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For the degree of Master of Science

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DUAL FUNCTIONS OF THE PROTEIN MGTE IN *PSEUDOMONAS AERUGINOSA*

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of

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by

Barbara M. Coffey

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LIST OF ABBREVIATIONS

ABC	Adenosine Triphosphate Binding Cassette
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
°C	Degrees Celsius
CF	Cystic Fibrosis
CFBE	Cystic Fibrosis Bronchial Epithelial
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CO_2	Carbon Dioxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
ΔF508	Deletion of Phenylalanine at Position 508
FBS	Fetal Bovine Serum
HCl	Hydrogen Chloride
HRP	Horseradish Peroxidase
LB	Luria-Bertani or Lysogeny Broth
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide

MEM	Minimal Essential Medium
Mg^{2+}	Magnesium
$MgSO_4$	Magnesium Sulfate
µg	Microgram
µL	Microliter
mL	Milliliter
mM	Millimolar
MM281	*Salmonella enterica* Typhimurium MM281
NAD^+	Nicotinamide Adenine Dinucleotide
nm	Nanometers
PA14	*Pseudomonas aeruginosa* Strain 14 (wild type)
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
pH	Potential Hydrogen
RPM	Revolutions Per Minute
SDS	Sodium Dodecyl Sulfate
T3SS	Type III Secretion System
TE	Tris-EDTA Buffer
TMD	Transmembrane Domain
YEPD	Yeast Extract Peptone Dextrose

ABSTRACT

Coffey, Barbara M. M.S., Purdue University, August 2011. Dual Functions of the Protein MgtE in *Pseudomonas aeruginosa*. Major Professor: Gregory G. Anderson.

The Gram-negative bacterium *Pseudomonas aeruginosa* is an opportunistic pathogen which readily establishes itself in the lungs of people with cystic fibrosis (CF). Most CF patients have life-long *P. aeruginosa* infections. By modulating its own virulence and forming biofilms, *P. aeruginosa* is able to evade both host immune responses and antibiotic treatments. Previous studies have shown that the magnesium transporter MgtE plays a role in virulence modulation by inhibiting transcription of the type III secretion system, a mechanism by which bacteria inject toxins directly into the eukaryotic host cell. MgtE had already been identified as a magnesium transporter, and thus its role in regulating cytotoxicity was indicative of dual functions for this protein. This research focused on a structure-function analysis of MgtE, with the hypothesis that the magnesium transport and cytotoxicity functions could be exerted independently. Cytotoxicity assays were conducted using a co-culture model system of cystic fibrosis bronchial epithelial cells and a Δ*mgtE* strain of *P. aeruginosa* transformed with plasmids carrying wild type or mutated *mgtE*. Magnesium transport was assessed using the same *mgtE* plasmids in a *Salmonella* strain deficient in all magnesium transporters. Through analysis of a number of *mgtE* mutants, we found two constructs – a mutation in a putative magnesium binding

site, and an N-terminal truncation – which demonstrated a separation of functions. We further demonstrated the uncoupling of functions by showing that different *mgtE* mutants vary widely in their ability to regulate cytotoxicity, whether or not they are able to transport magnesium. Overall, these results support the hypothesis of MgtE as a dual function protein and may lead to a better understanding of the mechanisms underlying *P. aeruginosa* virulence. By understanding virulence mechanisms, we may be able to develop treatments to reduce infections and pave the way to better health for people with cystic fibrosis.

CHAPTER 1: INTRODUCTION

1.1 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disease caused by a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). In the United States, CF occurs in approximately 1 in 3900 births, with the disease being most common among Caucasians at a rate of 1 in 2500 births [1]. Currently, the average life expectancy for an individual with CF is 37 years [2].

In CF, non-functional CFTR fails to conduct chloride ions and results in numerous pathologies throughout the body. In the lungs, CFTR dysfunction leads to the build-up of a thick mucus layer, which impairs cilia movement and diminishes the ability to clear pathogens. The bacterium *Pseudomonas aeruginosa* is able to resist both the host's immune system and antibiotic treatment, and as a consequence, chronic pulmonary infection is a major cause of morbidity and mortality for individuals with CF. Although several bacteria are known to cause lung infections, *P. aeruginosa* is the most predominant pathogen found in CF patients [2, 3]. Adding yet another layer of complexity to the interaction between *P. aeruginosa* and its host, the bacterium undergoes phenotypic changes in the CF lung as the infection evolves from acute to chronic, during which time *P. aeruginosa* regulates its own virulence mechanisms in

order to persist in its host [4, 5]. These include changes in secretions of toxins and exopolysaccharides, and formation of biofilms.

There is currently no cure for cystic fibrosis. One of the limitations of research on CF is the lack of a good animal model. Mice have been used in CF research, but the murine lung does not express a CF phenotype similar to humans. More recently, ferrets and pigs with CF have been developed, but there are enormous challenges to breeding and maintaining these animals [6]. In the absence of a practical animal model, tissue culture is a highly valuable research tool for CF lung infection. Potential for medical treatment of chronic lung infection and improved health for CF patients lies in better understanding of *P. aeruginosa* virulence mechanisms, much of which we hope to understand through *in vitro* experimentation.

1.2 Cystic Fibrosis Transmembrane Conductance Regulator

Encoded on human chromosome 7, the CFTR protein contains 1480 amino acids and has a molecular weight of 168 kDa [7]. It is a chloride channel located in epithelial cells throughout the body, and belongs to the ATP-binding cassette transporter (ABC-transporter) family of proteins. Over 1700 mutations of CFTR have been found, with the mutation ΔF508 being most often identified and attributed to approximately 75% of CF cases [2]. ΔF508 is a deletion of the amino acid phenylalanine at position 508 in the protein. This mutation leads to a misfolded and defective protein, which is quickly degraded [8].

Some reports have suggested that defective CFTR impairs innate immune response, thereby implicating this protein in the facility with which *P. aeruginosa* initially infects

the CF lung [9-11]. These studies assert that normal CFTR is able to recognize the pathogen, signal epithelial cells to activate transcription factor NF-κB, and initiate an immune response. Abnormal CFTR is unable to induce this response and therefore leads to immunological deficiency.

The tissue cultures used in this research are grown from human-derived cystic fibrosis bronchial epithelial (CFBE) cells that express the CFTR ΔF508 mutation.

1.3 The Bacterium *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative bacterium commonly found in both natural and man-made environments. It is a versatile, adaptable opportunistic pathogen with a large genome (6.3 million base pairs) encoding approximately 5500 genes [12]. Although typically non-virulent to healthy individuals, *P. aeruginosa* causes numerous types of infections in immunocompromised individuals, including burn infections, nosocomial infections such as pneumonia and catheter-related urinary-tract infections, and chronic, antibiotic-resistant lung infections in people with CF [13]. Chronic *P. aeruginosa* infection has been recognized in CF patients since the 1970s, and the presence of different phenotypes was also noted [14]. It has since been elucidated that *P. aeruginosa* undergoes phenotypic changes and differentially regulates its own virulence factors during the course of infection in the CF lung [15]. Bacterial gene expression varies according to whether the infection is acute or chronic, such that the bacterium which initially enters the host and establishes infection is markedly different from the bacterium that maintains itself and persists, possibly for decades, in the same host [16, 17]. It has been found that as the infection endures and the patient's age progresses, the lung

microbial community loses diversity and becomes increasingly dominated by *P. aeruginosa,* although within a single patient, there may exist multiple *P. aeruginosa* phenotypes [18, 19].

"Conversion to mucoidy" is a term which refers to the transition of the bacteria from a planktonic, free-floating state to a colonizing, alginate-producing phenotype. The mucoid form of *P. aeruginosa* secretes an exopolysaccharide that aids in protection against the host's immune cells, forms a barrier against antibiotics, and helps in the initiation of biofilm formation. Mucoid *P. aeruginosa* in the lungs of CF patients is indicative of deteriorating lung function and declining patient condition [14, 20-22].

The *P. aeruginosa* strain used in this study is PA14, identified by Rahme *et al.* in 1995 [23]. This strain was initially discovered among a screen of 30 human clinical isolates and was shown to elicit pathogenicity in both mice and *Arabidopsis*. PA14 was selected for further study due to its unique characterization as a dual plant-animal pathogen. PA14 is a non-mucoid strain, but has been shown to form biofilms [24]. Both mucoid and non-mucoid *P. aeruginosa* can form biofilms, but the biofilms formed by mucoid *P. aeruginosa* are impossible to eradicate from the CF lung [25]. The numerous phenotypes of *P. aeruginosa* found in the various stages of infection make the study of this bacterium even more challenging.

The progression of CF lung disease and the accompanying microbiology are tremendously complex. It remains to be fully understood why *P. aeruginosa* dominates over other bacteria in the CF lung. For this reason, there is ample need to continue research efforts toward illuminating *P. aeruginosa* virulence mechanisms in the CF lung environment.

1.4 Biofilms

Biofilms are a remarkably successful microbial survival mechanism. A biofilm is a colony of bacteria that has transitioned from a planktonic (free-swimming) state to a fixed, surface-attached state. The surface to which the biofilm attaches may be biotic or abiotic. The process of biofilm formation takes place in a number of distinct stages, brought about through differential expression of bacterial genes in response to their environment [26].

The components of a biofilm vary depending on the environment, but biofilms are generally comprised of living bacteria, exopolysaccharides, and macromolecules arranged within an intricate matrix that provides a protective structure as well as a system of channels allowing for the diffusion of water, nutrients, and metabolic waste [27-29]. Recent analysis has shown that the extracellular matrix of *P. aeruginosa* PA14 is composed largely of DNA and lipopolysaccharides (LPS) [24]. In this study and numerous others, PA14 has been used for laboratory research due to its strong biofilm-forming ability.

When the transition to a biofilm state occurs within a human host, the infection condition evolves from acute to chronic. As a biofilm forms in the lungs of a CF patient, a complex bacterial community develops that is highly resistant to the host's immune system and antibiotic treatment. It is thought that the longer the biofilm remains, the more antibiotic resistant it becomes [30]. Much remains to be understood about how bacteria regulate this process, and why *P. aeruginosa* biofilms in particular thrive in the environment of the CF lung.

Biofilm formation and chronic lung infection is a serious problem for CF patients, causing permanent lung damage that leads to decline in patient condition and ultimately death. Although biofilms lack the virulence factors attributed to planktonic bacteria, they are nevertheless highly destructive to the host. The decreased cytotoxicity of the bacteria in biofilms is one of the adaptations that allows them to persist. Previous studies indicate that deletion of the gene encoding the protein MgtE from *P. aeruginosa* increases the cytotoxicity of biofilms, although it does not impact biofilm formation [31].

1.5 MgtE

MgtE is a magnesium-transport protein found in all domains of life. The groundwork already done to understand the role of MgtE in prokaryotes has been carried out in several bacterial species, and although *P. aeruginosa* MgtE is thought to function in a similar manner, it has not been fully characterized. When first identified in 1995 in the Gram-positive bacterium *Bacillus firmus,* MgtE was immediately recognized as a unique protein, unrelated to any other previously characterized family of magnesium transporters [32]. The crystal structure (Figure 1) was resolved in *Thermus thermophilus* [33], and the peptide sequence is 29% identical in *P. aeruginosa;* therefore, our current understanding of MgtE in *P. aeruginosa* is by analogy.

P. aeruginosa MgtE has a molecular mass of 54 kDa and is suggested to function as a homodimer. The carboxy-terminal transmembrane domain of the monomer includes five alpha-helices which form a transmembrane pore when dimerized. The cytosolic amino-terminus includes several globular domains which work cooperatively to sense intracellular magnesium levels. The transmembrane and cytosolic domains are joined by

a third region called the connecting, or plug, helix. The current model of MgtE suggests a significant conformational change between the magnesium-bound and unbound states. The binding of magnesium to the cytosolic domains affects movement of the connecting helices, which then leads to opening or closing of the transmembrane pore [34]. It has been shown that the MgtE pore is highly specific for magnesium ions and is not regulated by other divalent cations such as calcium, although there is some evidence of sensitivity to Co^{2+} [32, 34].

P. aeruginosa expresses other magnesium transporters. CorA is constitutively expressed and is the primary mediator of magnesium influx. CorA has also been shown to mediate magnesium efflux in Gram-negative bacteria when intracellular magnesium concentrations approach 1mM [35-38]. Two other proteins, MgtA and MgtC are thought to mediate magnesium influx only, but MgtE is unrelated to these proteins [32, 39]. Although magnesium is essential to life, magnesium transport proteins and the regulation of magnesium homeostasis are not yet fully understood.

While well-established as a magnesium transporter, MgtE in *P. aeruginosa* has also been shown to play a role in regulating virulence, and it has been suggested that the two functions, magnesium transport and regulation of cytotoxicity, may be separable. This was initially demonstrated by Anderson *et al.* in experiments with an *mgtE* construct containing a C-terminal His_6 tag. This mutant was unable to transport magnesium; however, it did regulate cytotoxicity. These studies connected the effect of increased cytotoxicity to an increase in the expression of the type III secretion system [31].

1.6 Type III Secretion System

The type III secretion system (T3SS) in *P. aeruginosa* is a large protein complex, often described as a needle-like structure, which enables the pathogen to inject cytotoxic effector molecules directly into its eukaryotic host. This system mediates acute infections such as hospital-acquired pneumonia, but has been found to be diminished in adult CF patients with long-term *P. aeruginosa* infection. In simplest terms, the longer *P. aeruginosa* infection persists, the less it expresses T3SS [4].

Currently, there are four known effectors of the *P. aeruginosa* type III secretion system: ExoS, ExoT, ExoU, and ExoY. It appears that all four are not usually encoded in the genome of a single strain. Their cytotoxic effects on host cells are achieved through a variety of mechanisms including phospholipase, adenylate cyclase, and GTPase-activating protein (GAP) activities, as well as numerous other disruptions of host cell functions. Among the four effectors, ExoU is the most cytotoxic and rapidly causes host cell death. Consistent with the idea that *P. aeruginosa* downregulates its virulence as infection persists, ExoU-producing strains are not often found in chronically infected CF patients [13, 40].

1.7 Research Goals

The goal of this research was to better understand the functional interactions of the MgtE domains and how they relate to magnesium transport and cytotoxicity, with the hypothesis that the magnesium transport and cytotoxicity functions of *P. aeruginosa* MgtE can work independently of each other. Cytotoxicity, more specifically, should be

tested in the context of the CFTR mutation, since our interest is focused on understanding the unique virulence behaviors of *P. aeruginosa* in people with CF.

The hypothesis was tested by doing a structure-function analysis of MgtE and demonstrating which regions of the protein were essential for magnesium transport and which were essential for regulation of cytotoxicity. All assays for cytotoxicity were performed on cystic fibrosis bronchial epithelial cells (CFBE) that express the CFTR ΔF508 mutation. The research process was guided by three specific aims:

- Specific Aim 1: Test the effect of C-terminal truncations on the ability of MgtE to transport magnesium and inhibit cytotoxicity toward CFBE.
- Specific Aim 2: Test the effect of N-terminal truncations on the ability of MgtE to transport magnesium and inhibit cytotoxicity toward CFBE
- Specific Aim 3: Test the effect of mutations in the magnesium binding sites on the ability of MgtE to transport magnesium and inhibit cytotoxicity toward CFBE.

In addition to these three aims, we also evaluated the kinetics of cytotoxicity and the effects of extracellular magnesium concentration. Overall, our results confirm the role of MgtE in regulation of cytotoxicity and begin to elucidate the importance of certain regions either for magnesium transport, cytotoxicity, or both. By better understanding MgtE and *P. aeruginosa*, we are working toward our overarching goal of finding avenues toward improved health and quality of life for people with CF.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial Strains and Cell Cultures

Four bacterial strains were used for this research (Table 1). Bacterial cultures were grown overnight in LB at 37°C with shaking, and antibiotics were used at the following concentrations to maintain selectivity for the desired transformants: 50µg/mL gentamicin for *P. aeruginosa*; 10µg/mL gentamicin for *E. coli* and *Salmonella*. The addition of 100mM $MgSO_4$ was necessary for maintenance growth of *Salmonella* MM281 without plasmids.

2.2 Plasmids

Plasmids used in this study are listed in Table 2 and illustrated in Figure 2. New recombinant plasmids were created by utilizing homologous recombination in yeast, described below. Full-length and mutant *mgtE* constructs were ligated into expression vector pMQ72 [41], which includes a gentamicin resistance gene to allow for selectivity of the desired transformants.

2.3 Yeast Transformation

Plasmid pBC101 is a replacement of the transmembrane domain of MgtE with the heterologous transmembrane protein DgkA, a diacylglycerol kinase found in *P.*

aeruginosa. The recombinant plasmid was created as follows: *dgkA* was PCR amplified from strain PA14 using primers DgkAfusfwd and DgkAfusrev (Table 3) and verified by gel electrophoresis on 1% agarose gel (Figure 3). Plasmid pGA200 was digested with HindIII. The *dgkA* fragment and digested plasmid were then joined by homologous recombination in yeast *Saccharomyces cerevisiae* using the following method known as "Lazy Bones" Protocol [42]. Yeast cultures were grown overnight in 5mL YEPD. The culture was transferred to 1.5mL tubes and centrifuged to pellet cells. Supernatant was drawn off, and the pellet was washed with 500µL TE. TE was drawn off, and 500µL of Lazy Bones Solution (40% polyethylene glycol, 0.1M lithium acetate, 10mM Tris-HCl pH 7.5, 1mM EDTA) [42] was added. Carrier DNA (sheared salmon sperm DNA) was heated for 10 minutes at 100°C, and then 20µL was added to the transformation reaction. 10µL of digested plasmid pGA200 and 20µL of amplified *dgkA* fragment were added. The mixture was vortexed for one minute and then incubated at room temperature overnight for two nights. The tube was heated at 42°C for 12 minutes to heat shock cells, and then centrifuged to pellet. Supernatant was drawn off, and the pellet was resuspended in 1mL TE. The sample was centrifuged again, and all but 100µL of the supernatant was drawn off. Remaining sample was plated on uracil dropout media and incubated at 30°C for 4 days. A control reaction was also prepared, which included all reagents as described above, except no DNA was added from salmon sperm, pGA200 plasmid digest, or *dgkA* fragment. Transformants were selected by growth on uracil dropout media. Control plate had no growth.

Plasmids pBC102, pBC103, and pBC104 are a series of truncations of the MgtE N-terminus. These were constructed in the same manner just described, except that *mgtE* fragments were ligated into expression vector pMQ72.

2.4 Plasmid Purification from Yeast

Recombinant plasmids were purified from *S. cerevisiae* as follows. Transformed yeast colonies were scraped from the agar plate and resuspended in 1mL YEPD. The culture was centrifuged for one minute, and supernatant was drawn off. The pellet was resuspended in 500µL sterile ddH_2O, centrifuged, and supernatant was drawn off. The pellet was resuspended in 250µL QIAGEN Buffer P1 from QIAprep Spin Miniprep Kit (Catalog #27106, QIAGEN Inc., Valencia, CA). Two hundred fifty microliters of QIAGEN Buffer P2 and 250µL of glass beads were added. The sample was vortexed for 2 minutes and allowed to incubate on ice for 5 minutes. Next, 350µL of chilled QIAGEN Buffer N3 were added, mixed by inverting, then incubated on ice for 5 minutes. The sample was centrifuged for 10 minutes at maximum speed in a tabletop centrifuge. Supernatant was applied to the QIAprep spin column, and remaining steps were followed according to the kit protocol to elute the plasmid DNA.

2.5 Bacterial Transformation

Plasmids purified from yeast were transformed into *E. coli* S17 [43] by a rapid electroporation method, described by Choi *et al* [44]. Transformed cultures were plated on selective media and grown overnight at 37°C. Individual colonies were then streaked for isolation on the same selective media and again grown overnight. From this plate, an

individual colony was selected and grown in 5mL liquid LB with selective antibiotic. Following overnight growth, transformations were verified by PCR and gel electrophoresis. In some cases, transformations were also verified by sequencing. Once verified, plasmids were purified from *E. coli* using a QIAprep Spin Miniprep Kit. Purified plasmids were transformed by electroporation into PA14, GGA52 (Δ*mgtE*), and MM281.

2.6 Tissue Culture

Tissue cultures of human-derived cystic fibrosis bronchial epithelial (CFBE) cells [45] were maintained in 750mL polystyrene culture flasks at 37°C in 5% CO_2, and media was changed every 2 to 3 days. Media was prepared by filter sterilization of minimal essential medium (MEM 1X, Cellgro® Minimal Essential Medium Eagle, Mediatech Inc., Manassas, VA) plus 10% fetal bovine serum, 50U/mL penicillin, and 50µg/mL streptomycin. After cells were grown to confluence (Figure 4), typically in 7 to 10 days, the culture was divided into new flasks and clear polystyrene multi-well tissue culture plates as needed for assays. To divide the confluent monolayer, the existing media was aspirated, and cells were washed with 20mL PBS. Eight milliliters of trypsin (Cellgro® Trypsin EDTA 1X, Mediatech Inc., Manassas, VA) were added to the flask, and cells were incubated for 15 minutes at 37°C. This allowed detachment of the CFBE cells from the flask. Cells were removed by pipette, placed in a 15mL conical tube with 4mL standard tissue culture media to deactivate trypsin, and centrifuged for 5 minutes at 4400 RPM to obtain a pellet. All but 2mL of supernatant was drawn off, and the pellet was resuspended in the remaining supernatant. Cell concentration was determined by count

on hemacytometer, and the volume of cells needed to seed new flasks and plates was calculated. New flasks were typically seeded at a concentration of 2×10^6 cells/mL, and 24-well plates were seeded at a concentration of 2×10^5 cells/mL. Work with tissue cultures was performed in a sterile hood using aseptic technique.

2.7 Co-culture Model System and Cytotoxicity Assay

A great deal of our understanding of biofilms has been gained through studies of formation on abiotic surfaces. Living tissue, and in particular the CF lung, provides a dramatically different environment for bacterial growth. The co-culture model system was developed by Anderson *et al.* in order to provide a means to study *P. aeruginosa* virulence toward CF airway epithelial cells [46]. Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Part# G1780, Promega, Madison, WI) was used for all assays. This colorimetric assay measures levels of lactate dehydrogenase (LDH), a cytosolic protein which is released into the culture supernatant when cells are lysed. The color results from two coupled enzymatic reactions. First, in the presence of LDH, NAD^+ and lactate are converted to pyruvate and NADH. Next, in the presence of diaphorase and tetrazolium salt (Promega Substrate Mix, proprietary composition), NADH is oxidized to NAD^+, and formazan forms, which is red or dark pink in color. Darker shades are indicative of more LDH release and therefore higher cytotoxicity (Figure 5). Cytotoxicity can then be analyzed quantitatively using a spectrophotometer to gather absorbance data.

In preparation, CFBE cells were grown in multi-well tissue culture plates for 7 to 10 days to reach confluence, and bacterial cultures were grown overnight. To verify even

growth of bacterial cultures, serial dilutions were plated to obtain approximate count of colony forming units. CFBE cells were washed with 500µL PBS (Cellgro® Dulbecco's Phosphate-Buffered Saline without calcium and magnesium, Mediatech Inc., Manassas, VA) and then given 500µL fresh media containing MEM without phenol red (Cellgro® Minimal Essential Medium Eagle, Mediatech Inc., Manassas, VA), plus 2mM glutamine. Next, 3µL of bacteria were added to each well, and the assay plate (Figure 5) was placed in a 37°C incubator. After one hour, media was replaced with the same media with addition of 0.4% arginine. At timepoints, 300µL samples of supernatant were taken, placed in microcentrifuge tubes, and centrifuged for 2 minutes at 13,200 RPM. Fifty microliters of this supernatant were then transferred to a clear, 96-well flat bottom plate, and 50µL of Substrate Mix were added to each sample. Plates were incubated in the dark at room temperature for 30 minutes to allow assay color to develop. Fifty microliters of Stop Buffer were added, and any bubbles that had formed were popped with a needle. Plate was placed in a SpectraMax M2 spectrophotometer and absorbance read at 490nm. Data was normalized to maximum release of LDH by CFBE cells treated with Triton® X-100 Lysis Solution (supplied in assay kit).

2.8 Magnesium Transport Assay

The ability of *mgtE* mutants to transport magnesium was measured using *Salmonella enterica* Typhimurium MM281, a strain created for the purpose of testing magnesium transport constructs for their ability to restore growth without magnesium supplementation [47]. MM281 contains mutations in all of its magnesium transporters

and is unable to grow unless supplemented with 100mM magnesium or transformed with a functional magnesium transporter.

In this study, MM281 was transformed with *mgtE* plasmids. Mutants were grown overnight in 3mL LB supplemented with 100mM magnesium and 10µg/mL gentamicin to maintain selectivity for transformed bacteria. Following overnight growth, 5µL of the culture was plated on N-minimal media [48] containing 10µg/mL gentamicin, and plates were incubated at 37°C for 1 to 2 days.

Growth indicated complementation of magnesium transport (Figure 6). All plates included MM281pGA200 (full-length *mgtE*) as a positive control, and MM281pMQ72 (empty vector) as a negative control. If an *mgtE* mutant construct did not grow in this assay, we concluded that the mutated region was essential for magnesium transport (Table 4). Inversely, if growth occurs, then the mutated region of *mgtE* was not essential for magnesium transport.

CHAPTER 3: RESULTS

3.1 Regions of MgtE Essential to Magnesium Transport

Magnesium transport was assessed using *Salmonella enterica* Typhimurium MM281, which is unable to transport magnesium (see Materials and Methods, Section 2.8). We transformed MM281 with plasmids carrying various mutations of *mgtE* (Table 2, Figure 2), and samples of the transformed cultures were plated on minimal media without magnesium. Cultures would grow only if the *mgtE* plasmid restored ability to transport magnesium (Figure 6). For the purpose of structure-function analysis, we concluded that if a particular *mgtE* mutation failed to grow, then the mutated region was essential for magnesium transport. Inversely, if an *mgtE* mutant was able to restore growth in MM281, then the mutated region was non-essential to magnesium transport function. Results of magnesium transport assays are summarized in Table 4.

It was predicted that C-terminal truncations would not transport magnesium because mutations in this region would be unlikely to form a functional transmembrane pore. A total of six C-terminal mutations of MgtE were tested: The five transmembrane alpha-helices were truncated one at a time, and the entire transmembrane domain was also replaced with the heterologous transmembrane protein DgkA (Figure 2). As anticipated, these mutations failed to complement magnesium transport in *Salmonella* MM281, with one interesting exception, pGA203.

There was anomalous, spotty growth for the construct pGA203, which is a truncation of transmembrane domains 3, 4, and 5 (see Figure 6A). Results were replicated four times. It was suspected that the spotty growth of MM281pGA203 may have been the result of a contaminated or mixed culture, or perhaps a spontaneous mutation in the lab stock culture. To examine this, we sub-cultured and re-plated several generations. We found that the spotty growth persisted through sub-cultures of the original stock. However, when individual spot colonies were selected from a plate, and either streaked for isolation or grown overnight in liquid media and then plated, solid growth occurred. Of the six C-terminal mutations of *mgtE*, pGA203 was the only construct shown to transport magnesium, and also demonstrated the lowest level of cytotoxicity compared to the other C-terminal mutations.

To further investigate the possibility of a mutation in the MM281pGA203 culture, DNA sequencing was performed on a stock sample and two samples from magnesium transport assays. Data obtained from the Indiana University School of Medicine DNA Sequencing Core Facility indicated no spontaneous mutation in *mgtE*. Although there was some growth shown by pGA203, it is unlikely that this construct is able to form a functional transmembrane pore; therefore, I have concluded that the full transmembrane domain is essential for magnesium transport.

It was anticipated that individual magnesium binding site mutations would not cause complete disruption of magnesium transport. There are a total of seven proposed magnesium binding sites in the MgtE monomer, one in the transmembrane domain and six in the cytosolic domain [34], and since they work cooperatively, it seems that mutation in one of the cytosolic sites would not necessarily result in complete

dysfunction of the protein. Binding site 1 is located in the transmembrane pore, while sites 2 through 6 are in the cytosolic region. Results demonstrated that magnesium binding sites 2 through 6 (cytosolic region) were not individually essential for magnesium transport, although binding site 1 (pore region) was essential. Our tests did not include mutations in the seventh putative magnesium binding site, which is also in the cytosol. Given that other studies have shown the MgtE pore to be highly specific for magnesium ions [34], it is not surprising that a mutation in this region would impair magnesium transport function. Magnesium binding site 1 has also been shown to be less conserved between bacterial species than the other binding sites [49]. Mutations of binding sites 2 and 3, which are located in the connecting helix, were combined in one plasmid (pGA207), and still this construct was able to transport magnesium. The connecting helix region is of particular interest because mutation of this region demonstrated separation of function between magnesium transport and regulation of cytotoxicity, which will be discussed further in the next section.

Results of magnesium transport assays suggest that the entire N-terminal intracellular domain is essential for full complementation of magnesium transport (Figure 6). This is consistent with the findings of Hattori *et al.* which state that the cytosolic domain of MgtE functions to sense intracellular magnesium levels and regulate the opening and closing of the transmembrane pore [34]. Although it was anticipated that N-terminal truncations would impair or eliminate magnesium transport function, we obtained an interesting result, which was replicated in triplicate. The shortest N-terminal truncation (pBC102) resulted in weak growth compared to full-length *mgtE* (Figure 6D). No growth was seen with a longer truncation which included the globular N-domain. And spotty

growth, similar to that seen for C-terminal truncation pGA203, appeared from the longest truncation. Possible variation in results due to plating technique was addressed by plating duplicate samples of each culture. Samples were also checked by PCR to assure that they were not cross-contaminated.

3.2 Regions of MgtE Essential to Regulation of Cytotoxicity

Our goal was to determine which regions of MgtE were critical to regulating *P. aeruginosa* cytotoxicity. Specifically, we wanted to measure cytotoxicity toward CFBE cells. Cytotoxicity was assessed using a colorimetric assay to measure LDH release from CFBE cells incubated with bacteria.

To determine which regions of MgtE are essential for the regulation of cytotoxicity, the *P. aeruginosa* strain GGA52 (PA14 Δ*mgtE*), was transformed with plasmids carrying various mutations of *mgtE* (see Table 2). Levels of cytotoxicity were compared to wild type *P. aeruginosa* PA14 with empty vector pMQ72, GGA52 with overexpression of full-length *mgtE* (pGA200), and GGA52 with empty vector pMQ72. Results of cytotoxicity assays are represented in Figures 7, 8, 9.

Results support previous research demonstrating that overexpression of MgtE inhibits cytotoxicity of *P. aeruginosa* toward CFBE cells [31]. Anderson *et al.* demonstrated that this occurs through inhibition of transcription of the type III secretion system.

As expected, C-terminal truncations had a significant effect on the regulation of cytotoxicity (Figure 7), as did the replacement of the transmembrane domain (Figure 8). C-terminal *mgtE* mutants were assayed over 20 times, and although there was

considerable variation in the raw data, there was a consistent trend showing mutants' loss of cytotoxicity regulation compared to overexpression of *mgtE*.

Magnesium binding site point mutations had widely different effects on cytotoxicity, depending on their location in the protein (Figure 9). A mutation in the transmembrane pore (pGA206) resulted in loss of inhibitory function, but the greatest loss of function was observed in mutation pGA207, which is in the connecting helix region. This was the only mutant which retained magnesium transport function while losing regulation of cytotoxicity.

In seven independent experiments of N-terminal *mgtE* mutants, it was observed that progressive truncations resulted in progressive loss of regulation of cytotoxicity (Figure 8). The shortest truncation (pBC102), which is a deletion of 37 amino acids, demonstrates regulation of cytotoxicity comparable to overexpression of *mgtE* (pGA200). The magnesium transport function was diminished in this mutant, making pBC102 our second construct that displays a separation of functions: it regulates cytotoxicity, but has impaired magnesium transport function.

3.3 Separation of Functions

Two *mgtE* mutants were identified which demonstrated a separation between the functions of magnesium transport and regulation of cytotoxicity. The mutations were expressed in plasmids pGA207 and pBC102. Plasmid pGA207 carries point mutations in magnesium binding sites 2 and 3, located in the connecting helix. Plasmid pBC102 is an N-terminal truncation of 37 amino acids.

Plasmid pGA207 was able to complement magnesium transport function, demonstrated by restored growth of *Salmonella* MM281; however, in cytotoxicity assays, pGA207 was unable to regulate cytotoxicity, demonstrating levels of cytotoxicity similar to the *mgtE* deletion mutant GGA52. In other words, mutation of the connecting helix region of MgtE does not impair magnesium transport function but does eliminate cytotoxicity regulating function. Previous research on the structure of MgtE has suggested that in the magnesium-bound state, the magnesium binding sites in the connecting helix may maintain stability of the closed conformation, and that dimerization is facilitated by the N-terminal globular domains [34]. Thus, our structure-function analysis supports these findings by suggesting that dimerization can occur, but when magnesium is unable to bind in the connecting helix region, MgtE remains in an open conformation and allows the passage of ions.

In plasmid pBC102, the N-terminal truncation of 37 amino acids resulted in diminished magnesium transport function (Figure 6D), while regulating cytotoxicity at levels comparable to over-expression of full-length *mgtE* (Figure 8). This result is the inverse of that seen for pGA207, described above. This means that we have shown magnesium transport without cytotoxicity regulation, and we have also shown cytotoxicity regulation without magnesium transport. Combined, the results from these two *mgt*E mutants support the hypothesis that the functions of MgtE are separable.

3.4 Effects of Magnesium Concentration

Low serum magnesium levels are common in CF patients [50]. The concentration of magnesium in the CF lung has been shown to be 1.0-2.0mM [51], with serum magnesium

levels in the range of 0.46-1.03mM (normal range 0.74-1.1mM) [50]. We were interested to see if the concentration of magnesium in the growth media would affect the level of cytotoxicity of *P. aeruginosa* toward CFBE cells. In other words, do extracellular magnesium levels impact the interaction between bacteria and host? In the standard media for cytotoxicity assays, we varied the magnesium concentrations ranging from 1µM to 10mM. The standard magnesium concentration in our media is 0.8mM. In the 1µM to 1mM range, the cytotoxicity of wild type *P. aeruginosa* strain PA14 toward CFBE cells increased along with increases in magnesium concentration. However, in the 2mM to 10mM magnesium range, cytotoxicity levels remained fairly consistent and did not exceed the 1mM levels (Figure 10).

This data suggests that extracellular magnesium levels affect host-pathogen interaction, although it does not tell us if magnesium levels affect cellular functions of the host, the pathogen, or both. It would be interesting to test *mgtE* mutants' response to varying concentrations of extracellular magnesium. This might give us a clue as to which regions of the protein are most sensitive to extracellular magnesium levels.

3.5 Kinetics of Cytotoxicity

The cytotoxicity assays were 6-hour experiments. We wanted to see if levels of cytotoxicity increased gradually over time, or if cytotoxicity reached a peak and then leveled off. Also, we wanted to compare the kinetics of cytotoxicity in wild type PA14 to our *mgtE* mutants. Representative results are shown in Figure 11. Cytotoxicity increased over time up to 6 hours, with no plateau in any of the samples tested. In *mgtE* mutants, levels of cytotoxicity increased markedly throughout the timecourse, while in wild type

PA14 and over-expressed full-length *mgtE*, cytotoxicity levels remained comparatively low until 4 hours, after which point they increased significantly. Overall, these results suggest that cytotoxicity, if unregulated by MgtE, increases steadily over time; whereas, when the cytotoxicity-regulating function is intact, cytotoxicity may be inhibited to a greater degree in the earliest stages of host-pathogen interaction. This could be due to different rates of bacterial growth or to expression levels of virulence factors.

CHAPTER 4: DISCUSSION

This research has demonstrated that MgtE functions as both a magnesium transporter and a regulator of cytotoxicity. The goal to perform a structure-function analysis of MgtE was achieved, resulting in a better understanding of which regions of the protein are critical for magnesium transport and regulation of cytotoxicity. The three specific aims of this project – to assess magnesium transport and regulation of cytotoxicity in three groups of *mgtE* mutants – were addressed experimentally through cytotoxicity assays and magnesium transport assays. Additionally, work was completed to assess the kinetics of cytotoxicity and the effect of extracellular magnesium concentration.

Previous studies have shown that the plasmid pGA200 (full-length MgtE) localizes to the inner membrane [31]. To support the results found in this study, I would like to assess membrane localization and protein expression levels of the *mgtE* mutants. This would require a cell lysis method that is effective for Gram-negative bacteria and allows preservation of protein association with the inner membrane. It will be particularly important to localize and quantitate the *mgtE* mutants that currently support my hypothesis of separation of functions.

The hypothesis that the two functions of MgtE could be separated was supported by two mutant constructs. Mutant pGA207, containing a point mutation in the connecting helix region, was able to transport magnesium, but did not regulate cytotoxicity. Mutant

pBC102, a truncation of 37 amino acids from the N-terminus, inhibited cytotoxicity at levels similar to full-length *mgtE*, but showed only weak magnesium transport.

The spotty growth of two *mgtE* mutants in magnesium transport assays was not considered as full growth comparable to wild type *mgtE*, and therefore these mutants were not considered to be transporting magnesium. Our tests did not reveal an explanation for the spotty growth, but one possibility is that some cells in the culture developed suppressor mutations. Suppressor mutations are spontaneous mutations, either in the gene of interest or elsewhere in the genome, which in essence override the effect of the intended mutation. Suppressor mutations revert the phenotype to wild type [52].

The complete transmembrane (C-terminal) domain was shown to be essential for full complement of magnesium transport as well as regulation of cytotoxicity. This may be due to the necessity of all five TMDs for pore formation, insertion in the membrane, dimerization, or all of these. Only one magnesium binding site was essential to magnesium transport; this site was located in the pore. Since the remaining magnesium binding sites are cytosolic and thought to work cooperatively, it was not surprising to see magnesium transport function unaffected by their individual mutation.

The cytosolic N-domain, however, tells a more complicated story. None of the N-terminal truncations were able to transport magnesium at a level comparable to full-length *mgtE*, but the shortest truncation showed weak magnesium transport, and the longest truncation showed spotty growth. With the first N-terminal truncation, inhibition of cytotoxicity was comparable to overexpression of full-length *mgtE*, but cytotoxicity-regulating function was diminished with progressive truncations of the N-domain. It has been previously shown that MgtE inhibits transcription of T3SS [31], but the mechanism

remains to be elucidated. I propose that the cytosolic domain of MgtE contains an inhibitory region which may interact with the T3SS pathway to regulate release of cytotoxic effector molecules. The need for further investigation of this interaction is reflected in the statement of Hattori *et al.*, "…the function of the N domain remains elusive." [34]

Much of our structure-function analysis in *P. aeruginosa* is consistent with the findings of Hattori *et al.* in their work on the crystal structure of MgtE in *Thermus thermophilus*. This is exciting because it suggests we are on track toward a better understanding of the role of MgtE in magnesium homeostasis.

While there appears to be a correlation between magnesium transport and cytotoxicity, this research suggests that they are not necessarily inextricably linked. A key finding of this study is that disruption of one function of MgtE does not always eliminate the other.

In interpreting the results of these experiments, there are a couple of variables to consider. First, the passage number of the CFBE cells can affect results. In cytotoxicity assays, we observed that cells from higher passage numbers showed increased levels of LDH release. In part, this is addressed by calculating cytotoxicity as a percentage of maximum LDH release rather than using raw spectrophotometer data. Nevertheless, it is important to be mindful of the CFBE cell passage number when comparing data from independent experiments. Second, it is difficult to assure uniformity of CFBE cell growth in every well of a 24-well plate, and in some cases up to three plates were used in each experiment. Due to potential human error in seeding the plates, it is possible that a well could contain no CFBE cells, or twice the amount. This problem can be addressed

at least in part by assaying samples in triplicate, but this limits the range of samples that can be assayed in a single experiment. In an attempt to test a wide range of plasmid constructs at one time, some assays were run using 96-well plates, but it proved to be problematic to use a sample size unintended for this protocol. Although efforts were made to adjust concentrations of bacteria and assay reagents to accommodate the smaller sample size, results were inconsistent with previous experiments.

At present, there remains a lack of understanding of magnesium homeostasis and magnesium transport proteins, although the role of magnesium levels in pathogenesis has been recognized since studies of toxic shock syndrome in the 1980s [53, 54]. The future direction of this research could involve further investigation in several areas: magnesium homeostasis in bacteria and both healthy and immunocompromised hosts; structure and function of currently identified magnesium transport proteins, and perhaps others to be discovered; bacterial regulation of cytotoxicity in various environments; and immunogenicity and pathogenesis pathways.

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TABLES

Table 1: Experimental Organisms.

Organism	Description or Purpose	Source
Bacterium *Escherichia coli* S17-1	laboratory cloning strain	Simon *et al.* 1983
Bacterium *Pseudomonas aeruginosa* PA14	wild type, clinical isolate	Rahme *et al.* 1995
Bacterium *Pseudomonas aeruginosa* GGA52	PA14 Δ *mgtE*	Anderson *et al.* 2010
Bacterium *Salmonella enterica* Typhimurium MM281	*corA45* ::MudJ *mgtA21* ::MudJ *mgtB10* ::MudJ testing magnesium transport complementation	Hmiel *et al.* 1989
Human-derived cystic fibrosis bronchial epithelial cells	CFTR ΔF508	Cozens *et al.* 1994
Yeast *S. cerevisiae* InvSc1	*ura3-52 / ura3-52* ; creation of plasmids by homologous recombination	Invitrogen

Table 2: Description of Plasmids.

Plasmid	Description	Source
pBC101	TMD replacement	This project.
pBC102	N-terminal deletion (-111 base pairs)	This project.
pBC103	N-terminal deletion (-303 base pairs)	This project.
pBC104	N-terminal deletion (-324 base pairs)	This project.
pBS23	Mg^{2+} binding site point mutation	Anderson *et al.*
pGA200	full-length MgtE	Anderson *et al.*
pGA201	C-terminal deletion, alpha helices 1-5	Anderson *et al.*
pGA202	C-terminal deletion, alpha helices 2-5	Anderson *et al.*
pGA203	C-terminal deletion, alpha helices 3-5	Anderson *et al.*
pGA204	C-terminal deletion, alpha helices 4-5	Anderson *et al.*
pGA205	C-terminal deletion, alpha helix 5	Anderson *et al.*
pGA206	Mg^{2+} binding site 1 (pore), point mutation	Anderson *et al.*
pGA207	Mg^{2+} binding sites 2/3 (connecting helix), point mutation	Anderson *et al.*
pGA208	Mg^{2+} binding site 4 (cytosol), point mutation	Anderson *et al.*
pGA209	Mg^{2+} binding site 5 (cytosol), point mutation	Anderson *et al.*
pGA210	Mg^{2+} binding site 6 (cytosol), point mutation	Anderson *et al.*
pMQ72	control empty expression vector with gentamicin-resistance gene	Shanks *et al.*

Table 3: Primers.

Primer	Description	Sequence (5' - 3')
729	verify *mgtE* mutants	CAGACCGCTTCTGCGTTCTG
730	verify *mgtE* mutants	GCAACTCTCTACTGTTTCTCC
913Nleftfor	construct *mgtE* mutants	TCGAGATAACCGTTGGCCTC
913Nrightrev	construct *mgtE* mutants	CAGACCGCTTCTGCGTTCTG
913Nrightfor	construct *mgtE* mutants	GAATTCAGGAGGAGCGCGCTATGGAAGGCCAGCA CCATGACCG
913Nleftrev	construct *mgtE* mutants	CGGTCATGGTGCTGGCCTTCCATAGCGCGCTCCTCC TGAATTC
DgkAfusfwd	construct *mgtE* mutants	GCAAGTGCTGGAAGTCATGGCCACCGACCCGGTGA CCTTCGTGTCGCCTTCCCCCTTCAA
DgkAfusrev	construct *mgtE* mutants	CTGTATCAGGCTGAAAATCTTCTCTCATCCGCCAA AACAGTCAGCCCAGCAGGATGGTCG
MgtNCBSDelRfor	N-terminal truncations	GAATTCAGGAGGAGCGCGCTATGGAAGTCCTCAA CATGGCCGGTC
MgtNDomDelRfor	N-terminal truncations	GAATTCAGGAGGAGCGCGCTATGGAAAGCCTCGA CGCGCAGCAG
MgtNDomDelLrev	N-terminal truncations	CTGCTGCGCGTCGAGGCTTTCCATAGCGCGCTCCTC CTGAATTC
MgtNCBSDelLrev	N-terminal truncations	GACCGGCCATGTTGAGGACTTCCATAGCGCGCTCC TCCTGAATTC

Table 4: Summary of Magnesium Transport Assays.

Plasmid	Brief Description	Transports Magnesium
pBC101	TMD (C-terminal) replacement	NO
pBC102	N-terminal deletion	WEAKLY
pBC103	N-terminal deletion	NO
pBC104	N-terminal deletion	SPOTTY
pBS23	Mg^{2+} binding site point mutation	NO
pGA200	full-length MgtE	YES
pGA201	C-terminal deletion	NO
pGA202	C-terminal deletion	NO
pGA203	C-terminal deletion	SPOTTY
pGA204	C-terminal deletion	NO
pGA205	C-terminal deletion	NO
pGA206	Mg^{2+} binding site point mutation	NO
pGA207	Mg^{2+} binding site point mutation	YES
pGA208	Mg^{2+} binding site point mutation	YES
pGA209	Mg^{2+} binding site point mutation	YES
pGA210	Mg^{2+} binding site point mutation	YES
pMQ72	control empty vector	NO

FIGURES

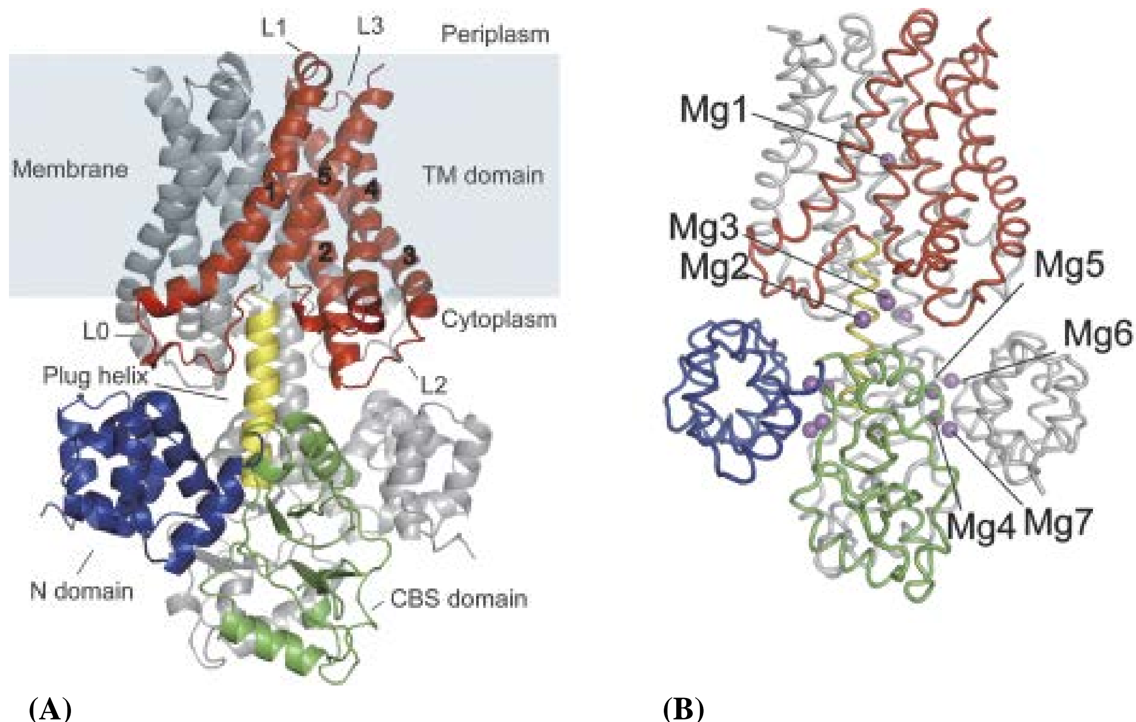


Figure 1: Structure of MgtE. The crystal structure of MgtE was resolved in *Thermus thermophilus* in 2007 by Hattori *et al.* **(A):** MgtE is believed to function as a homodimer. Figures depict one monomer in color, with the transmembrane domain in red, the connecting (plug) helix in yellow, the CBS domain in green, and the N domain in blue. The other monomer is shown in gray. **(B):** Figure illustrates seven putative magnesium binding sites, with magnesium ions shown as purple balls.

Hattori, M., et al., *Mg(2+)-dependent gating of bacterial MgtE channel underlies Mg(2+) homeostasis.* EMBO J, 2009. **28**(22): p. 3602-12.

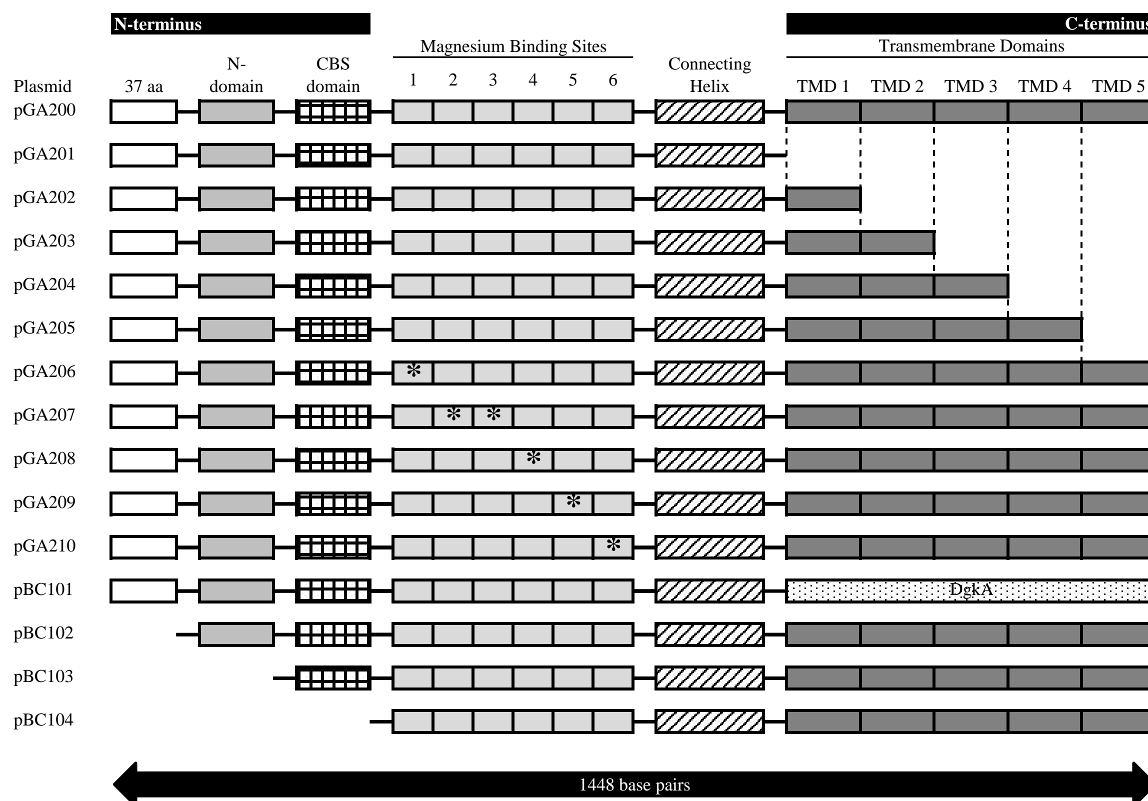


Figure 2: Schematic of MgtE Mutations. Mutations were made to the *mgtE* gene and cloned into the pMQ72 expression vector. Plasmid pGA200 carries full-length *mgtE* with no mutations. Plasmids pGA201 through pGA205 carry C-terminal truncations. Plasmids pGA206 through pGA210 carry point mutations in the magnesium binding sites. pBC101 is a replacement of the entire transmembrane domain. pBC102 through pBC104 are N-terminal truncations. As shown, relative lengths and positions of protein domains are purely diagrammatic and not to scale.

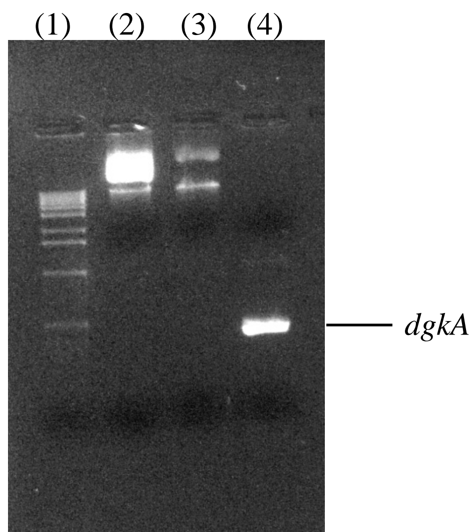
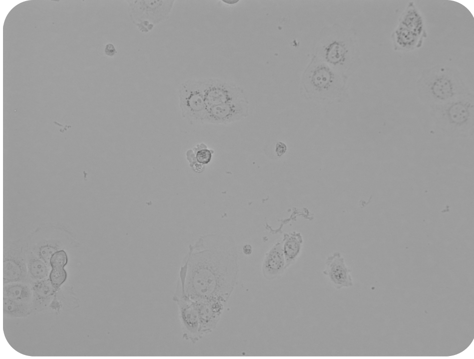
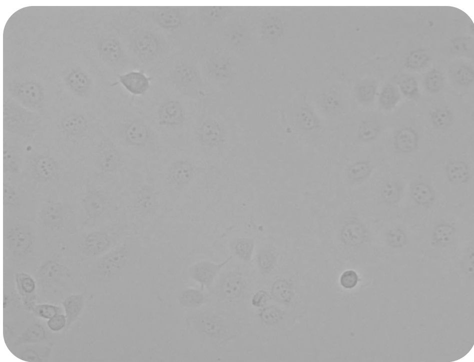


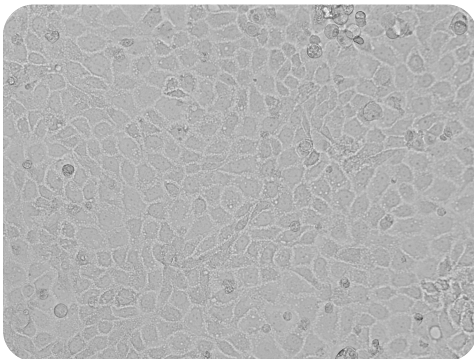
Figure 3: PCR of DkgA. DgkA is a heterologous transmembrane protein in *P. aeruginosa* which was used to replace the transmembrane domain in MgtE. *dgkA* was PCR amplified from strain PA14 using primers DgkAfusfwd and DgkAfusrev. Lane 1: Molecular weight marker. Lane 2: Plasmid pGA200. Lane 3: Plasmid pGA200 digested with HindIII. Lane 4: PCR amplified fragment of *dgkA*. Expected fragment size is 372 base pairs. Gel electrophoresis was performed on 1% agarose gel.



2 days' CFBE cell growth



4 days' CFBE cell growth



7-10 days, confluent monolayer of CFBE cells

Figure 4: Cystic Fibrosis Bronchial Epithelial Cells. Immortalized human-derived cells express CFTR ΔF508, the mutation most often found to be the cause of cystic fibrosis.

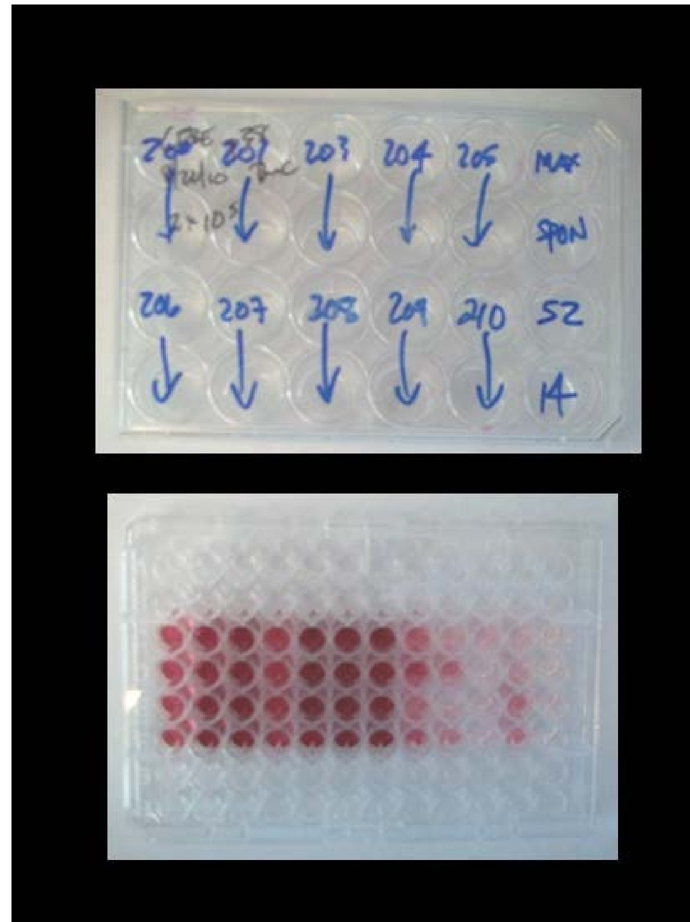


Figure 5: Cytotoxicity Assay. Top image is a 24-well tissue culture plate in which CFBE cells have been growing for 7-10 days to reach confluent monolayer. Bacterial cultures were added for assay. Wells are labeled according to the plasmid constructs being tested. Bottom image is 96-well microtiter plate containing assayed samples. Darker color indicates higher level of cytotoxicity, measured by LDH release.

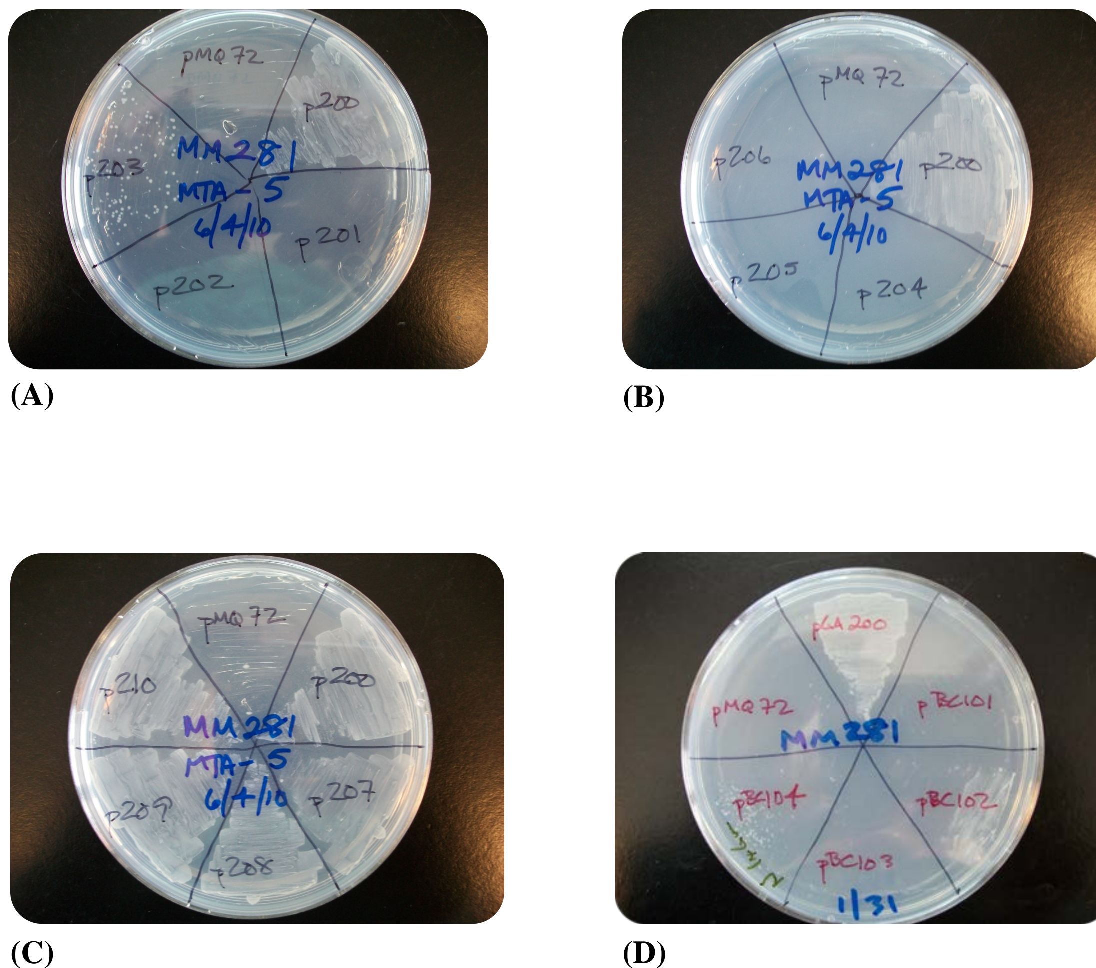


Figure 6: Magnesium Transport Assays. Ability of *mgtE* mutants to complement magnesium transport was assessed by growth on minimal media. Growth is observed as white, cloudy areas. pMQ72 is empty vector used as negative control. pGA200 is full-length *mgtE* used as positive control. **(A), (B):** Magnesium transport complementation by *mgtE* with C-terminal truncations of transmembrane domains, plasmids pGA201 through pGA205. Anomalous spotty growth is visible in pGA203. **(B), (C):** Magnesium transport complementation by *mgtE* with magnesium binding site mutations, plasmids pGA206 through pGA210. **(D):** Magnesium transport complementation by *mgtE* with N-terminal truncations, plasmids pBC101 through pBC104. Light growth is seen in pBC102, and spotty growth is visible in pBC104.

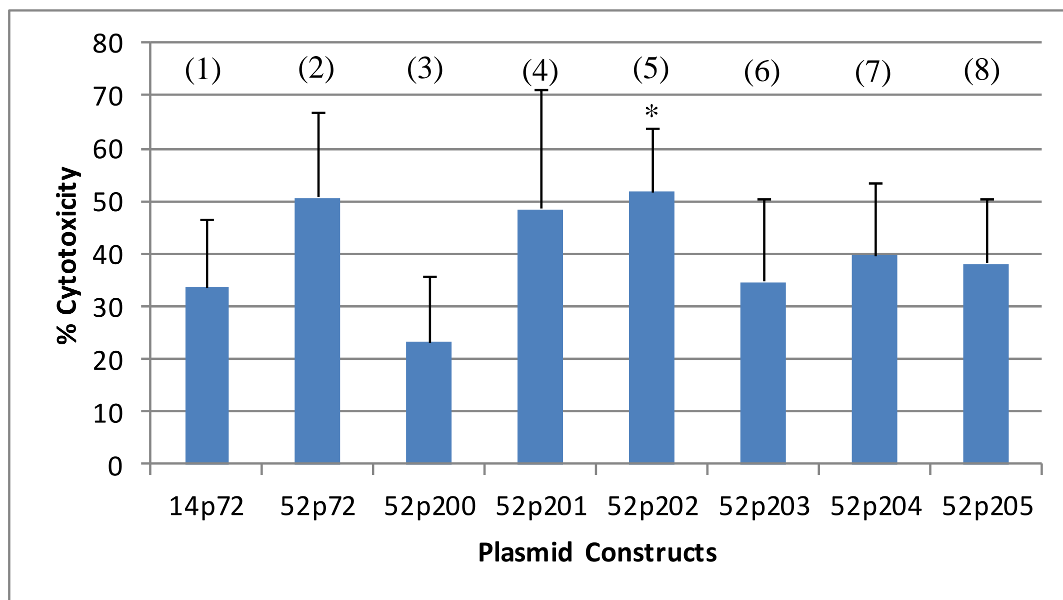


Figure 7: Cytotoxicity Assays, C-Terminal Truncations. C-terminal truncations (lanes 4-8) demonstrate decreased regulation of cytotoxicity compared to overexpression of full-length MgtE (lane 3). These results suggest that the full C-terminal transmembrane domain is required for regulation of cytotoxicity. The X-axis displays controls and plasmid constructs. On the Y-axis, percent cytotoxicity is normalized as a percentage of maximum LDH release by CFBE cells following treatment with Triton® X-100. Lane 1 is wild type PA14 with empty vector. Lane 2 is GGA52 (PA14Δ*mgtE*) with empty vector. Lane 3 is GGA52 with pGA200 (full-length MgtE). Lanes 4, 5, 6, 7, and 8 are progressive truncations of the five C-terminal transmembrane alpha-helices. Data is representative of 20 independent experiments. *, $P < 0.05$.

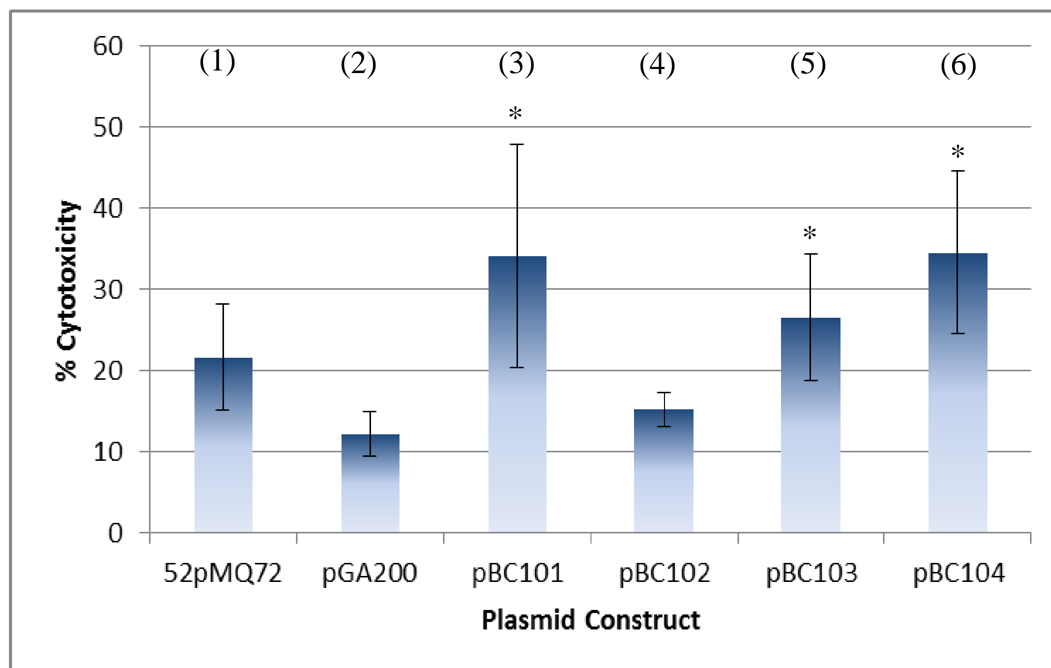


Figure 8: Cytotoxicity Assays, N-Terminal Truncations and TMD Replacement. Results demonstrate that increasing truncations of the N-terminal region of MgtE result in increasing loss of regulation of cytotoxicity. Replacement of the C-terminal transmembrane domain with a heterologous transmembrane protein also results in inability to regulate cytotoxicity. The X-axis displays controls and plasmid constructs. On the Y-axis, percent cytotoxicity is normalized to maximum LDH release by CFBE cells following treatment with Triton® X-100. Lane 1 is GGA52 (PA14 Δ*mgtE*) with empty vector. Lane 2 is GGA52 with pGA200 (full-length MgtE). Lane 3 (pBC101) is a replacement of the transmembrane domain with the heterologous transmembrane protein DgkA. Lane 4 (pBC102) is a truncation of 37 amino acids (111 base pairs) from the N-terminus. Lane 5 (pBC103) is a deletion of 303 N-terminal base pairs. Lane 6 (pBC104) is a truncation of 324 N-terminal base pairs. Data is representative of 7 independent experiments. *, $P < 0.05$.

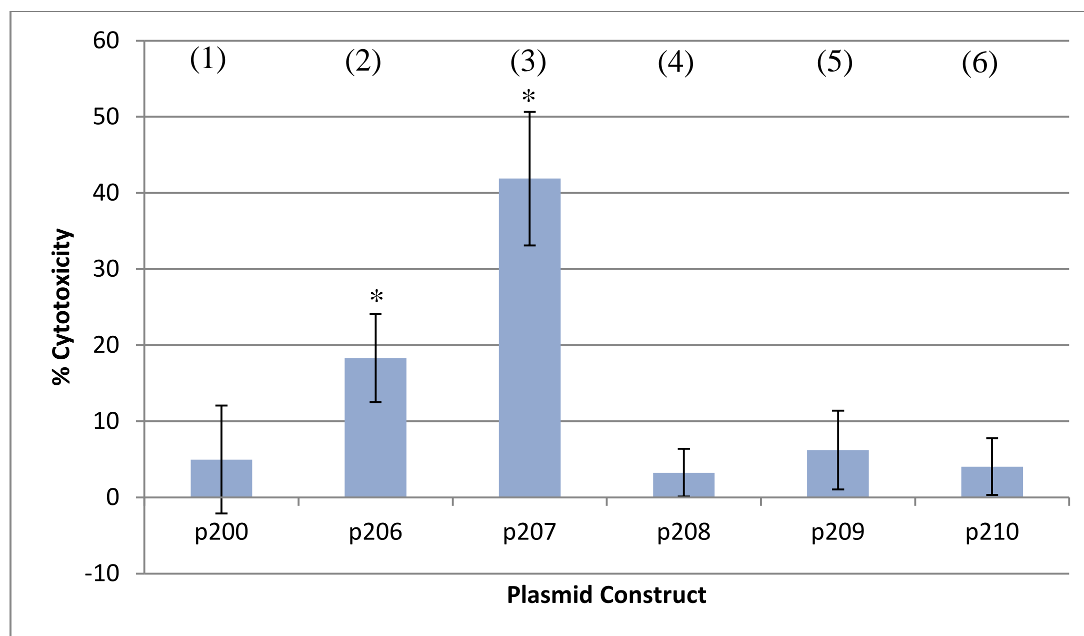


Figure 9: Cytotoxicity Assays, Magnesium Binding Site Point Mutations. Results demonstrate varying effects of magnesium binding site mutations on the regulation of cytotoxicity. Data also suggests that the magnesium binding site in the transmembrane pore (pGA206, lane 2) is essential for regulating cytotoxicity. The X-axis displays controls and plasmid constructs. On the Y-axis, percent cytotoxicity is normalized to maximum LDH release by CFBE cells following treatment with Triton® X-100. All lanes are GGA52 (PA14 Δ*mgtE*) with plasmids. Lane 1 is full-length MgtE. Lane 2 is magnesium binding site 1, located in the transmembrane domain. Lane 3 is binding sites 2 and 3, located in the connecting helix. Lanes 4, 5, and 6 are point mutations in the CBS (cytosolic) domain. Data is representative of 20 independent experiments.
*, $P < 0.05$.

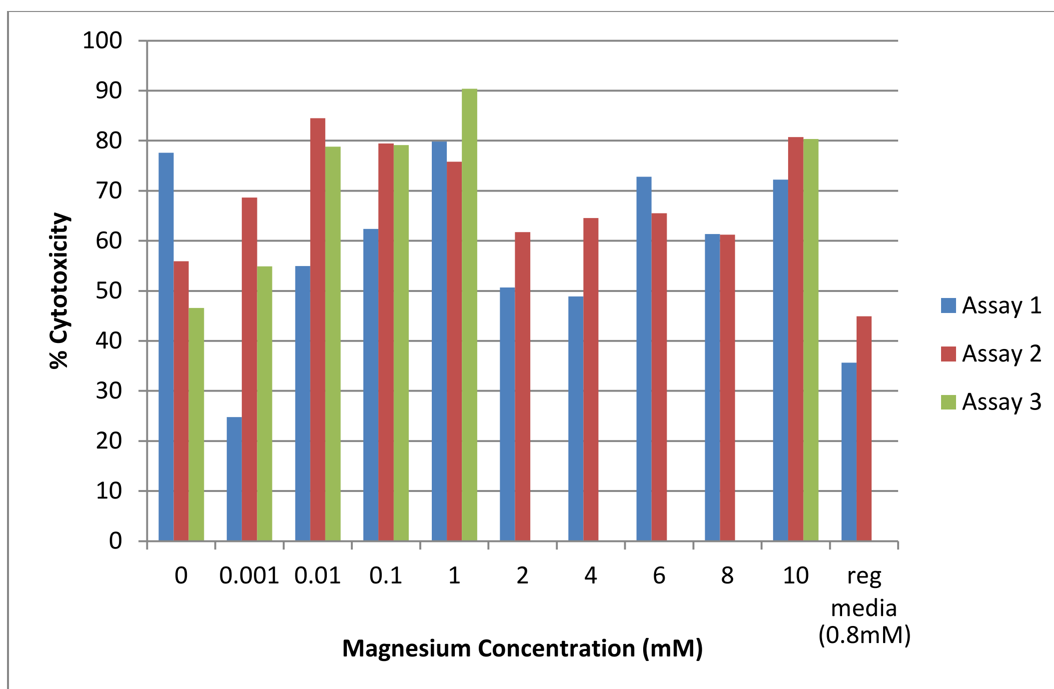


Figure 10: Magnesium Concentration. Levels of extracellular magnesium affect wild type *P. aeruginosa* PA14 cytotoxicity toward CFBE cells. As magnesium concentrations in the media were increased from 1μM to 1.0mM, a corresponding increase in cytotoxicity was observed. No further increases in cytotoxicity were noted at concentrations of 2mM to 10mM.

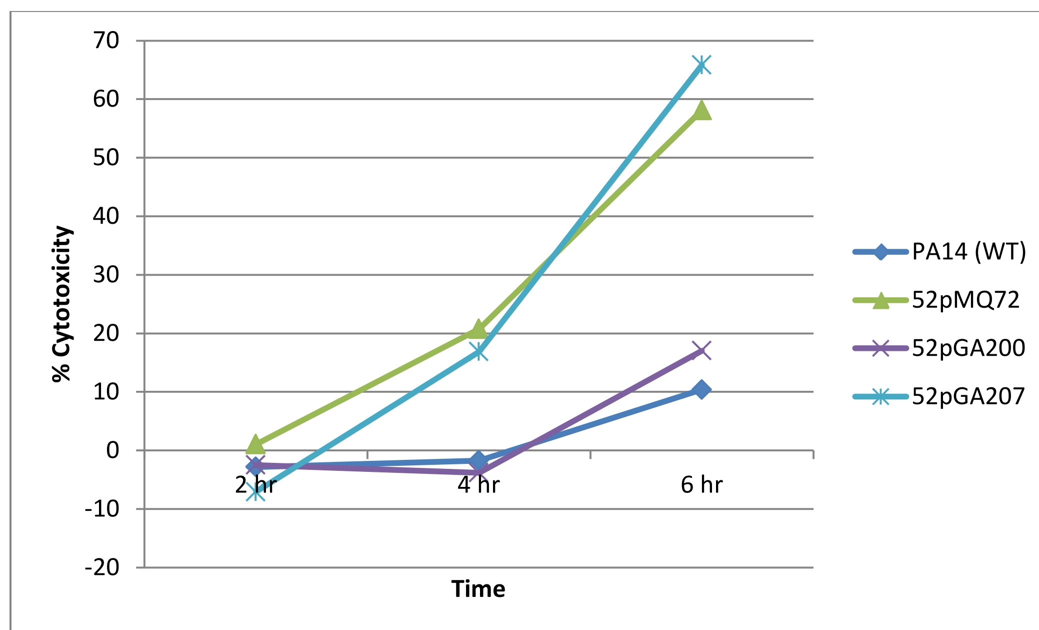


Figure 11: Kinetics of Cytotoxicity. Levels of cytotoxicity increase over time. In *mgtE* mutants, cytotoxicity increases more rapidly between 2 and 4 hours compared to wild type and over-expression of full-length *mgtE*. Construct 52pGA200 is over-expression of full-length *mgtE*. 52pGA207 is a mutation in magnesium binding sites 2 and 3, shown in previous experiments to lack cytotoxicity-regulating function. 52pMQ72 is empty expression vector. Results are representative of three independent experiments.