99).

Twitching motility. Twitching-motility assays were performed by dipping a pipette tip into overnight cultures of the indicated bacterial strain and then stabbing the tip to the bottom of an LB agar plate, as described elsewhere (169). Plates were incubated at 37^0C for 24 h, then for an additional 24 h at room temperature. Then, the agar was removed, and the plate was stained with 0.1% crystal violet for 10 min. Twitching zone sizes were determined by measuring three diameters for each zone and averaging. Each strain was tested in three independent experiments with three to six replicates each.

Rhamnolipid production. To detect rhamnolipids, we prepared M8 medium supplemented with 1 mM MgSO4, 0.2% glucose, 0.5% casamino acids, 0.2% cetyltrimethylammonium bromide (CTAB), 0.0005% methylene blue, and 1.5% agar, as described elsewhere (170). Overnight culture of each strain (5 ml) was spotted on the surface of the agar and the plates were incubated at 37^0C for 24 h, then for an additional 24 h at room temperature. Secreted rhamnolipid is precipitated by the CTAB and thus

forms a halo around each colony, with the diameter related to the amount of rhamnolipid produced. Diameters were measured as above for twitching. Each strain was tested in three independent experiments with three to six replicates each.

Pyocyanin production. Pyocyanin production was assessed by chloroform extraction of filter-sterilized overnight culture supernatants, as previously described (91). OD_{376} was measured in a SpectraMax M2 spectrophotometer as a measure of relative pyocyanin levels. Each strain was tested in three independent experiments with three to four replicates each.

AlgR biochemical studies. AlgRD54E, a phosphomimetic form of AlgR (164) was used to test whether AlgR directly binds to the mgtE promoter *in vitro*. Expression and purification of AlgRD54E was previously described (164). For the electrophoretic mobility shift assay (EMSA), the previously described protocol was used (164). For the assay, 6 different concentrations of purified AlgRD54E were used (0.15 µM, 0.3 µM, 0.6 µM, 1.25 µM, 5 µM and 10µM). The test oligonucleotide encompassed the *mgtE* promoter region containing the putative AlgR binding site (shown in bold: 5'-CAGTTTC**CCGTTTGAC**CAATCAC-3'). The *algD* promoter was used as positive control (5'-TGGCGCTA**CCGTTCGTC**CCTCCGA-3', AlgR binding site shown in bold. These experiments were performed by Dr. Michael Schurr, University of Colorado.

Statistical analysis. Statistical significance was determined via a P value <0.05 using a two-tailed Student's t-test with Sidak adjustment for multiple comparisons.

4.3 Results

MICs are unaffected by changes in magnesium concentration. It is possible that increased expression of *mgtE* could affect antibiotic resistance. Because MgtE is a magnesium transporter, and because magnesium limitation increases *mgtE*expression (171), we wondered whether incubation in decreased magnesium levels resulted in greater antibiotic resistance. We assayed the MIC of tobramycin, gentamicin, ciprofloxacin and imipenem, as representatives of the three modes of action of the antibiotics that were tested previously (2) (protein synthesis inhibition, replication inhibition, cell wall inhibition). We found that the MIC remained the same (Table 4.3), despite growth in magnesium concentrations that stimulate (0.1 mM) or repress (1 mM) *mgtE* transcription (171). Combined with our previous observation that antibiotic sensitivity was unaffected by isogenic deletion of *mgtE* (91), it seems that *mgtE* expression exerts only a minor role, if any, on antibiotic resistance. The antibiotic-resistance phenotype is likely multifactorial.

Table 4.3. Minimum Inhibitory Concentrations of *P. aeruginosa* in Different Magnesium Levels

	Tobramycin	Gentamicin	Ciprofloxacin	Imipenem
1 mM Mg^{2+}	3	4	0.5	1.5
0.1 mM Mg^{2}	3	4	0.5	1.5
0.01 mM Mg^{2}	3	4	0.5	1.5

Effect of various stresses other than antibiotics on *mgtE* transcription. Since *mgtE* transcription was upregulated in antibiotic treated *P. aeruginosa* biofilms, we tested whether *mgtE* transcription upregulation was part of a general stress response pathway in *P. aeruginosa*, or whether the response was largely restricted to antibiotic pressure. To

this end, we constructed an *mgtE* transcriptional reporter in *P. aeruginosa* by fusing a *lacZ* gene downstream of the proposed *mgtE* promoter. This reporter strain was grown under various stress conditions (Table 4.4) and *mgtE* transcription was measured by beta-galactosidase activity. Treatment with 5X M63 salts resulted in increased beta galactosidase activity, while 20% glucose and growth at 55^0Cinhibited transcription. Activity after treatment with 10% ethanol, 100 mM KCl, and 100 mM $MgSO_4$ remained unchanged compared to untreated controls. This data indicates that *mgtE* transcription is upregulated only under very specific types of stresses and may not be part of a generalized stress response pathway in *P. aeruginosa*.

Table 4.4. Activity of P_{mgtE}-*lacZ* under various stresses. Values represent average of at least 3 independent experiments (+/- standard deviation). ns= not significant, compared to untreated

Stress Condition	**Miller Units**	**Significance**
Untreated	469 (+/-109)	
10% Ethanol	460 (+/-66)	**ns**
100 mM KCl	543 (+/-98)	**ns**
100 mM MgSO4	460 (+/-142)	**ns**
20% Glucose	244 (+/-81)	$p<0.05$
5X M63 Salts	870 (+/-183)	$p<0.05$
55^0C	176 (+/-92)	$p<0.05$

algR* genetically interact with *mgtE* to affect T3SS gene transcription in *P. aeruginosa. A previous study from our lab showed that *algR* abrogates the cytotoxicity phenotype of *mgtE* (Fig. 1.2) (2). To investigate the mechanism behind this observation, we tested

whether this effect on cytotoxicity is mediated through effects on T3SS gene transcription; cytotoxicity in *P. aeruginosa*, in large part, is due to the T3SS (91). For this experiment, we used the previously described P_{exsD}-*lacZ* T3SS transcriptional reporter in *P. aeruginosa* (see Chapter 3) and assayed the effects of the WT, Δ*mgtE*, Δ*algR* and Δ*mgtE*/Δ*algR* on T3SS gene transcription. As evident from Fig 4.1, mutation in *algR* disrupts the enhancing effect of the *mgtE* mutant on T3SS gene transcription, indicating that the effect of the Δ*mgtE*/Δ*algR* double mutant on cytotoxicity in *P. aeruginosa* is likely mediated through effects on T3SS gene transcription. To further elucidate the nature of the *algR*-*mgtE* genetic crosstalk, we tested whether expression of *mgtE* in a Δ*algZR* mutant can inhibit T3SS gene transcription, like that in the WT *P. aeruginosa* (Chapter 3). *mgtE* still significantly reduced P_{exsD}-*lacZ* reporter values even in the Δ*algZR* mutant (Fig 4.2), indicating complex genetic interaction between *mgtE* and *algR*.

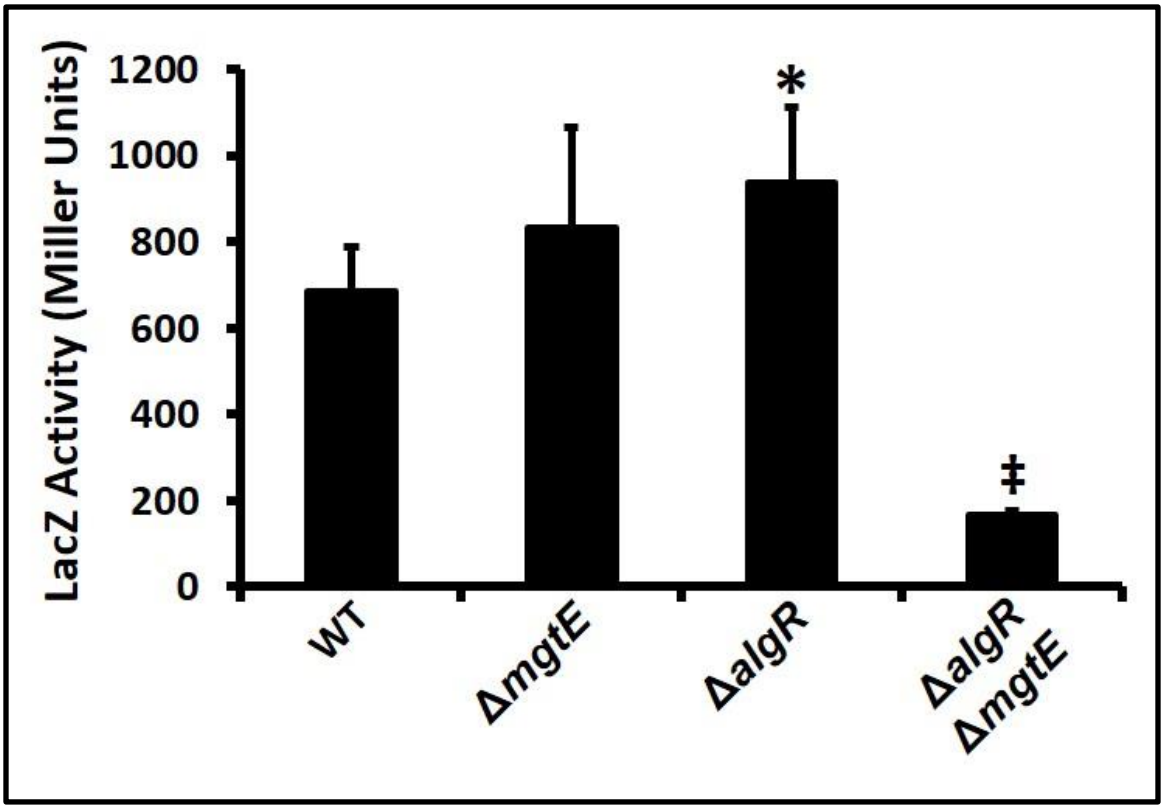

Figure 4.1. Mutation in *algR* disrupts the enhancing effect of the *mgtE* mutant on T3SS gene transcription.

For examining the T3SS promoter activity in the PA14 strain under different mutant conditions, beta-galactosidase activity (Miller units) was measured from the P_{exsD}–*lacZ* construct in the WT, Δ*mgtE*, Δ*algR* and Δ*mgtE*/Δ*algR* strains grown as planktonic, broth cultures. The data are representative of five independent experiments. *p<0.05, compared to WT; #p<,0.05, compared to all other strains.

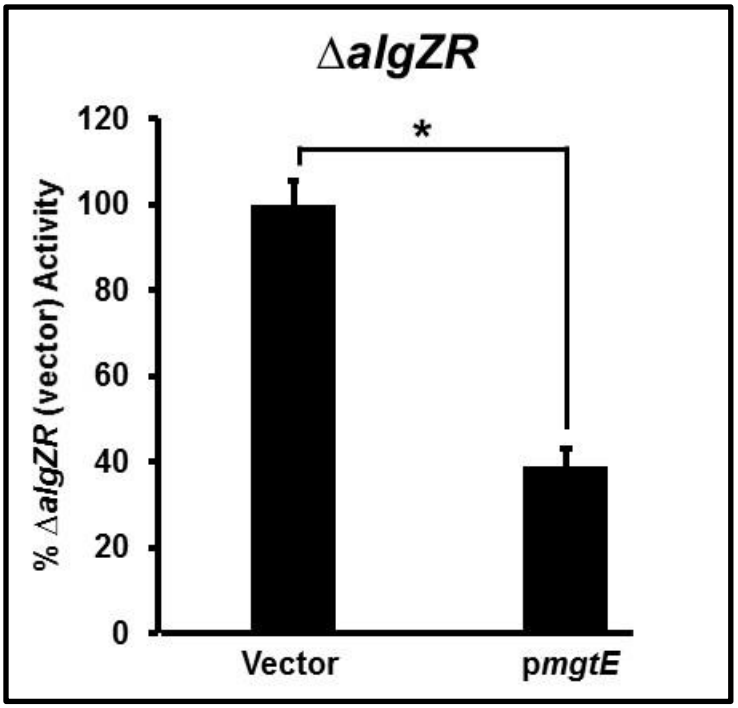

Figure 4.2. *mgtE* inhibits T3SS gene transcription in a Δ*algZR* background. Δ*algZR* strains with either vector control or p*mgtE* were assayed under T3SS inducing (+EGTA) conditions for beta-galactosidase activity from the P_{exsD}-*lacZ* reporter construct. Percent activity was calculated considering the P_{exsD}-*lacZ* activity in the Δ*algZR* strain with blank vector as 100%. *p<0.0005.

The Δ*mgtE*/Δ*algR* mutation does not inhibit transcription from the proximal P_{exsA} promoter. As mentioned in Chapter 3, ExsA is transcribed from a polycistronic operon and it regulates its own expression from the P_{exsC} promoter. However, there is a small proximal promoter P_{exsA} immediately upstream of the *exsA* gene that is independent of ExsA. Since the Δ*mgtE*/Δ*algR* mutation inhibits T3SS gene transcription, one possibility is that this double mutation inhibits *exsA* transcription from the proximal promoter P_{exsA}. To test this possibility, we assayed whether the Δ*mgtE*/Δ*algR* mutation reduced *lacZ* expression from the P_{exsA}-*lacZ* transcriptional reporter, as compared to what is seen in the WT. As shown in Fig. 4.3, the Δ*mgtE*/Δ*algR* mutation does not inhibit *exsA* transcription from the P_{exsA} promoter.

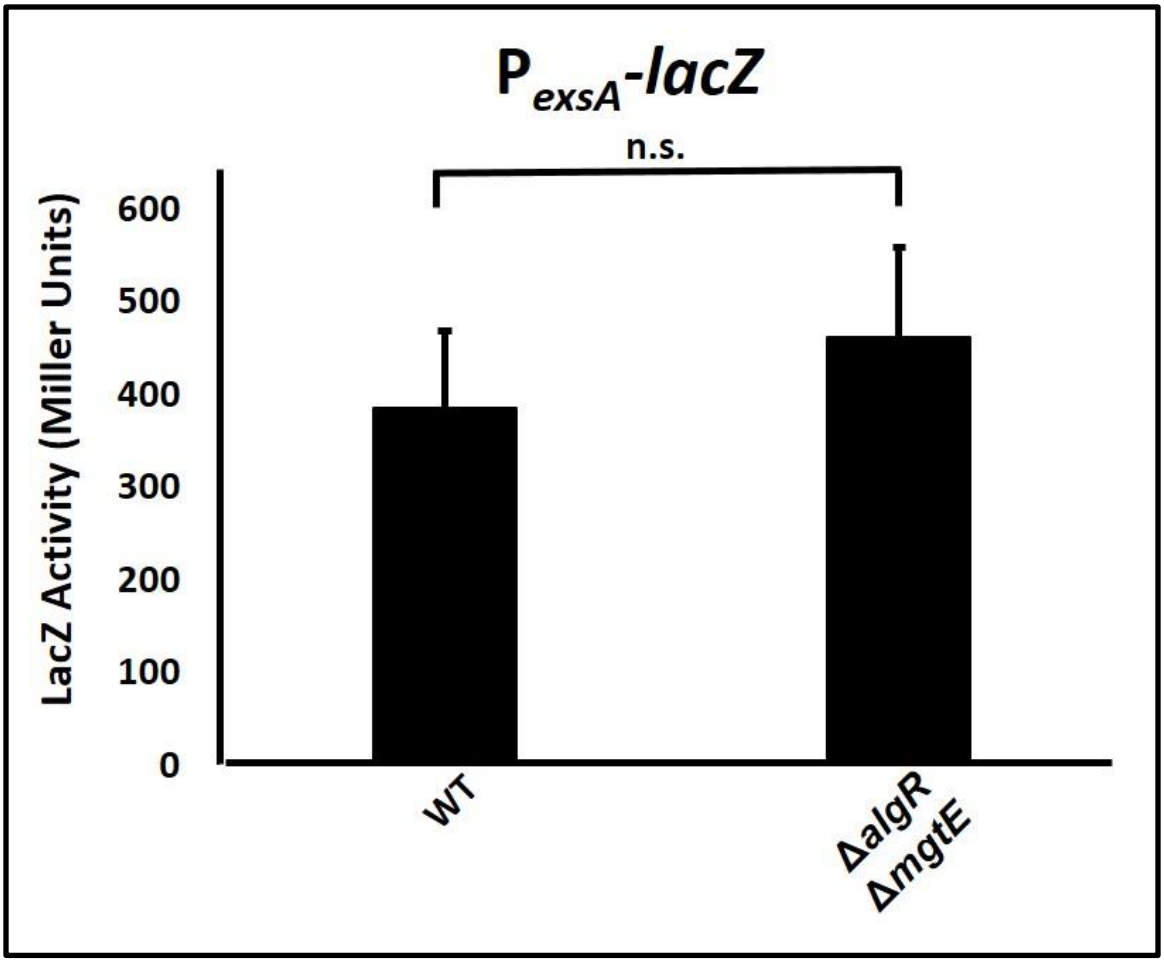

Figure 4.3. Δ*mgtE*/Δ*algR* does not inhibit *exsA* transcription from the P_{exsA} promoter. WT and ΔmgtE/ΔalgR mutant strains were assayed under T3SS inducing (+EGTA) conditions for beta-galactosidase activity (expressed as Miller units) from the P_{exsA}-*lacZ* reporter construct. n.s.=not significant.

***algR* is an inhibitor of *mgtE* transcription.** To further analyze the relationship between MgtE and AlgR, we investigated whether mutation of one gene led to altered expression of the other. Toward that end, we generated *lacZ* transcriptional fusions to the putative promoters of *mgtE* and *algR*, as described in Material and Methods, and measured beta-galactosidase production in WT and mutant strains. In this manner, we found little difference in *algR* promoter activity between WT and Δ*mgtE* strains (Fig. 4.4A). However, transcription from the *mgtE* promoter was greatly enhanced by mutation of *algR* compared

to the WT strain (Fig. 4.4B). Interestingly, *in silico* analysis found a putative AlgR binding sequence around 65 bases upstream of the *mgtE* start codon (Fig 4.5A). To further investigate whether AlgR represses *mgtE* transcription by directly binding to the *mgtE* promoter region, electrophoretic mobility shift assay (EMSA) was performed (courtesy Dr. Michael Schurr, University of Colorado) using a phosphomimetic AlgR (AlgRD54E) which has a high affinity for DNA binding and the oligonucleotide 5'-CAGTTTC**CCGTTTGAC**CAATCAC-3', which represents the segment of the *mgtE* promoter region containing the putative AlgR binding sequence (Fig. 4.5B). The AlgR binding site in the *algD* promoter region (164) was used as the positive control (Fig. 4.5C). There appears to be a faint bandshift when 10μM AlgRD54E is used (Fig. 4.5B), indicating that AlgR directly binds, albeit weakly, to the *mgtE* promoter region *in vitro*.

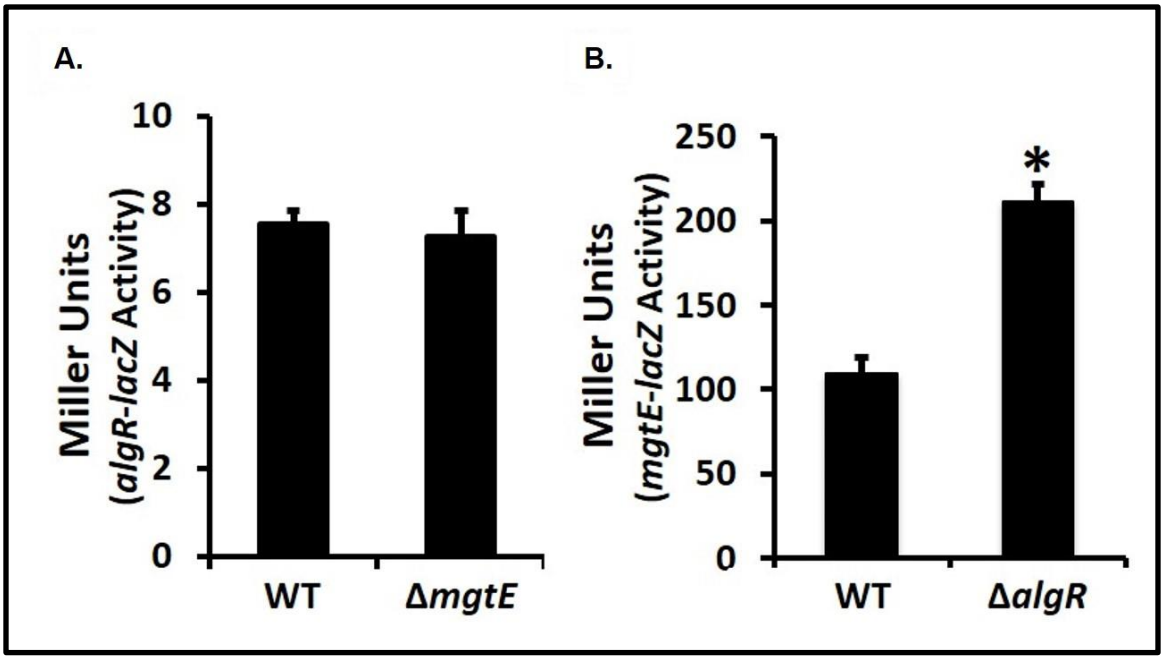

Figure 4.4. AlgR negatively regulates *mgtE* promoter activity.
(A) Beta-Galactosidase activity (Miller units) was measured from the P_{algR}–*lacZ* construct in the WT and Δ*mgtE* strains grown as planktonic, broth cultures. Data are representative of three independent experiments, each containing triplicate or quadruplicate samples. (B) Transcriptional activity of the *mgtE* promoter was analyzed by the beta-galactosidase assay (Miller units) using the P_{mgtE}–*lacZ* construct in the WT and Δ*algR* strains grown as planktonic, broth cultures. Data are representative of three independent experiments, each containing triplicate or quadruplicate samples. *$p<0.05$, compared to WT.

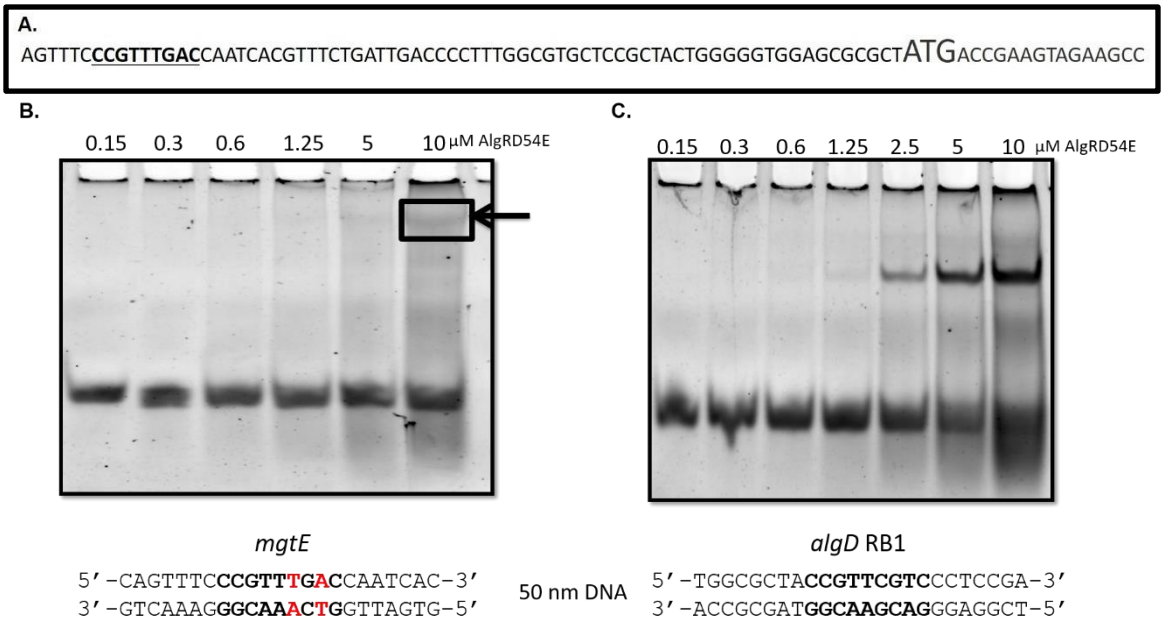

Figure 4.5. AlgR weakly binds to the *mgtE* promoter region.
(A) *In silico* analysis reveals a putative AlgR binding site (shown in bold) in the *mgtE* promoter region. The *mgtE* start codon is demarcated by large font. (B) EMSA was performed using varying concentrations (0.15-10µM) of purified AlgRD54E protein with the oligonucleotide containing the putative AlgR binding site in the *mgtE* promoter or with the oligonucleotide containing the AlgR binding site in the *algD* promoter (C). 50nm oligonucleotide was used in both experiments. The base pairs in the putative AlgR binding region in the *mgtE* promoterthat are mismatched from the consensus AlgR binding sequence are marked in red. The faint bandshift highlighted with the box is indicative of weak but direct binding of AlgR to the putative binding site in the *mgtE* promoter. (Courtesy Dr. Michael Schurr).

Examination of other *algR* and *mgtE* regulated phenotypes. Since the genetic crosstalk between *algR* and *mgtE* was evident on regulation of T3SS gene transcription, we tested whether this genetic interaction affected other virulence phenotypes as well in *P. aeruginosa*. For this, we tested the effects of the WT, Δ*mgtE*, Δ*algR* and Δ*mgtE*/Δ*algR* on twitching motility, rhamnolipid production and pyocyanin synthesis in *P. aeruginosa*. As shown in Fig 4.6, the *mgtE-algR* genetic interaction is not evident in regulation of these virulence phenotypes, indicating that this crosstalk is specifically directed towards T3SS regulation in *P. aeruginosa*.

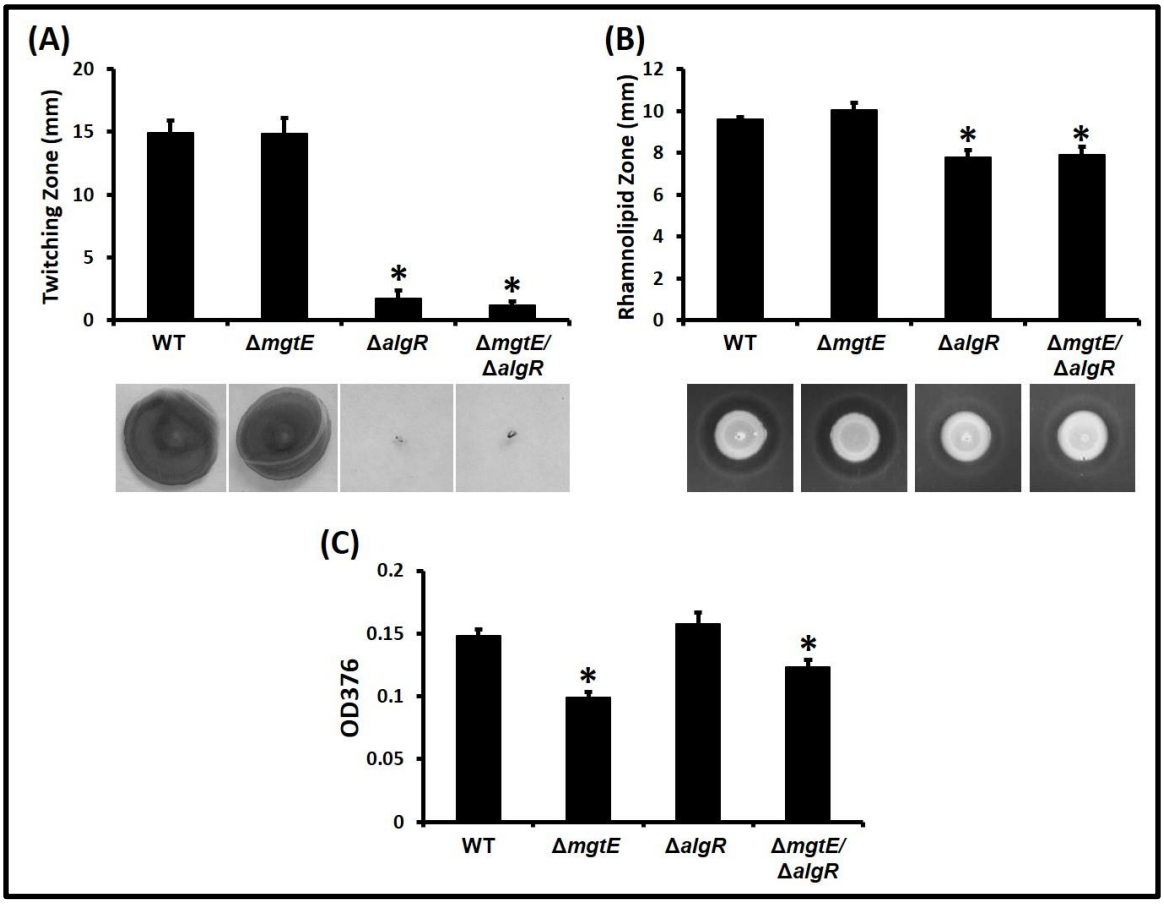

Figure 4.6. Examination of other AlgR and MgtE regulated phenotypes.
(A) Twitching motility was assessed for the individual mutants and for the Δ*mgtE*/Δ*algR* double mutant, compared to WT. In the top panel, mean twitching zones for each strain are reported. *p<0.05, compared to WT and Δ*mgtE*. The bottom panel depicts representative images of crystal violet-stained twitch zones for each strain. (B) Rhamnolipid secretion of mutants was assessed by zone size of CTAB/rhamnolipid precipitant, compared to WT. In the top panel, mean zone size for each strain is reported. *p<0.05, compared to WT. The bottom panel depicts representative images of colonies and CTAB/rhamnolipid precipitant zones. (C) Pyocyanin production of WT and mutants was assessed as described in Methods. *p<0.05, compared to WT and Δ*algR*.

4.4 Discussion

MgtE, a magnesium transport protein, has been identified as a virulence modulatory protein in *P. aeruginosa* (91, 99), and a virulence and biofilm modulatory protein in several other bacteria (156, 157, 172). However, the regulation of *mgtE* and the environmental signals that influence *mgtE* transcription (and thus affect downstream virulence pathways) are

poorly understood. In previous studies, we found that *mgtE* could affect bacterial cytotoxicity through modulation of T3SS (99) and that tobramycin and various other antibiotic treatments resulted in a significant increase in *mgtE* transcript levels (91). Importantly, isogenic deletion of *mgtE* resulted in WT levels of antibiotic sensitivity (91), suggesting that *mgtE* induction might play only a minor role in antibiotic resistance. In accordance with these previous findings, we also observed that incubation in decreased levels of magnesium, which can stimulate *mgtE* transcription, has little effect on MIC (Table 4.3). Instead, we hypothesized that antibiotics act as signals that induce *mgtE* transcription, as part of an effort to downregulate production of factors associated with acute infections (e.g. T3SS) during biofilm development.

AlgR is a regulatory protein that functions in many different pathways, most notably the alginate biosynthesis pathway. Induction of this pathway results in a mucoid *P. aeruginosa* phenotype, due to production of copious amounts of alginate. The mucoidy phenotype is evident during chronic infection of the CF airway and it enhances biofilm formation. AlgR is part of the AlgR–AlgZ two component regulatory system, which interacts with AlgU (92), the sigma factor that activates many genes essential for induction of the alginate pathway, including *algR.* Furthermore, *algR* is an important gene for *P. aeruginosa* pathogenesis, as an *algR* mutant is less virulent than a WT strain in an acute septicemia mouse model of infection (173). AlgR is also required for twitching motility, which is associated with increased virulence (174).

This evidence, as well as proteomic analysis of an *algR* mutant (173), suggests that AlgR is a global regulator impacting the expression of many different genes, including the T3SS genes. When AlgR is mutated, an increased expression of certain T3SS genes, such as *exsA* and *exoS*, is observed. ExsA is the master regulator for the T3SS in *P. aeruginosa* (55), and prior and recent findings (Chapter 3) suggest that MgtE may be indirectly affecting this protein to inhibit T3SS. Indeed, increased expression of *mgtE* leads to decreased T3SS production and lowered cytotoxicity. These results imply important but complex interactions of AlgR with other virulence pathways in *P. aeruginosa*.

Our findings also support a possible interaction between *algR* and *mgtE* (Fig. 1.1), because deletion of *algR* in the Δ*mgtE* strain led to decreased transcription from the P_{exsD} promoter (Fig. 4.1). Also, our results suggest that *algR* has negative regulatory effects on *mgtE* transcription (Fig. 4.4). Interestingly, *in silico* analysis has identified a putative AlgR binding site upstream of the *mgtE* promoter (Fig 4.5A). Further, in collaboration with Dr. Michael Schurr, we have identified a weak but direct binding of AlgR to the *mgtE* promoter in vitro (Fig 4.5B). Ongoing studies are aimed at further investigating this interaction between AlgR and the *mgtE* promoter.

Altogether, these data imply that there is a peculiar cross-talk between *algR* and *mgtE* in regulating the T3SS, and this effect seems to be specific to T3SS (Fig. 4.6). Because reduction in T3SS is an important component of *P. aeruginosa* biofilms in the CF lung, these data support the hypothesis that *mgtE* can influence the biofilm phenotype in response to CF-relevant signals. We speculate that MgtE and AlgR both inhibit the function of a

T3SS repressor, either separately or in concert. In this scheme, mutation of either *mgtE* or *algR* is compensated by the presence of the other, but when both are mutated, high-level activity of the repressor prevents T3SS transcription and cytotoxicity (Fig 4.7). However, it is also possible that MgtE and AlgR could be acting independently on T3SS. Further studies are needed to fully characterize the relationship between these two proteins, and whether MgtE regulation of T3SS is mediated by the observed *mgtE* regulation by AlgR (Figs. 4.4 and 4.5). Among efforts directed at elucidating the nature of the *algR-mgtE* genetic interaction in *P. aeruginosa*, we are performing transcriptomic (RNAseq) and proteomic analysis of the WT, Δ*mgtE*, Δ*algR*, and Δ*mgtE*/Δ*algR* strains.

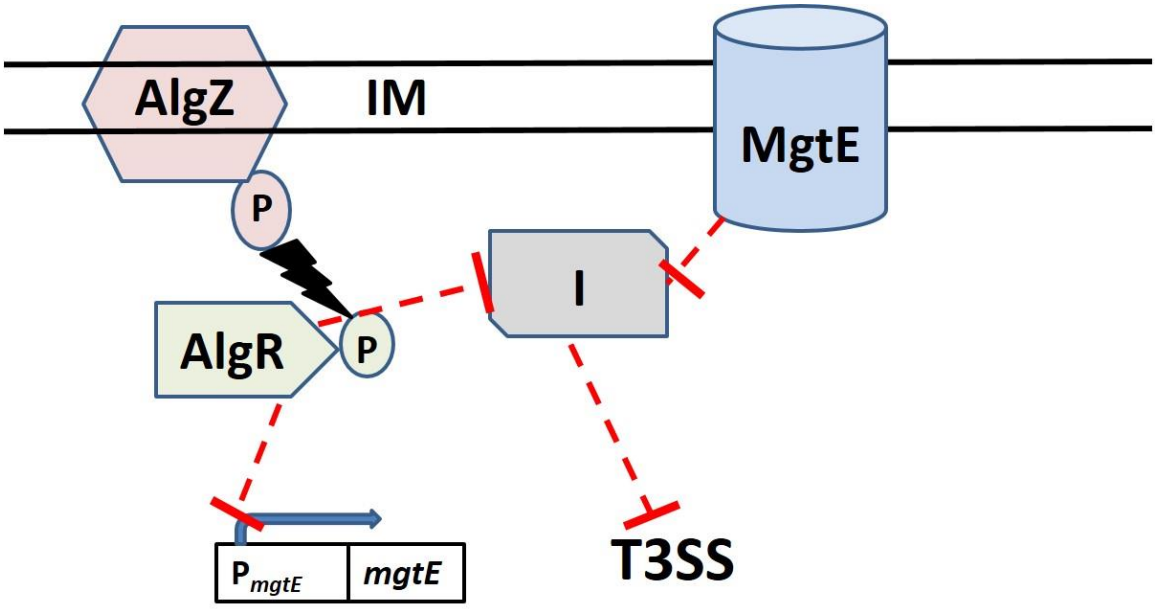

Figure 4.7. Hypothetical model to account for the inhibition of T3SS gene transcription in the Δ*mgtE*/Δ*algR* strain of *P. aeruginosa*.
AlgZR and the inner membrane (IM) Mg^{2+} transporter MgtE, by an unknown mechanism inhibits a third cryptic inhibitor (I), which inhibits T3SS gene expression. This inhibitor is inhibited by both *mgtE* and *algR*. Therefore, when both *mgtE* and *algR* are mutated, this inhibitor gets upregulated (synthetically or functionally) and inhibits T3SS gene transcription. Dashed red lines indicate inhibition. Additionally, AlgR, putatively by directly binding to the *mgtE* promoter, downregulates *mgtE* transcription, as we have shown in this Chapter.

Previous studies in our lab have clearly demonstrated that numerous antibiotics can enhance *mgtE* transcription, possibly as the result of activation of stress responses. Indeed, AlgR is regulated by the sigma E homologue AlgU, which responds to heat shock, among other envelope stresses. Thus, inhibition of *mgtE* transcription at 55^0C (Table 4.4) could be the result of repression by elevated levels of AlgR under these conditions. Additionally, it is possible that *mgtE* is activated by the stringent response (175), as evidenced by increased *mgtE* transcription in the nutrient-limited M63 medium (without carbon or amino acids sources) (Table 4.4). Enhanced *mgtE* expression could result in biofilm phenotype changes by decreasing T3SS (99) and possibly interacting with other known virulence pathways as shown in Chapter 3. A greater understanding of *mgtE* regulation could lead to novel methods of inhibiting biofilm formation and bacterial toxicity. Our data suggest that AlgR might act as a link between external signals like antibiotics and *mgtE* transcriptional responses. As we dissect the complex relationship between *mgtE*, biofilm formation and toxicity, we can devise novel methods for treatment of this recalcitrant microbe.

CHAPTER 5. FINAL DISCUSSION AND FUTURE PERSPECTIVES

A major global health problem is the rise in antibiotic resistance in pathogenic bacteria (176). *Pseudomonas aeruginosa* is one of the antibiotic recalcitrant ESKAPE (***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa,* and ***E****nterobacter* species) superbugs (177). Antibiotic-resistant *P. aeruginosa* is listed as one of the top 18 drug-resistant threats in the United States (listed as Hazard Level Serious) by the Centers for Disease Control and Prevention (178), and one of the top 12 Priority Pathogens for development of new antibiotics (Priority 1: Critical) by the World Health Organization (179). In addition to its inherent and acquired antibiotic resistance mechanisms, the capability to form clinically relevant biofilms is an important weapon possessed by *P. aeruginosa*, that renders it even more immune to antibiotic therapies. Though the regulatory pathways governing biofilm formation, maintenance, and dispersal in *P. aeruginosa* are diverse and complicated, the common feature is a reciprocal regulation between high cytotoxicity and increased biofilm forming lifestyles (88). It is important to elucidate these regulatory mechanisms to identify novel therapeutic targets in *P. aeruginosa* to both i) prevent it from forming antibiotic resistant biofilms and ii) reverting it to the planktonic lifestyle which is more susceptible to antibiotics.

This current study describes two different mechanisms by which *P. aeruginosa* downregulates its cytotoxicity, thereby making it adapt to the biofilm lifestyle (Fig. 5.1). T3SS is a predominant feature behind cytotoxicity in *P. aeruginosa* (91). In Chapter 3, I

describe one molecular pathway through which *mgtE* inhibits the T3SS: through Rsm-mediated translation inhibition of ExsA. These results largely illuminate cytotoxicity regulatory cascades in *P. aeruginosa* and once again confirm *mgtE* as a virulence regulator in *P. aeruginosa*. In Chapter 4, I have established *algR* as a regulator of *mgtE* expression and signaling, adding to its already reported pleiotropic role in *P. aeruginosa*. This AlgR data also suggest a second means for regulating T3SS: a potential unknown inhibitor that is inhibited by both AlgR and MgtE (Fig. 5.1).

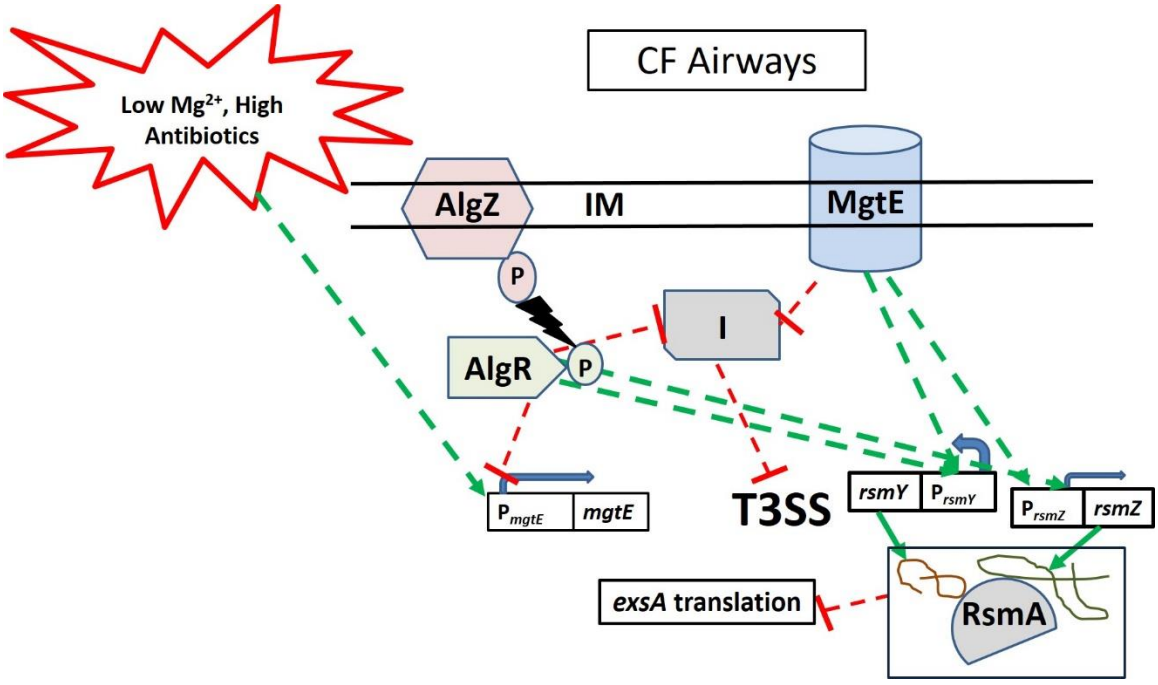

Figure 5.1. Pleiotropic effects of MgtE in *P. aeruginosa* pathogenesis.

Low Mg^{2+} and high antibiotic conditions (as in CF lungs) activate *mgtE* transcription by an unknown mechanism. This results in higher levels of MgtE in the inner membrane (IM), which activates *rsmY* and *rsmZ* expression, through GacAS (Chapter 3), resulting in reduction in ExsA translation. AlgR, upon being phosphorylated by AlgZ, also activates RsmY and RsmZ expression (3). On the other hand, as discussed in Chapter 4, AlgR inhibits *mgtE* transcription. Also, AlgR and MgtE seem to simultaneously inhibit a third cryptic inhibitor (I), because of which a double mutation in *algR* and *mgtE* results in upregulation of this inhibitor and reduction in T3SS. Broken and solid green arrows depict activation by unknown and known mechanisms respectively; broken red lines indicate inhibition by a poorly understood mechanism.

It is unclear how these two pathways intersect to regulate T3SS. I have presented two key pieces of data to elucidate this issue. First, *mgtE* expression loses T3SS inhibiting effect in a Δ*rsm* strain (Fig. 3.6), suggesting that *mgtE* acts exclusively through Rsm alone. It was recently shown that AlgR impacts T3SS activation by modulating the Rsm pathway (3), and it is tempting to speculate that MgtE figures into this process. However, our data (Fig 4.2) demonstrate that T3SS transcription can be inhibited by *mgtE* expression even in a Δ*algR* mutant. Thus, while it is possible that MgtE acts through an AlgR-mediated pathway to affect Rsm, and hence T3SS activation, this mechanism would likely be a minor activity.

Alternatively, MgtE could impact T3SS through two separate avenues. In addition to inhibiting T3SS through a GacAS-Rsm pathway to decrease ExsA translation, MgtE could also be inhibiting a T3SS transcriptional inhibitor. In this scenario, AlgR would also be inhibiting this hypothetical inhibitor. Thus, mutation of either *mgtE* or *algR* could still lead to T3SS transcriptional enhancement, due to sequestration of the inhibitor by the other molecule, but deletion of both factors would lead to freeing of the inhibitor, resulting in T3SS downregulation (Fig. 4.7).

In either case, we see a clear effect of decreased T3SS transcriptional activation upon *mgtE* expression. While there are numerous two-component, quorum sensing, adenylate cyclase, and other regulators that impact T3SS and biofilm establishment in *P. aeruginosa*, the signals that initiate these signaling cascades remain undefined. Research from our laboratory has shown that antibiotics as well as limiting magnesium conditions stimulate *mgtE* transcription. Furthermore, my research has shown that *mgtE* expression intimately

ties into the biofilm signaling networks (Fig. 3.7). Thus, we propose that limiting magnesium and high antibiotic levels are two factors that activate biofilm pathways, shifting the bacterium toward a chronic, biofilm lifestyle. Importantly, these are both signals present in the CF lung environment; patients are treated with high levels of antibiotics, and also exhibit decreased lung and serum magnesium levels, compared to non-CF individuals (149, 150). Thus, MgtE could be a key factor modulating biofilm signaling during infections (Fig. 5.1).

Future experiments would aim to further characterize *mgtE* expression and signaling in *P. aeruginosa*. Specifically, the precise protein-protein interactions that mediate *mgtE* regulation of GacASsignaling (Fig. 3.5) is an important milestone for research in our laboratory. Another important goal would be to characterize the mechanism behind *algR*-driven *mgtE* transcription inhibition. Preliminary studies (Fig. 4.5) indicate that AlgR might be directly binding to the *mgtE* promoter. Ongoing studies to further characterize this DNA-protein interaction are underway. Also, it will be important to elucidate the exact mechanism behind the unique cytotoxicity inhibitory phenotype in the Δ*algR*/Δ*mgtE* double mutant in *P. aeruginosa*.

mgtE has been implicated as a virulence modulator in other organisms as well (106). Thus, the mechanism by which *mgtE* signals in these pathogens is an intriguing avenue for research. Interestingly, *rsm* homologues exist in some of the organisms where *mgtE* is implicated in pathogenesis (159). If future studies indicate that *mgtE* signals through a

conserved mechanism, that would set an important paradigm in membrane transporter biology.

Given the role that *mgtE* plays in cytotoxicity regulation, it might be an interesting drug target, though extensive future studies are required to explore this proposition. Taken together, this research, provides clues for identification of novel therapeutic targets in *P. aeruginosa*. Drugs that modulate the biofilm phenotype (possibly through MgtE) such that bacteria emerge from the biofilm phenotype (and are thus killable) but maintain a low toxicity phenotype would revolutionize biofilm therapies. Thus, my work can serve as the foundation for studies to ameliorate the morbidity and mortality of patients affected by *P. aeruginosa*.

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