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By Carly Virginia Redelman

Entitled
Antibiotic Treatment of Pseudomonas aeruginosa Biofilms Stimulates Expression of mgtE, a Virulence Modulator

For the degree of Master of Science

Is approved by the final examining committee:

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ANTIBIOTIC TREATMENT OF *Pseudomonas aeruginosa* BIOFILMS STIMULATES EXPRESSION OF *mgtE*, A VIRULENCE MODULATOR

A Thesis

Submitted to the Faculty

of

Purdue University

by

Carly Virginia Redelman

In Partial Fulfillment of the

Requirements for the Degree

of

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There are many people who have both influenced and supported me in achieving this degree including my family, my parents Robert and Karen Asher and my sister and brother, Nicole and Benjamin Asher. Also, I need to thank my "family at IUPUI", fellow graduate students and friends, for laughs and camaraderie that made hard days in the lab seem not so bad. Lastly, this thesis is dedicated to my "kids", Stuart, Sheldon, Elsa, Izzy, and especially my husband, Ryan Redelman. He has been an unwavering support throughout my two years at IUPUI. He has given me the gifts of unconditional understanding and inconceivable strength. He has helped me develop the perseverance needed to be successful, and I am eternally grateful.

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NOMENCLATURE

SYMBOL	DESCRIPTION
Δ	Deletion, in reference to a gene
ASL	Airway Surface Liquid
CF	Cystic Fibrosis
CFBE	Cystic Fibrosis Bronchial Epithelial cells, CFBE41o-
CFTR	Cystic Fibrosis Transmembrane Conductance Regulatory protein
HRP	Horseradish peroxidase
LB	Lysogeny Broth
LDH	Lactate Dehydrogenase
MIC	Minimal Inhibitory Concentration
PA14	Wild Type *Pseudomonas aeruginosa* lab strain
RT-PCR	Semi-quantitative reverse transcriptase polymerase chain reaction
QRT-PCR	Quantitative real time polymerase chain reaction
WT	Wild type

ABSTRACT

Redelman, Carly Virginia. M.S., Purdue University, August 2011, Antibiotic Treatment of *Pseudomonas aeruginosa* Biofilms Stimulates Expression of *mgtE*, a Virulence Modulator. Major Professor: Gregory G. Anderson.

Pseudomonas aeruginosa is a gram negative opportunistic pathogen with the capacity to cause serious disease by forming biofilms, most notably in the lungs of cystic fibrosis (CF) patients. Biofilms are communities of microorganisms that adhere to a solid surface, undergo global regulatory changes, secrete exopolysaccharides, and are innately antibiotic resistant. Virulence modulation is an important tool utilized by *P. aeruginosa* to propagate infection and biofilm formation in the CF airway. Many different virulence modulatory pathways and proteins have been identified including the protein, MgtE. MgtE has recently been discovered and has been implicated in virulence modulation, as an isogeneic mutation of *mgtE* leads to increased cytotoxicity. To further elucidate the role of MgtE in *P. aerugionsa* infections, transcriptional and translational regulation of this protein following antibiotic treatment has been explored. I have demonstrated that *mgtE* is transcriptionally upregulated following antibiotic treatment of most of the twelve antibiotics tested utilizing RT-PCR and QRT-PCR. A novel model system was employed, which utilizes cystic fibrosis bronchial epithelial (CFBE) cells homozygous for the ΔF508 mutation for these studies. This model system allows *P. aeruginosa*

biofilms to form on CFBE cells modeling the *P. aeruginosa* in the CF airway. Translational effects of antibiotic treatment on MgtE have been attempted via Western blotting and cytotoxicity assays. Furthermore, to explore the possibility that *mgtE* is interacting with a known regulatory pathway, a transposon-mutant library was utilized and the regulatory proteins, AlgR and NarX, among others have been identified as possibly interacting with MgtE. Lastly, an MgtE homologue from *Staphylococcus aureus* was utilized to further demonstrate the virulence modulatory effects of MgtE by demonstrating the expression of the homologue results in decreased cytotoxicity, exactly like expression of the native *P. aeruginosa* MgtE. This research explores a newly discovered protein that impacts cytotoxicity and biofilm formation and provides valuable information about *P. aeruginosa* virulence.

CHAPTER ONE INTRODUCTION

1.1 What is a Biofilm?

It is becoming increasingly clear that bacteria persist in environmental and host niches as complex, heterogeneous communities called biofilms. Following the early characterizations of biofilms in the 1970s by William Costerton and other researchers[1], these microcolony-derived structures have been identified as ubiquitous entities in nature as well as in industrial and clinical environments[2]. Furthermore, fossilized biofilm microcolonies, identified via morphology, indicate biofilm formation occurring in certain environments, such as hydrothermal niches, dating back 3.2-3.4 billion years ago. These findings implicate biofilm formation as an innate and evolutionarily selected trait in prokaryotes[3,4]. It is likely that biofilm formation evolved to protect the prokaryote against the changing surroundings of primitive earth[5]. Convergent prokaryotic biofilm evolution is revealed as phenotypic similarities that are consistent in similar environments. Changes in the environments in which biofilms grow, such as moving waters versus quiescent waters, results in phenotypic changes in the biofilm, such as filamentous streamer formation versus mushroom-like appendages, respectively[6]. Undoubtedly, as biofilm formation is still apparent in many different environments and causes exacerbated medical conditions, this evolutionary trait allowing adherence to solid surfaces still provides a selective advantage to prokaryotes.

Biofilms are traditionally described as sessile bacteria adhered, most likely permanently, to a solid surface followed by global regulatory changes leading to morphologic, phenotypic, and genetic changes in the microcommunity that is formed in comparison to planktonic, or free swimming bacteria. Biofilms are architecturally complex and are analogous to microbial towns, in which bacteria work together to survive. Biofilms can be composed of a single species of bacteria, such as *P. aeruginosa* biofilms infecting the cystic fibrosis (CF) airway, or as multi-species biofilms, such as dental plaque, which can contain up to 700 species[7]. *P. aeruginosa* biofilms have been shown to cause a chronic airway infection in patients with CF; therefore, this particular biofilm forming bacterium is the focus of much research in an attempt to understand how this microorganism accomplishes this effect. Proteomic studies have demonstrated *P. aeruginosa* biofilms form in four distinct stages (Figure 1).

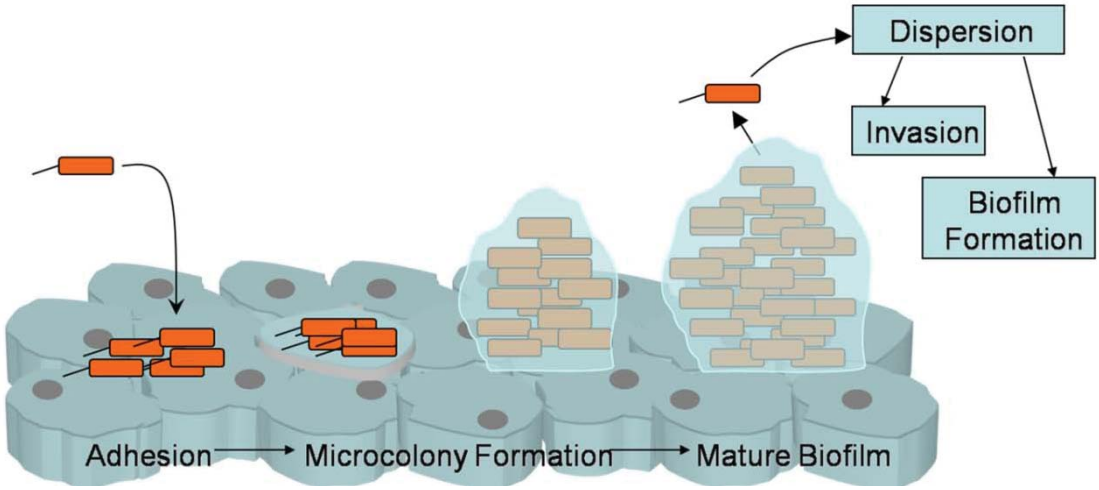

Figure 1. Biofilm formation and cycling. *P. aeruginosa* forms biofilms in four stages leading to the formation of a mature biofilm. Stage one is the initial adhesion of the bacteria followed by microcolony formation in stage two. Stage three results in the formation of a mature biofilm, and stage four occurs when planktonic bacteria disseminate from the biofilm in a process called dispersion. Dispersion may lead to further biofilm formation which is known as biofilm cycling.

Biofilm formation begins in stage one by loose, transient binding of planktonic bacteria to a solid surface followed by significant adhesion. Stage two involves formation of microcolonies via aggregation of bacterial cells followed by growth and maturation leading to the formation of a mature biofilm in stage three[8]. Significant changes in gene expression occur throughout the biofilm maturation process. Phenotypically, a mature biofilm may be mushroom shaped or flat depending on nutrient availability and environmental conditions, such as water flow[9]. Lastly, stage four is marked by shedding from the mature biofilm, where bacterial constituents are able to leave the biofilm as either planktonic bacteria or as microcolonies, causing acute exacerbations and/or further colonizing the host with biofilms. This stage is commonly described as dispersal[8]. Dispersal is an advantageous strategy. As the biofilm ages, nutrient acquisition may become limited and waste may accumulate. When this occurs, it is beneficial for biofilm constituents to be able to disseminate from the biofilm, and there are certain specific agents that allow them to do so[10]. As discussed later, biofilm attachment and dispersal are both exciting and important endeavors being pursued by many researchers in studying disease causing biofilms and will be discussed in more detail later in this chapter.

Regardless of phenotypic or bacterial composition, biofilms exhibit similar characteristics that allow these communities to successfully survive in many different environments. These characteristics include the formation of water channels and secretion of exopolysaccharides. Both have been observed using confocal laser microscopy where it is possible to visualize sessile bacteria surrounded by a matrix of polysaccharides with water channels forming internally[11]. Water channels are part of the

complex architecture of biofilms. They allow nutrient movement around the biofilm to the sessile bacterial inhabitants and removal of waste away from the bacterial constituents[12], linking form with function. Exopolysaccharide secretion not only adds volume to the biofilm, but also protects the biofilm from a potentially harmful environment. Exopolysaccharides are able to trap molecules such as antibiotics and biocidals preventing interaction of these harmful compounds with bacterial constituents[13] and contributing to biofilms exhibiting an innate antibiotic resistance. These observations indicate the complexity of biofilms, revealing an order and function to the biofilm community.

Both genetic and environmental factors influence biofilm structure, attachment, and development. Random transposon mutations and deletion mutations have identified genes that play important roles in biofilm formation. These studies compare mutants to the wild type background strain looking for impairment in the mutant's ability to form biofilms. Genes that have been identified as crucial to biofilm formation include those that regulate or express surface adhesion proteins, genes encoding pili, flagella or extracellular matrix material, and many regulatory pathway proteins[14-17]. Although deletion mutations of many of these genes do not fully impair biofilm formation, they may either retard or limit biofilm growth. This demonstrates the convergence of many genes and pathways in the prokaryote's ability to form biofilms and, once again, conveys the complexity of biofilm formation and maintenance.

1.2 Biofilms and Human Disease: A Selective Advantage

Biofilm formation protects microbial inhabitants, and biofilms are innately resistant to biocidal agents. This type of resistance is especially important in medically relevant biofilms. As alluded to earlier, bacteria in biofilms secrete an exopolysaccharide matrix which protects bacterial constituents from reactive, charged, or large antimicrobial agents through neutralization or binding, therefore diluting these agents to nonlethal concentrations[5]. Many antimicrobial agents can penetrate this first protective mechanism only to find the bacteria within the biofilm to be in a stationary phase of growth rendering many antimicrobials ineffective, particularly certain antibiotics[18]. Bacterial constituents of a biofilm often experience limited nutrient availability, which promotes entrance into a stationary phase in which there is little to no growth of the bacteria[19] (Figure 2).

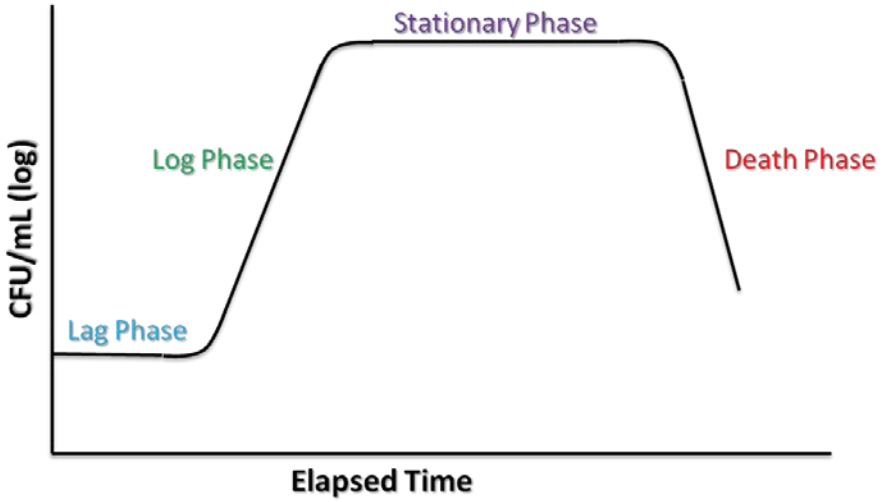

Figure 2. Bacterial growth curve. Bacteria undergo phases of growth from an exponential growth to stationary growth to death.

Growth rate is a primary modulator of antibiotic action; therefore, bacteria that have left the logarithmic stage of growth are less susceptible to antibiotic induced killing[20]. Furthermore, biofilm growth is a perfect environment for gene transfer leading

to antibiotic resistant communities. Lastly, sessile bacteria know as "persisters" are another important contribution to the persistence of biofilms as their name indicates[18]. They are a dormant phenotype evident in many different bacterial biofilms. Persisters are a separate group of bacterial constituents distinct from both growing and stationary phase biofilm constituents. They are tolerant to antibiotics, and, generally, will be the only bacterial cells in a biofilm to survive high antibiotic dosages. Their mechanism of tolerance is distinct from antibiotic resistance[21]. Overexpression of persister genes leads to this phenotype, and these genes shut down important cellular functions rendering the antibiotic useless[22]. All of these components, including exopolysaccharide secretion, growth phase changes, gene transfer, and persister cells, join to result in biofilm persistence in many different environments, probably the most important environment being the infected host organism.

1.3 Modeling Biofilms

There are numerous examples of biofilm involvement in many different infections. In fact, more than 60% of bacterial infections treated in the developed world are believed to involve biofilm formation[23], and there is a constant stream of new information elucidated through ongoing biofilm pathogenesis research. Bacterial biofilms are implicated in a striking number of chronic disease states, including dental disease[24], medical device-related infections[25], infective endocarditis[26], otitis media[27], rhinosinusitis[28], chronic wounds[29], urinary tract infections[30], gastrointestinal tract infections[31], and respiratory infections such as *Pseudomonas aeruginosa* infections in the

CF airway[32]. These chronic infectious states are caused by many different types of bacteria and even yeast, demonstrating that many different microbes have the capability to form biofilms as a pathogenic strategy. To maintain consistency when identifying and examining biofilm induced disease states, four criteria have been proposed to define biofilm etiology during an infection[2]. These criteria include: surface associated pathogenic bacteria, clustered bacteria encased in a matrix substance as revealed by direct examination, localization of the infection, and antibiotic resistance. There are different co-culture assays designed to study biofilm formation in a pathogenic setting by modeling the infection using immortalized or primary animal and human cells.

Bacterial/human cell biofilm co-culture assays are generally divided into three different techniques: static systems, flow systems, and human model infections. The static co-culture system is demonstrated by Anderson *et al.* where standard tissue culture plates are seeded with CF-derived human airway cells, Cystic Fibrosis Bronchial Epithelial (CFBE) cells[33]. The epithelial cells are allowed to grow to confluency, and then inoculated with a broth culture of *P. aeruginosa*[33]. *P. aeruginosa* biofilms form within 6-8 hours after inoculation (Figure 3). The static co-culture system has been used to ascertain antibiotic resistance as well as effects of antibiotics on gene regulation[33] and cytotoxicity[34]. As shown below, there are many other examples of static co-culture biofilm systems (Table 1).

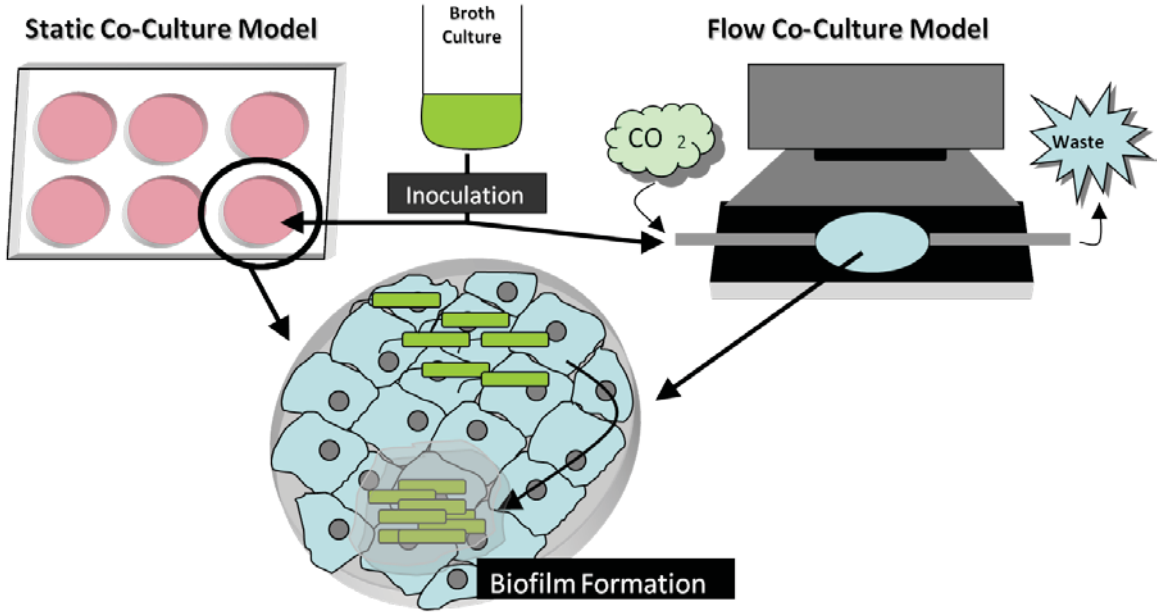

Figure 3. Co-culture biofilm model systems. Two co-culture model systems employed to pattern biofilm pathogenesis. The main difference between the two systems is how the conditions for growth are delivered, whether statically in an incubator or as part of a flow apparatus.

Table 1. Static and flow human cell co-culture model systems.

Co-culture Models	Organism	Cell Line	Reference
Static	*Pseudomonas aeruginosa*	Cystic Fibrosis Bronchial Epithelial cell line, CFBE41o-	[33]
	Fusobacterium nucleatum *Porphyromonas gingivalis*	Periodontal Ligament Fibroblasts	[35]
	Streptococcus pneumoniae	Conjunctival Epithelial cell line, HCjE and HME-HCjE	[36]
	Fusobacterium nucleatum *Porphyromonas gingivalis*	Gingival Epithelial cell line, Ca9-22 Aortic endothelial cell line, HAEC	[37]
	Francisella tularensis	Epithelial cell lines, HEp-2 and A549 Bronchial cell line, HBE	[38]
	Streptococcus agalactiae	Brain Microvascular Endothelial cell line, HBMEC	[39]
	Campylobacter jejuni	Intestinal Epithelial cell line, Caco-2 Embryonic Intestinal Epithelial cell line, INT407	[40]
	Escherichia coli, uropathogenic (UPEC)	Bladder Epithelial cell line, ATCC HTB-9 5637	[41]
	Neisseria gonorrhoeae	Endocervical Epithelial cell lines, ME-180 and HeLa	[42]
	Neisseria gonorrhoeae	Endocervical Epithelial cell line, End/E6E7	[43]
	Streptococcus salivarius	Bronchial Epithelial cell line, 16HBE14o-	[44]
	Lactobacillus rhamnosus *Prevotella bivia* *Gardnerella vaginalis* *Candida albicans*	Normal Endocervix cell line, End1/E6E7 ATCC-CRL-2615, Ectocervix cell line, Ect1/E6E7 ATCC-CRL-2614, Vaginal cell line, VK2/E6E7 ATCC-CRL-2616	[45]
	Lactobacillus helveticus *Campylobacter jejuni*	Colon T84 cell line and Intestinal 407 cell line	[46]
	Fusobacterium nucleatum *Streptococcus gordonii*	Immortalized Gingival Keratinocytes, HIGK	[47]
	Porphyromonas gingivalis	Oral Keratinocytes, TERT-2	[48]
Flow	*Pseudomonas aeruginosa*	Cystic Fibrosis Bronchial Epithelial cells, CFBE41o-	[49]
	Salmonella Typhimurium	HEp-2 cell line	[50,51]
	Salmonella Typhimurium *Escherichia coli*	HEp-2 cell line	[52]

The flow co-culture systems are modifications of standard biofilm flow cell apparatuses, wherein the abiotic biofilm substratum (glass or plastic coverslip or capillary) is replaced by a coverslip supporting a monolayer of cultured human cells. For instance, Moreau-Marquis *et al.* employed the FCS2 closed system to grow *P. aeruginosa* biofilms on human CFBE cells under flow[53] (Figure 3). They used this model to investigate the integrity of the CFBE monolayer and the growth of *P. aeruginosa* on the monolayer (Table 1). These studies were aided by the ability to acquire high-quality images by confocal microscopy[49].

The literature review and research presented in this thesis will focus on chronic *Pseudomonas aeruginosa* infection in the CF airway. This chronic state is consistent with the above biofilm criteria, and has been characterized by an abundance of literature as a biofilm infectious state. Furthermore, the static co-culture biofilm method is used to understand *P. aeruginosa* infection in the CF airway[2,23,54].

1.4 Infection Strategies Utilized by *P. aeruginosa*

P. aeruginosa is a gram negative bacterium implicated in a multitude of diseases ranging from burn infections[55] to airway infections[56]. CF airway infections, by this bacterium, have elicited much interest by researchers, because *P. aeruginosa* dominates as the primary infectious agent in this genetic disease by late childhood leading to increased morbidity and mortality[56]. *P. aeruginosa* has a multitude of virulence factors including the type three secretion system (T3SS), biofilm formation, certain regulatory pathways (alginate pathway among others), and quorum sensing (Table 2).

Table 2. Common virulence factors expressed by *P. aeruginosa*.

Virulence Factor	Effect on Host
Type III Secretion System	Syringe-like appendage from gram-negative bacteria and functions to translocate toxins from bacterial cells across host cell membrane
Biofilm Formation	Community of bacteria undergoing global regulatory changes and implicated in chronic disease states
Quorum Sensing	Autoinducers released by bacteria as means of communication and implicated in biofilm formation and toxin production
Two-Component Regulatory Systems	Composed of an inner membrane histidine kinase and a cytoplasmic response regulator and implicated in biofilm formation, toxin production, and antibiotic resistance

The T3SS is mainly found in gram-negative bacteria, and the expression of the T3SS is thought to contribute to the bacterium's ability to successfully evade phagocytosis, an immune response by host cells following bacterial infection. The T3SS is composed of a macromolecular complex that is able to translocate effector proteins (toxins), named ExoS, ExoT, ExoU, and ExoY[57], across the eukaryotic host epithelial cell membrane barrier[58]. These toxins, once injected, have anti-host properties. ExoS and ExoT disrupt actin and cytoskeleton rearrangement, focal adhesins, and signal transduction cascades in host phagocytes thereby inhibiting their function[59]. ExoU and ExoY are cytotoxins that localize to host organelle membranes and exact their detrimental effects on the host in different ways. ExoU has phospholipase A_2 activity that can induce cell death. ExoY induces inflammatory reactions by generating arachidonic acid which is the substrate for prostoglandin generating pathways[57,59]. T3SS expression in *P. aeruginosa* is influenced by certain environmental factors, mainly contact with host cells and extracellular calcium concentrations in the micromolar range[59]. These environmental factors influence strict transcriptional regulation through the global activator regulatory protein, ExsA, and three interacting proteins, ExsC, ExsD,

ExsE, that form alternate binding interactions in the on and off states[60]. Interestingly, secretion of T3SS proteins is associated with increased morbidity in patients with *P. aeruginosa* airway infections demonstrating that the T3SS is both a pathogenic and prognostic indicator[57].

Quorum sensing (QS) is a virulence factor that *P. aeruginosa* employs during biofilm formation and toxin production. QS allows individual *P. aeruginosa* bacteria to communicate with one another. Small compounds called autoinducers are released into the environment by a bacterium, and other bacteria in the same environment are able to sense these compounds at certain concentrations. Autoinducers communicate both the presence of the bacterial population and gene expression between neighboring bacteria[57]. Quorum sensing in *P. aeruginosa* is mediated through three interconnecting systems, the Las system, the Rhl system, and the Pqs system. These systems sense three distinct autoinducers and play specific roles in biofilm regulation and development such as the characteristic cap of the mushroom shaped biofilm, regulation of certain known components of the biofilm channel maintenance and extracellular matrix (such as rhamnolipids and extracellular DNA, respectively[61]) and essential modulation of metabolic functions know to be required for biofilm formation, such as anaerobic nitrate respiration[62].

Biofilm formation in *P. aeruginosa* follows the general steps described in section 1.2. However, it is important to highlight the specific mechanism in which *P. aeruginosa* is able to form a biofilm. Initial attachment involves a variety of factors including flagella, type IV pili[63], extracellular DNA[64], and Psl polysaccharide[65]. Initial reversible attachment by the flagella does not commit the bacterium to irreversibly attach. When *P.*

aeruginosa attaches irreversibly to a surface, the bacterium will attach on its long axis to the solid surface (Figure 4)[66].

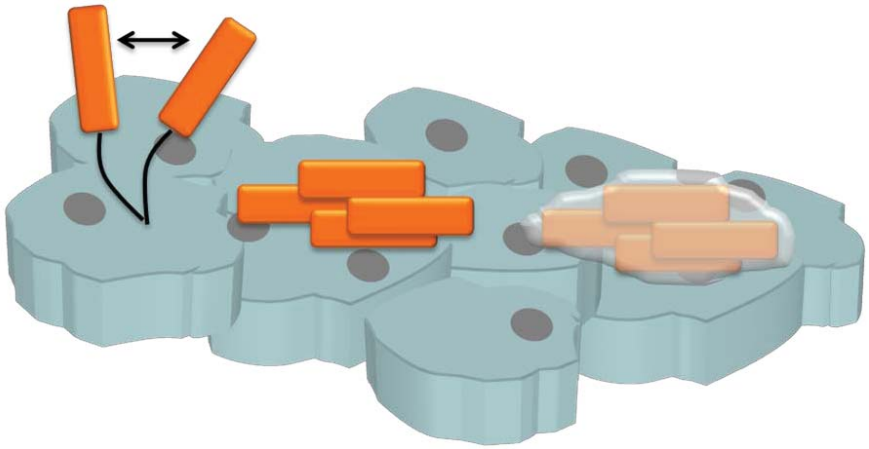

Figure 4. Reversible to irreversible attachment of *P. aeruginosa* to a solid surface during biofilm formation. Initially, reversible attachment occurs as the flagellum binds to a solid surface, such as epithelial cells. The bacterium will still be able to move with the flagella bound, and reversible attachment does not commit the bacterium to forming a biofilm through irreversible attachment. Irreversible attachment is marked by attachment of the bacterium to the solid surface via its long axis. Following this act, microcolony formation ensues, marked by exopolysaccharide secretion.

Exopolysaccharide matrix production is an important and highly regulated aspect of *P. aeruginosa* biofilm formation and maintenance. This matrix is composed of mainly polysaccharides, proteins, and nucleic acids[64], although the exact composition of this matrix will vary between *P. aeruginosa* biofilms based on the age of the biofilm and certain environmental factors. These environmental factors can be sensed by known sensor kinase/response regulators LadS, RetS, and GacS[67,68].

Following adherence and formation of the mature biofilm, dispersal from the *P. aeruginosa* biofilm by subpopulations of motile bacterial cells occur either as a necessary action of the infection to elicit further population of the host or as a result of unfavorable conditions, such as nutrient loss[69] or decreased carbon availability[70]. In a mature biofilm, a wall-forming subpopulation of non-motile cells constitutes the outer parts of the

microcolonies of the biofilm. For dispersion to occur, the core subpopulation of motile cells rapidly move toward this “wall ” and make their way out of the individual microcolony in which they were trapped. This results in a microcolony with a central void[71]. Dispersion is part of the natural process of biofilm formation and biofilm cycling, where bacteria are able to leave the biofilm and cause expansion of biofilm growth or acute exacerbations in a clinical setting.

Regulatory protein systems can impact virulence modulation, such as biofilm formation and expression of the T3SS by *P. aeruginosa*, and these systems are often categorized as two component regulatory systems. These systems generally comprise an inner membrane spanning sensor histidine kinase and a cytoplasmic response regulator[72], and are divided into three groups based on complexity (Figure 5). The basic mechanism of these regulatory systems is that the histidine kinase is auto-phosphorylated upon stimulation and activated at its N-terminal input domain. Following phosphorylation, the histidine kinase's transfer domain can phosphorylate and activate the response regulator via its conserved aspartate domain, which activates the output domain of the response regulator and leads to specific functions, such as activation or repression of transcription. Some regulatory pathways require an external phosphotransfer protein, thereby adding complexity to the basic pathway schematic[73].

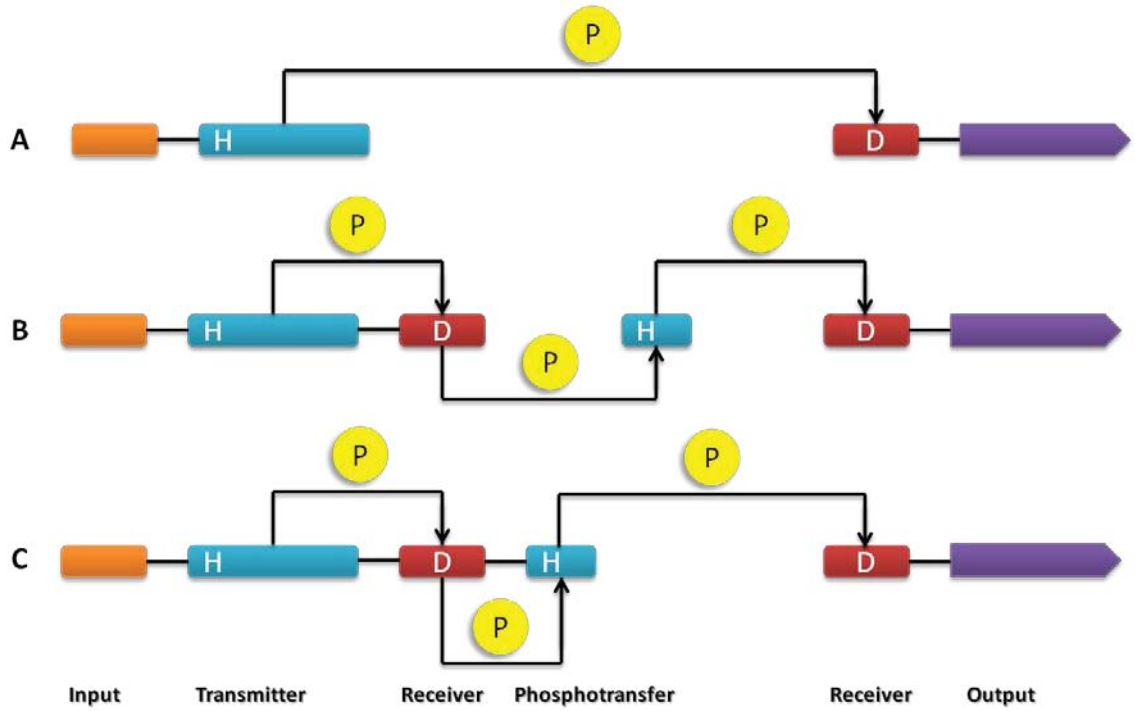

Figure 5. Two component regulatory pathway schematic. Two component regulatory systems can be divided into three categories based on the complexity of the system. The most basic system is the top system drawn (A), with the complexity increasing with the second (B) and third systems drawn (C).
(blue transmitter protein: histidine kinase with N-terminal input domain, red receiver protein: response regulator with conserved aspartate domain and output domain, and yellow circle: phosphorylation).

Table 3. Common two-component regulatory systems expressed by *P. aeruginosa*.

Regulatory System	Function
PhoP-PhoQ Regulatory System	Mediates response to low extracellular magnesium levels and controls resistance to aminoglycosides; PhoQ is involved in biofilm formation
PmrA-PmrB Regulatory System	Mediates response to low extracellular magnesium levels and mediates antibiotic resistance
NarX-NarL Regulatory System	Mediates nitrate metabolism in anaerobic environments and likely mediates motility and biofilm formation
GacA-GacS Regulatory System	Unknown stimulus activates this pathway leading to increased biofilm formation and increased antibiotic resistance, especially of aminoglycosides and chlormaphenicol
LadS and RetS	LadS can phosphorylate and activate GacA leading to biofilm formation. RetS deactivates GacA leading to induction of the T3SS.
SadARS Regulatory System	Implicated in biofilm formation
PvrR	Regulatory protein implicated in biofilm formation
Alginate Biosynthesis Pathway	Proteins involved in complex feedback pathway resulting in biofilm formation

P. aeruginosa expresses a multitude of regulatory pathways which are involved in virulence modulation and antibiotic resistance. In this review, I will discuss the better studied two-component systems and their relevance in virulence modulation of *P. aeruginosa* (Table 3). The PhoP-PhoQ system mediates an adaptive response of *P. aeruginosa* to low extracellular magnesium concentrations and controls resistance to aminoglycoside antibiotics through the upregulation of the operon *arnBCADTEF*, inducing a net negative charge of the lipopolysaccharide (LPS), and LPS associates with a variety of cations. The association with other cations results in LPS-LPS crossbridging and limiting interactions with polycationic antibiotics[74]. LPS is an endotoxin that is able to bind to the toll-like receptor, TLR4, and activates the secretion of pro-inflammatory cytokines, therefore acting as an exogenous pyrogen. Also, PhoQ is involved in biofilm

formation; a PhoQ mutant leads to increased cytotoxicity because biofilm formation is impaired[75]. Another regulatory system, PmrA-PmrB, is involved in virulence modulation in a similar model to PhoP-PhoQ. PmrA-PmrB activates the same operon to induce resistance to antimicrobial peptides following the same stimulus, low extracellular magnesium[74]. The NarX-NarL two component regulatory system, where NarX is the histidine kinase and NarL is the response regulator, functions in nitrogen metabolism and biofilm formation through motility modulation[76]. Furthermore, this system is able to inhibit the energetically less favorable arginine fermentation pathway under anaerobic, denitrification conditions[77]. The GacA-GacS two component regulatory system involves GacS functioning as a sensor kinase phosphorylating and activating GacA, following an unknown stimulus, leading to increased antibiotic resistance, especially to aminoglycosides and chloramphenicol[78], and biofilm formation[79]. Individual sensor kinases also feed into the GacA-GacS pathway in contradictory roles. LadS can phosphorylate and activate GacA leading to biofilm formation[80]. The antagonist, RetS, also feeds into the GacA pathway, but instead of phosphorylating GacA, RetS deactivates GacA resulting in induction of the T3SS leading to the upregulation of acute infection virulence factors thus further implying the importance of GacA in biofilm formation[75]. Much like the GacA-GacS pathway, the SadARS regulatory pathway is associated with biofilm formation as demonstrated by mutations in the three proteins, SadA, SadR, and SadS. These mutations all lead to altered mature biofilm architecture[81]. Other regulatory pathways implicated in biofilm formation include the regulator, PvrR[82], and the alginate biosynthesis pathway. The alginate pathway, in particular, is a very complex pathway composed of a plethora of proteins and feedback mechanisms resulting in biofilm

formation. Key proteins in this pathway include AlgR, AlgZ, AlgU, AlgB, AlgD, and AlgA. All of the proteins listed play different roles in the overall alginate biosynthesis pathway but are key proteins to biofilm formation as a whole[83]. When *P. aeruginosa* starts overproducing alginate, the microorganism coverts to a mucoid phenotype. This is believed to occur in the lungs of CF patients during chronic *P. aeruginosa* infection, because the mucus filled lung environment is anaerobic and elicits alginate production by *P. aeruginosa*. When this conversion occurs, the CF lung infection is dramatically worsened for the patient, as antibiotic treatment becomes significantly less effective and a chronic, biofilm infection ensues[62].

The virulence factors discussed above create a picture of the impact *P. aeruginosa* infections can have on its human host, but also the impressive ability of this bacterium to acclimate to its surroundings efficiently. As demonstrated, *P. aeruginosa* has the ability to switch between acute and chronic lifestyles rather resourcefully as many pathways can induce one or both of the above lifestyle choices. Of course, an acute lifestyle indicates an acute infection, where *P. aeruginosa* would most likely be actively expressing and utilizing the T3SS and other toxins. In comparison to the chronic lifestyle choice, through which *P. aeruginosa* would be living as a biofilm inside the host organism and upregulating certain regulatory pathways, quorum sensing molecules, and excreting certain proteins, such as extracellular matrix proteins. Both acute and chronic lifestyles employed by *P. aeruginosa* are observed during *P. aeruginosa* infections in the CF airway and explain the multitude of literature on virulence modulation of *P. aeruginosa* in the CF airway.

1.5 Cystic Fibrosis: *P. aeruginosa* Infections Increase Complexity

Cystic fibrosis (CF) is the most common life threatening autosomal recessive disorder among Caucasian people, with a case rate of 1 in 2500 births and a total population of affected individuals at 90,000 people worldwide[84]. This disease is caused by a mutation of a single gene, encoding the cystic fibrosis transmembrane conductance regulatory protein (CFTR), on the long arm of chromosome 7[85]. The CFTR protein functions normally as an ion channel, specifically in chloride conductance, therefore implying a role in water movement across epithelial cell membranes[86]. The exact mutation in the single gene encoding the CFTR leading to CF can vary but all mutations result in the disease. The ΔF508 mutation (which results in a protein with a deletion of the phenylalanine at the 508th position) is the most common mutation, although 21 other mutations in the CFTR can cause CF. These other 21 mutations are found at highest frequency in certain ethnic groups, such as French Canadians and Askenazi Jewish populations[87]. *In vitro* physiologic studies have demonstrated that different mutations of the CFTR resulting in CF have a range of disruptive effects in epithelial cells, from complete loss of the CFTR protein to surface expression with poor chloride conductance[86]. This range is classified as five different types of mutations, although all are altering the function of CFTR and leading to CF (Table 4). Class I mutations result in premature transcriptional termination leading to a truncated CFTR without function which is degraded before translation. Class II mutations are usually missense mutations, such as the ΔF508 mutation, and result in protein misfolding and degradation before surface expression[88]. Class III mutations commonly implicate the two nuclear-binding domains therefore decreasing chloride channel activity due to abnormal ATP gating, but

this class of mutations in the CFTR will be expressed at the epithelial cell surface[89]. Class IV mutations of the CFTR are located in the membrane spanning domains that form the chloride channel and lead to reduced chloride secretion[90]. Class V mutations result in reduced quantities of CFTR making it into the epithelial cell membrane, resulting in decreased chloride secretion simply as a function of the quantity of normal CFTR protein inserted into the membrane[91]. There is overlap among the above class mutation definitions as a particular CF causing mutation may lead to more than one of the class mutation effects. Therefore a genotypic root mutation causing manifested CF may result in more than one molecular phenotypic result[86].

Table 4 Mutation classes in the CFTR.

Mutation Class	Mutation	Result
Class I	G542X	Truncated mRNA due to premature stop codon leading to immediate degradation
Class II	ΔF508	Misfolding of the protein in the endoplasmic reticulum resulting in ubiquination and degredation in the proteosome
Class III	G551D	Reaches the cell membrane but the channel is unable to be activated
Class IV	R347P	Reaches the cell membrane and activation of channel is successful but the channel is defective therfore preventing chloride movement
Class V	3849 + 10kb C➔T	Splicing defect where a reduced amount of normal CFTR proteins are produced leading to a milder CF phenotype

Although CF can manifest in many different organs in the body, leading to osmotic changes and altered organ function, the CF airway will be the focus of this review. Soon after birth, CF inflicted newborns will become infected with a variety of bacterial pathogens causing the mounting of an intense neutrophilic and inflammatory response. The bacterial inhabitants only contribute to an already induced inflammatory response, as several studies have demonstrated inflammatory responses in CF airways of culture negative patients[92]. Regardless of the possible causes of the inflammatory response, inflammation leads to mucus secretion and plugging of the small and medium sized bronchioles, and this response is only perpetuated as the CF patient matures and persistent neutrophilic infiltration leads to persistent inflammatory effects[93]. This persistent inflammation causes physiologic changes to the lungs such as hypertrophy of bronchial circulation and formation of bronchial cysts and pulmonary hypertension. Furthermore, inflammation endures as a result of bacterial infection which becomes chronic and biofilm forming[94] (Figure 6).

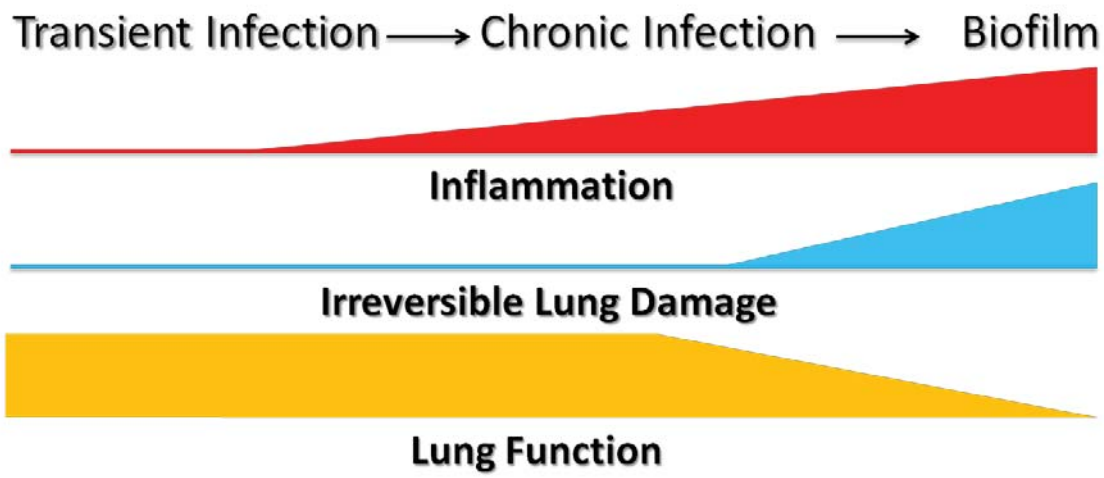

Figure 6. CF disease schematic. Overall schematic of the effects of *P. aeruginosa* bacterial infection in the CF airway. As the bacterial infection enters a mucoidy, chronic state, inflammation in the CF airway ensues. Biofilm formation induces severe inflammation and irreversible lung damage leading to significantly decreased lung function. Image adapted from Erickson *et al.*[95]

On a molecular level, the CFTR is a chloride transport protein. Functional impairment of the CFTR impacts osmotic pressures, and the volume of airway surface liquid (ASL) in the lungs changes. ASL exists in two layers above the epithelial surface, a mucus layer and a periciliary liquid layer which is the height of the extended cilia[96]. Therefore, the periciliary layer functions as a low viscosity liquid through which the cilia beat and to lubricate gel-forming mucins secreted from the cell surface[96]. The mucus layer consists of the secreted mucins, whose properties are easily altered with changes in water content and ion concentration. Under normal circumstances in a healthy airway, mucins are easily cleared from the airway[97]. Mucociliary clearance is impaired in the CF airway due to abnormal regulation of periciliary liquid volume[96]. Also, the reduced periciliary liquid volume promotes interaction between gel mucins in the mucus layer and cell surface mucins which impairs particle movement out of the CF airway. In concordance, hypersecretion of mucus and impaired mucociliary movement results in

large mucus plugs, and these mucus plugs contain steep hypoxic gradients due to viscosity changes[97] (Figure 7).

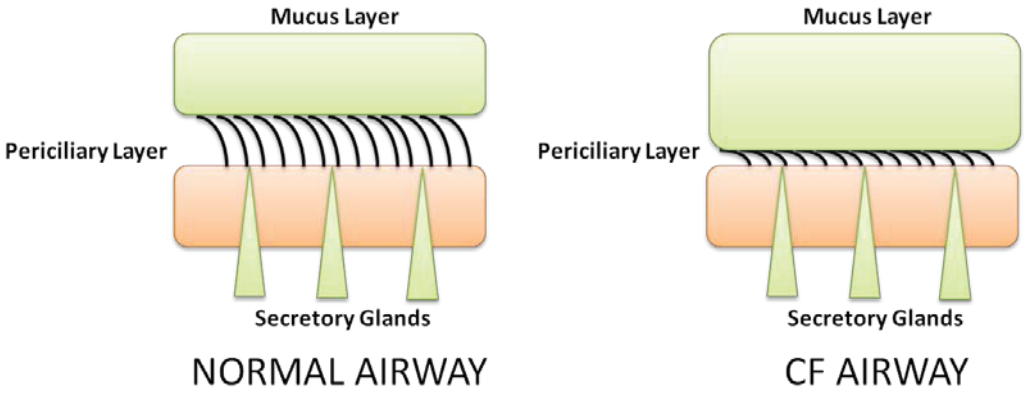

Figure 7. Physiologic changes in CF airway. On a physiologic level, mutation of the CFTR protein in the CF airway results in decreased volume of the periciliary layer producing impaired mucociliary clearance. Hypersecretion of mucus ensues and large mucus plugs form. Image adapted from Gibson *et al.*[56].

P. aeruginosa may be among many initial colonizers of the CF airway, but quickly becomes the dominating infectious agent. Upon deposition of *P. aeruginosa* on thick mucus surfaces, the bacteria are able to actively penetrate the mucus plugs and begin forming microcolonies. This occurs by initially adhering to the underlay of epithelial cells and aggregating to the final goal of forming mature biofilms. The bacteria within the mature biofilm adapt to the hypoxic environment, and a chronic infection ensues. Neutrophils in the CF airway contribute to the hypoxic lung environment. Upon death, neutrophils release their cellular constituent materials, which increase viscosity of the mucus plug furthering the survival of the *P. aeruginosa* biofilms via increased alginate production[98] (Figure 8).

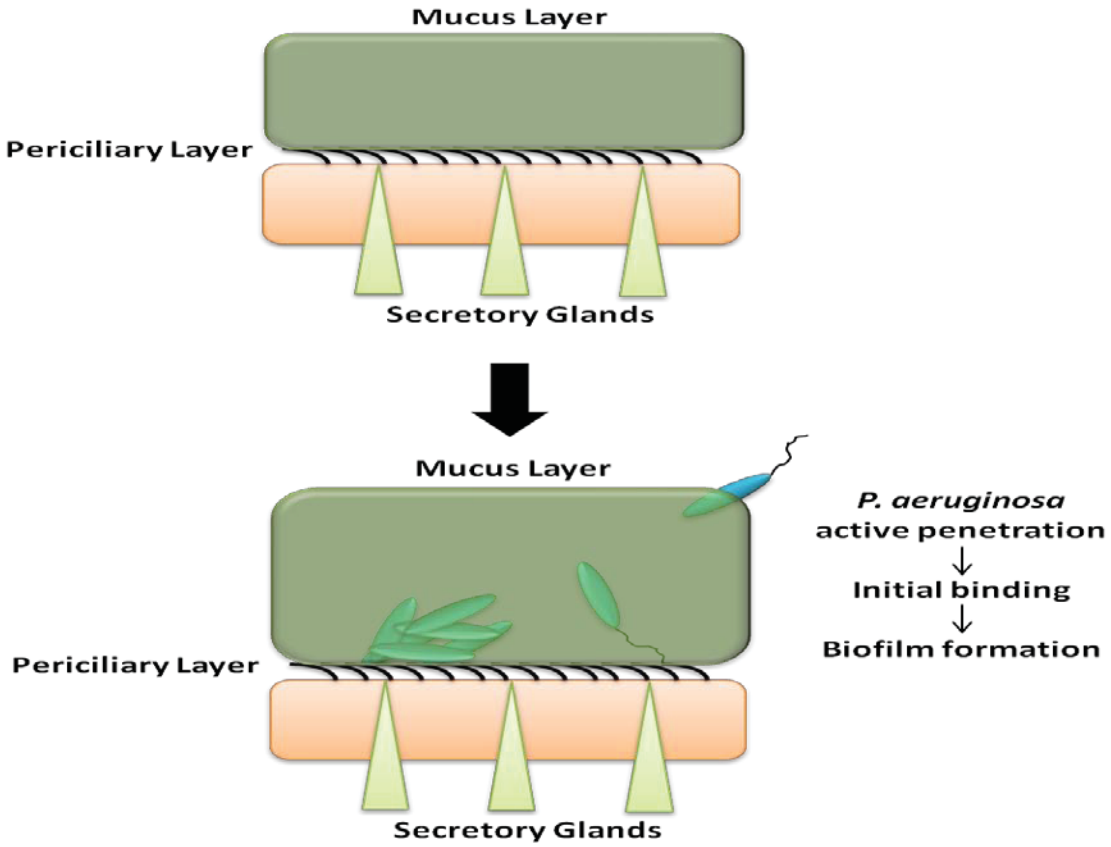

Figure 8. *P. aeruginosa* biofilm formation in the CF airway. *P. aeruginosa* bacteria are able to actively penetrate the thick mucus plugs in the CF airway and form biofilms. Image adapted from Gibson *et al.*[56].

Experimental evidence has confirmed the presence of *P. aeruginosa* biofilms as chronic infectious agents of the CF airway. *P. aeruginosa* quorum sensing molecules have been detected in the CF airway[54]. Both transmission and scanning electron microscopy have visualized *P. aeruginosa* microcolonies from CF patient sputum samples[99]. Therefore, *P. aeruginosa* colonization and biofilm formation poses a significant threat to patients with CF, as the persistent and acute aspects of *P. aeruginosa* infection increases morbidity and mortality in CF patients.

Many researchers are studying a wide variety of known virulence factors implicated in biofilm formation as well as trying to discover new virulence related

factors. Anderson *et al*. (2008) performed a microarray analysis employing a novel assay to identify biofilm gene expression particularly following tobramycin treatment of biofilms grown in a model system that represents the CF airway in comparison to planktonic cultures. The novel assay utilized a unique cell line, CFBE. This particular immortalized cell line originated from a CF patient homozygous for the ΔF508 mutation. In this experiment, the CFBE cells were allowed to form a confluent monolayer over a period of 7-10 days mimicking the CF airway. Upon formation of a confluent monolayer, the cells were inoculated with *P. aeruginosa* (lab strain PA14). Over a period of 6-8 hours following the addition of arginine at the 1 hour time point, the PA14 inoculum formed biofilms on the CFBE cells in a welled plate (whether 6, 24, or 96). The biofilms were treated with tobramycin (500 μg/mL) and a microarray analysis was performed. Results showed some expected genes were upregulated or downregulated, and many unexpected genes were impacted. One such gene encoded the putative magnesium transporter protein, MgtE. Upon further analysis utilizing cytotoxicity assays, deletion mutations and overexpressing the protein, MgtE has been implicated in virulence modulation (Figure 9) and will be discussed in detail in the next section.

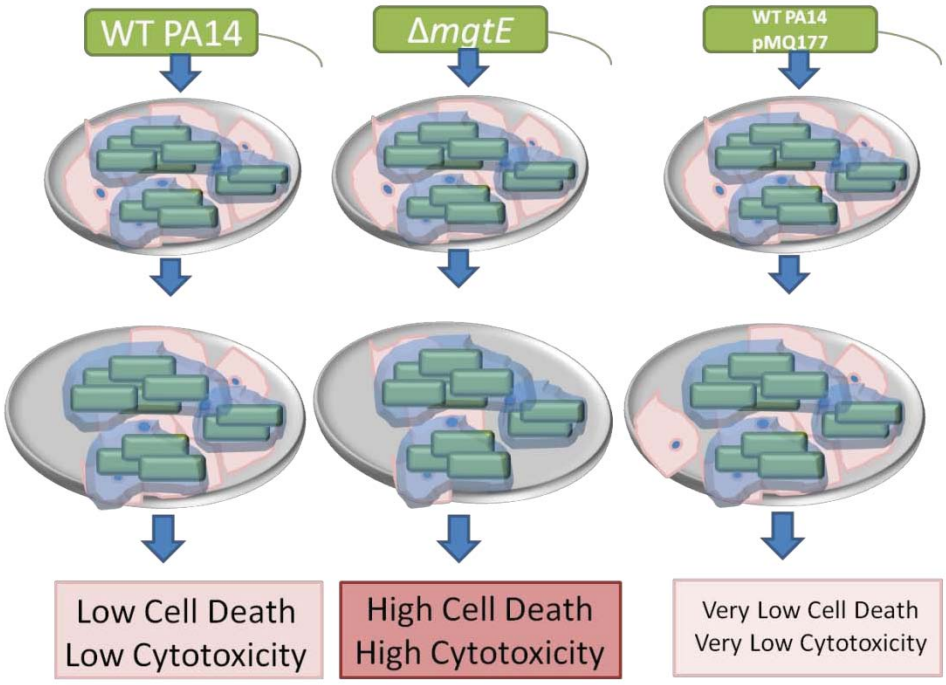

Figure 9. Cytotoxic effects of MgtE on the CFBE cell line. When *mgtE* is removed from the chromosome, the bacterial biofilms exhibit an increased cytotoxic phenotype toward the CFBE cell line resulting in increased epithelial cell death in comparison to the WT PA14 laboratory strain. When MgtE is overexpressed by a multicopy plasmid (pMQ177) in the WT PA14 laboratory strain, cytotoxicity toward the CFBE cell line further decreases below WT levels.

1.6 Prokaryotic Magnesium Transporters

In order to better understand the role MgtE is playing in virulence modulation, one needs to understand magnesium transport in *P. aeruginosa*. There are four magnesium transporters expressed and functioning in *P. aeruginosa*. Magnesium is a divalent cation that is essential for growth and maintenance of living cells, as well as, being utilized as a signaling molecule for certain regulatory pathways. Magnesium is not normally limiting in laboratory bacterial growth, as bacterial cells easily scavenge enough magnesium to survive. CorA was the first magnesium transporter to be identified in

prokaryotes and mediates both influx and efflux of magnesium for the prokaryotic cell[100]. CorA is conserved between eukaryotes, prokaryotes, and archea, therefore implying that this protein provides essential functionality for life[101]. Two other magnesium transporters in prokaryotes, MgtA and MgtB, are ATPases that actively transport magnesium. Both MgtA and MgtB mediate influx of magnesium, but not efflux[102]. Lastly, MgtE is the most recent magnesium transporter discovered in prokaryotes, and the crystallized structure has been elucidated, indicating that MgtE contains 5 transmembrane domains[103]. Although MgtE has the ability to transport magnesium via influx only as confirmed structurally through crystallization, we know that functionally CorA still performs the majority of magnesium influx in many prokaryotic organisms (approximately 90%). Therefore as magnesium transport does not seem to be the primary function of MgtE, it is not only plausible but likely that MgtE plays another important role in *P. aeruginosa.* One such role could be modulation of virulence, as demonstrated in other bacteria.

MgtE was initially identified in the bacteria, *Providencia stuartii* and *Bacillus firmus* OF4 in an attempt to search for members of the CorA family of magnesium uptake transporters[104]. Members of the MgtE family have been identified in Gram-negative and Gram-positive bacteria, archea and eukaryotes[105]. Humans have the protein, SLC41A1, which is homologous to certain bacterial MgtE magnesium transport proteins. SLC41A1 is responsive to magnesium levels and believed to be a magnesium transporter[106].

MgtE was isolated from both *P. stuartii* (Gram-negative) and *B. firmus* (Gram-positive). Individually, MgtE homologues from each organism were introduced into the magnesium transport mutant MM281 of *S. typhimurium*, which led to rescued growth and elicited magnesium uptake into the cell[104], proving that these homologues function in

magnesium transport. MgtE has also been implicated in the swarming ability of *Aeromonas hydrophila*. When MgtE is mutated in *A. hydrophila*, reduced ability to swarm in semisolid media is observed although no changes to the polar flagella or motility are observed. Also observed were a reduced ability to adhere to HEp-2 cells and decreased biofilm formation, which may be directly related to the reduced swarming ability. These observations could be connected to decreased magnesium, as divalent cations greatly contribute to the integrity and stability of the bacterial outer membrane, and LPS, which is able to bind divalent cations, and could affect both swarming and adherence ability[107]. *Campylobacter jejuni* has an MgtE homologue that exhibits a similar effect when mutated. This gene, Cj1496c, is required for attachment and invasion of INT-407 intestinal epithelial cells and the colonization of the chick gastrointestinal tract[108]. *Vibrio cholerae* is another bacterium in which MgtE is implicated in an aspect of virulence. In this organism, MgtE is an important gene for biofilm formation[109]. Therefore, these findings implicate a role for MgtE and magnesium in motility, adherence abilities, and biofilm formation in some bacteria.

Anderson *et al.* has recently implicated MgtE as playing a role in changes in cytotoxicity by *Pseudomonas aeruginosa* against a human CF cell line, CFBE cells[34]. An isogenic mutation of *mgtE* in *P. aeruginosa* biofilms results in increased cytotoxicity but does not affect biofilm formation. This effect requires a functional T3SS system. Furthermore, the antibiotic tobramycin led to transcriptional upregulation of *mgtE* by treated *P. aeruginosa* biofilms[34]. Based on these findings, this thesis will further elucidate the role of MgtE in *P. aeruginosa* virulence by analyzing the effects of different antibiotics on *mgtE* transcription and MgtE translation to better understand the regulation

of MgtE and further implicate this protein in virulence modulation by connecting MgtE to a regulatory pathway.

1.7 Conclusions and Study Design

This study employs both RT-PCR and QRT-PCR to observe transcriptional changes in *mgtE* expression following treatment of a mature *P. aeruginosa* biofilm grown on CFBE cells. Twelve different antibiotics of different classes and with different mechanisms of actions were investigated. In an attempt to understand translational effects of MgtE following antibiotic stimuli, Western blotting and cytotoxicity assays were utilized. As demonstrated, many bacteria express MgtE homologues, including *Staphylococcus aureus*[110]. This organism is an initial colonizer of the CF airway and is also able to form biofilms. Therefore in an attempt to implicate *S. aureus* MgtE homologues as having a virulence modulatory function, the genes were isolated, placed on a multi-copy plasmid, and transformed into SMC3604, PA14 Δ*mgtE*, our isogeneic mutant lab strain. Cytotoxicity assays were performed to identify changes in cytotoxicity against the CFBE cells. Finally, *P. aeruginosa* MgtE may be interacting with a regulatory pathway to exact the effects demonstrated. To identify possible pathways, regulatory proteins were screened to look for possible interactions via cytotoxic changes.

Overall, this research will contribute to a better understanding of *P. aeruginosa* virulence modulation under the context of CF airway infections, adding to a body of literature on the topic and, eventually, contributing to better therapeutic options for treatment of *P. aeruginosa* infections in the CF airway.

CHAPTER TWO MATERIALS AND METHODS

2.1 Bacteria Strains and Plasmids

The bacterial strains and plasmids used for the following studies are listed in Table 5, and a full list of primers used in this study are listed in Table 6. For all the studies, bacteria were grown overnight in a nutrient rich broth, LB (lysogeny broth), with appropriate concentrations of antibiotics when necessary.

Table 5. Strains and plasmids used.

Strain or plasmid	Genotype or description	Reference or source
P. aeruginosa strains		
PA14	Wild type	[111]
SMC3604	PA14 *ΔPA0913* (*mgtE*)	[33]
SMC3640	PA14 *ΔPA4635* (*mgtC*)	[33]
SMC3643	PA14 *ΔPA4825* (*mgtA*)	[33]
SMC3644	PA14 *ΔPA4635, ΔPA4635* (*mgtC, mgtA*)	[33]
SMC3646	PA14 *ΔPA4635, ΔPA0913* (*mgtC, mgtE*)	[33]
SMC3647	PA14 *ΔPA4635, ΔPA0913, ΔPA4825* (*mgtC, mgtE, mgtA*)	[33]
CVR3	SMC3604:pCR3	This study
E. coli strain		
S17-1	Laboratory strain for cloning	[112]
S. cerevisiae strain		
InvSc1	*In vivo cloning, ura3-52/ura3-52*	Invitrogen
S. aureus strains		
Newman	Laboratory strain	[113]
RN6390	Laboratory strain	[113]
Plasmids		
pMQ30	Allelic replacement vector; yeast cloning	[113]
pMQ70	P_{BAD} expression vector:Cb	[113]
pMQ72	P_{BAD} expression vector:Gm	[113]
pSMC233	Deletion of *mgtE*; pMQ30 backbone	[33]
pMQ177	Complement vector of *P. aeruginosa* PA14 *mgtE*; pMQ72 backbone	[113]
pSMC21	Complement vector of *P. aeruginosa* PA14 *mgtE*; pMQ70 backbone	[33]
pCR1	Deletion of *PA11990*; pMQ30 backbone	This study
pCR3	Histidine-tagged *mgtE* insert; pMQ30 backbone	This study
pCR9	Complement vector of Newman *mgtE*; pMQ72 backbone	This study
pCR10	Complement vector of DS762 m*gtE*; pMQ72 backbone	This study

Table 6. Primers used.

Primer	Sequence (5'-3')
PA5110forA	CCTACCTGTTGGTCTTCGACCCG
PA5110revA	GCTGATGTTGTCGTGGGTGAGG
MgtEforRTnew	*TGTTCGAAGGCTCCATCGAGAAAC*
MgtErevRTnew	*TTGCCGTAAAGATAGAAGGCCACC*
p30UP(1)	AATCTTCTCTCATCCGCCAAAACAGCCAAGCTCGCCATTCTTGTCCGCCACGACGGTCTC
MgtEPromEnd(2)	TCTTGGCTTCTACTTCGGTATGATGATGATGATGATGCATAGCGCGCTCCACCCCCAGTA
p30Down(3)	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTCTACATCAGGAAGATCGTCG
MgtEBegin(4)	CTGGGGGTGGAGCGCGCTATGCATCATCATCATCATCATACCGAAGTAGAAGCCAAGAAG
SAMgtECfor	
SAMgtErev	
p729	CAGACCGCTTCTGCGTTCTG
p730	GCAACTCTCTACTGTTTCTCC
913for	CCCATGGACTTACCCAGTAG
913rev	CCGTCGACGAGTATTTCGTC

2.2 Abiotic Static Biofilm Assay

Abiotic static biofilm assays compared biofilm growth under certain conditions. Overnight cultures of *P. aeruginosa* were diluted 1:100 into M63 minimal media with the addition of 0.4% arginine and 1 mM $MgSO_4$ and 100 µL of inoculated media was added into each well of a plastic 96 well plate[63]. For the biofilm assays that are conditionally testing the effects of varying levels of $MgSO_4$, the following concentrations of $MgSO_4$ were tested within two sets of ranges: 0.01 mM, 0.1 mM, 1 mM, 10 mM, and 1 µM, 10 µM, 100 µM, and 500 µM. The biofilm assays were incubated overnight (approximately 16 hours) at 37°C. Following incubation, the 96 well plates were stained with 0.1% crystal violet by filling each well with 125 µL of 0.1% crystal violet solution and incubating at room temperature for 12 minutes. The plates were then rinsed with

deionized water and qualitatively and quantitatively analyzed. Visualization of biofilms at the air liquid interface via staining allowed for qualitative analysis. For quantitative analysis, stained biofilms were dissolved in 30% acetic acid and analyzed via spectrophotometry (optical density at OD_{550}), which indicated biofilm presence by amount of light absorbed.

2.3 Static Co-culture Biofilm Assay

This assay allows for biofilms to be analyzed following growth on a human cell line and represents a novel model system for *P. aeruginosa* infection of the CF airway[33]. The immortalized human cells used are CFBE41o- cells (CFBE cells). For this model system, CFBE cells were seeded in 24 well or 6 well plates at a concentration of either 2 X 10^5 cells/well or 1 X 10^6 cells/well in 500 µL or 1.5 mL, respectively, of minimal essential medium (MEM) with 10% fetal bovine serum, 50 U/mL penicillin and 50 µg/mL streptomycin. The cells were incubated at 37°C at 5% CO_2 for 7-10 days allowing for a confluent monolayer of cells to form. For the co-culture biofilm assay, *P. aeruginosa* was inoculated at an approximate concentration of 2 X 10^7 CFU/mL in 1.5 mL or 1.2 X 10^7 in 500 µL of MEM/well with 2 mM L-glutamine (without fetal bovine serum, streptomycin, and penicillin) in 6-well or 24-well plates, respectively. These concentrations represent an approximate multiplicity of infection of 30:1 for both plate sizes. Following inoculation, the plates were incubated at 37°C and 5% CO_2 for 1 hour. Then, the media is removed and fresh MEM with 2 mM L-glutamine and the addition of 0.4% Arginine is added at the same concentration, 1.5 mL or 500 µL depending on plate size. Arginine promotes the formation of biofilms. These plates were incubated as

described for another 5-7 hours, a total incubation time of 6-8 hours. This length of time allows mature *P. aeruginosa* biofilms to form on the CFBE cells in each well. Following incubation, CFBE cell monolayer integrity was confirmed via microscopy. CFU/mL calculations of biofilms were performed. The cells in each well were washed with phosphate-buffered saline (PBS) to remove planktonic bacteria. Each well was treated with 0.1% Triton X-100 for 15 minutes which lyses the epithelial cells. The lysate was then vortexed for three minutes and serially diluted onto LB agar plates.

2.4 RT-PCR and QRT-PCR Analysis

For transcriptional analysis of *mgtE* using the static co-culture biofilm model system described above, wild type *P. aeruginosa* was allowed to form biofilms on CFBE cells, and these biofilms were allowed to grow for seven hours prior to antibiotic treatment at various concentrations (Table 7). Antibiotics were prepared according to manufacturer's specifications.

Table 7. Antibiotic treatment concentrations.

Class of Antibiotic	Antibiotic	Concentrations (ug/ml)
Aminoglycosides	Tobramycin	250, 500, 750
	Gentamicin	250, 500, 750
	Kanamycin	250, 500, 750
Carbapenem	Imipenem	5, 25, 50
Cephalosporin	Ceftazidime	5, 50, 100
Macrolide	Azithromycin	5, 10, 30
Monobactam	Aztreonam	100, 250, 500
Penicillin	Carbenicillin	250, 500, 750
Quinolone	Ciprofloxacin	5, 20, 40
	Nalidixic Acid	250, 500, 750
Tetracycline	Tetracycline	50,100,150
Other	Chloramphenicol	30, 90, 300

The cells were rinsed with PBS and fresh MEM with 2 mM L-glutamine (without fetal bovine serum, penicillin, and streptomycin) at the appropriate volume based on the plate with the appropriate concentrations of antibiotics. Antibiotic treatments were incubated for 30 minutes at 37°C and 5% CO_2. Following incubation with antibiotics, the cells were rinsed with PBS, and the RNeasy Plus Kit protocol (Qiagen) was followed for RNA isolation with modifications. Specifically, the cells were incubated in 600 µL of 1µg/mL lysozyme in Tris-EDTA buffer for 10 minutes. Then, the cells were lysed with 600 µL of RLT lysis buffer from the Qiagen RNeasy kit. The homogenized cells were pulled through a 20-gauge needle 10 times to shear genomic DNA and further homogenize the sample. 600 µL of 100% EtOH was added to the homogenized cell solution, and this solution was added to the RNeasy columns provided with the kit. The kit protocol was followed from this point forward. To prevent DNA contamination, an on-column DNA digestion and RNA clean up was performed following the Qiagen RNeasy optional protocol. The RNA clean up protocol was modified. Following the addition of the RLT lysis buffer and before the addition of 100% EtOH, I ran the RNA product through the provided genomic eliminator column, and then followed the provided protocol. Performing these optional procedures and adding the modification for the RNA clean-up protocol resulted in significant decrease in DNA contamination of the RNA product. I synthesized cDNA from the isolated bacterial RNA using a kit, the Superscript III First-Strand Synthesis System for RT-PCR, following the protocol provided (Invitrogen #18080-051). Semi-quantitative RT-PCR and quantitative RT-PCR (QRT-PCR) analysis was performed using the primers 5110forA, 5110revA, MgtEforRTnew, and MgtErevRTnew. Both RT-PCR and QRT-PCR were performed as previously described

with a modification in the RT-PCR protocol[114]. The modification involves increasing the amplification rounds during the RT-PCR reaction from 25 to 35 rounds. This was required for visualization of gene product.

2.5 Gene Constructs

2.5.1 Construction of Deletion Mutants

Isogenic deletion mutants were created as previously described[33,113]. Using the suicide vector, pMQ30, flanking regions of the gene to be deleted were amplified via PCR with specific primers and joined to pMQ30 via homologous recombination in *Saccharomyces cerevisiae*. These deletion vectors were then transformed into competent *Escherichia coli* S17 cells and confirmed via restriction digestion. Via conjugation, the deletion vector harbored by S17 transformants is inserted into either *P. aeruginosa* PA14 or other mutant laboratory strains depending on the study. Exconjugants are ascertained via selective agar plates (either gentamicin (50 μg/mL)/nalidixic acid (30 μg/mL) or carbenicillin (250 μg/mL)/nalidixic acid (30 μg/mL)). Following the selection, the exconjugants were grown overnight in LB, and spontaneous excision of the vector was selected by plating on 10% sucrose plates. Mutations were confirmed by PCR and sequencing. Deletion of *mgtE* was performed with the plasmids, pSMC233 and pSMC233-Amp, which have selective cassettes of either gentamicin or carbenicillin, respectively. Deletions of *mgtE* were confirmed via PCR using the primers, 913for and 913rev.

2.5.2 Construction of Histidine-tagged MgtE strain

The Histidine-tagged construct was designed in a similar fashion as the construction of the deletion mutants, therefore the suicide vector, pMQ30, was utilized. Specific primers, p30UP(1), p30Down(3), MgtEPromEnd(2), and MgtEBegin(4), for PCR amplification of the *mgtE* gene including the promoter region, were designed. These primers also incorporated a histidine-tag composed of 6 histidine amino acids following the AUG start codon in the *mgtE* reading frame. This construct was joined to pMQ30 via homologous recombination in *S. cerevisiae*. This vector, pCR3, was then transformed into competent *Escherichia coli* S17 cells and confirmed via restriction digestion. Via conjugation, the knockout vector harbored by S17 transformants was inserted into SMC3604. The selection process was the same as described in the previous section. This strain, CVR3, was confirmed via cytotoxicity analysis, Western blotting, and sequencing.

2.5.3 Construction of complementation plasmids and strains

The plasmids, pCR9 and pCR10, were created via PCR amplification using primers, SAMgtECfor and SAMgtECrev, of two *Staphylococcus aureus* strains, Newman and RN6390. The amplified regions were ligated onto EcoRI-digested pMQ70. Both plasmids, pCR9 and pCR10, were then transformed into the strain, SMC3604. The presence of the plasmid was confirmed via PCR amplification using the primers, p729 and p730.

The plasmid, pSMC21, was transformed into transposon-mutated strains where *mgtE* complementation was needed. This procedure involved transformation of various

Pseudomonas aeruginosa transposon mutated strains with the pSMC21. This presence of the plasmid was selected via selective media plates (carbenicillin). The presence of the plasmid was confirmed via PCR amplification using the primers, p729 and p730.

2.6 Cytotoxicity Assays

Cytotoxicity was assessed via the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega #G1781) which measures LDH, lactate dehydrogenase, release from epithelial cells. LDH release indicates disturbance of the epithelial cell membrane. In 24 well plates, CFBE cells were seeded at a concentration of 2×10^5 with 0.5 mL of MEM. They were grown until confluent for 7-10 days at 37°C and 5% CO_2. Medium was replaced every second day. Bacteria were inoculated at a concentration of 2×10^7 CFU/ml to only wells in which the epithelial cells were confluent as previously described[33]. Following inoculation, medium was replaced after one hour with media supplemented with 0.4% arginine and the samples were incubated for 5 more hours at 37°C and 5% CO_2. Following this incubation, 350 µl of the supernatant was harvested and cytotoxicity was assessed via manufacturer's specifications. In experiments testing wild type (WT) and mutant strains, bacteria were inoculated into separate wells in the same plates and analyzed in exactly the same fashion. Previous experiments have demonstrated this method results in similar bacterial growth[33]. As a positive control for these experiments, 0.1% Triton X-100 was added to a separate set of wells containing CFBE monolayers that were not inoculated with bacteria. These wells represented a maximal release of LDH or 100% cytotoxicity. As a negative control for these experiments, wells containing CFBE monolayers that were not inoculated with bacteria

or lysed with 0.1% Triton X-100 were assessed for cytotoxicity to ascertain the spontaneous release of LDH and represent 0% cytotoxicity. All experimental cytotoxicity levels were normalized to the spontaneous release values. Percent cytotoxicity was determined via the following equation: 100 X (experimental value - spontaneous value)/maximum release. Each assay was performed in triplicate.

2.7 Western Blotting

Via the static co-culture biofilm assay, biofilms of strain CVR3 were allowed to grow and were treated with various antibiotics at determined concentrations. Following 7 hours of growth and a 30 minute antibiotic treatment, the biofilms were extracted and dispersed utilizing a 0.1% Triton X-100 solution that lyses the CFBE cells. Following lysis of the CFBE cell monolayer, the samples were centrifuged for two minutes at full speed (15000 rpm). Bacterial pellets were broken apart via boiling. Supernatant was removed and protein concentrations were determined using a protein analysis kit (Pierce BCA Protein Assay Kit #23227). These results were used to control for protein loading. Western blotting was carried out utilizing a horseradish peroxidase (HRP)-nickel conjugate, the His Probe-HRP (Thermo Scientific #15165) and following the provided protocol. Western Lightning Chemiluminescence Reagent Plus was used according to the manufacturer's protocol to visualize the blot (Perkin Elmer, Boston, MA), according to the manufacturer’s protocol.

2.8 Statistical analysis

Statistical significance was determined via a p-value <0.05 using a Student's *t* test with Sidak adjustment for multiple comparisons which adjusts the p-value itself

CHAPTER THREE RESULTS

3.1 Semiquantitative RT-PCR Analysis of Antibiotic Treated Biofilms

Using the model system previously described by Anderson *et al.*, PA14 biofilms were formed over 7 hours on CFBE epithelial cells[33] and treated with antibiotics to investigate the effects of different antibiotics on *mgtE* transcription. Twelve antibiotics from nine antibiotic classes with different main modes of action were used in this study. All twelve antibiotics are used in the treatment of Gram-negative pathogens. A previous study demonstrated that transcription of *mgtE* is unregulated following tobramycin treatment of preformed PA14 biofilms at a concentration of 500 μg/mL[34]. Tobramycin was further analyzed at concentrations of 250 μg/mL and 750 μg/mL. Eleven other antibiotics were also analyzed from nine classes of antibiotics with three main modes of actions: inhibiting protein synthesis, inhibiting cell wall synthesis, and inhibiting DNA gyrase/topoisomerase (Table 8).

Table 8. Effect of antibiotic treatment on *mgtE* expression in *Pseudomonas* biofilms.

Class of Antibiotic	Mode of Action	Used	Concentrations Used (µg/ml)	RT-PCR Results	QRT-PCR Results
Aminoglycoside	Inhibit protein synthesis	Tobramycin	250, 500, 750	Upregulation	Upregulation
		Gentamicin	250, 500, 750	Upregulation	Upregulation
		Kanamycin	250, 500, 750 (SI)	Upregulation	Upregulation
Carbapenem	Inhibit cell wall synthesis	Imipenem	5, 25, 50 (SI)	Upregulation	Upregulation
Cephalosporin	Inhibit cell wall synthesis	Ceftazidime	5, 50, 100 (SI)	Upregulation	Downregulation
Macrolide	Inhibit protein synthesis	Azithromycin	5, 10, 30	Upregulation	Upregulation
Monobactam	Inhibit cell wall synthesis	Aztreonam	100, 250, 500 (SI)	Upregulation	No Change
Penicillin	Inhibit cell wall synthesis	Carbenicillin	250, 500, 750 (SI)	No Change	No Change
Quinolone	Inhibit DNA gyrase	Ciprofloxacin	5, 20, 40 (SI)	Upregulation	Upregulation
		Nalidixic Acid	250, 500, 750 (SI)	Upregulation	Upregulation
Tetracycline	Inhibit protein synthesis	Tetracycline	50,100,150	Upregulation	Upregulation
Other	Inhibit protein synthesis	Chloramphenicol	30, 90 (SI), 300	Upregulation	Upregulation

Subinhibitory antibiotic treatment levels indicated by (SI). All other unmarked antibiotic treatment levels are sublethal.

Varying concentrations of antibiotics were used depending on toxic effects toward the CFBE cells and changes in bacterial levels (measured as colony forming units (CFU)/mL) following treatment. A no-reverse transcriptase control was employed during the process of making cDNA from RNA samples, to ensure against DNA contamination. All antibiotic treated PA14 biofilms were controlled against untreated PA14 biofilms for each experiment, and a constitutively expressed gene fructose bisphosphate was used as a loading control to normalize samples. This gene, *fpb*, is expressed at a constant rate.

Results, which represent a single experiment, showed transcriptional upregulation of *mgtE* following most antibiotic treatments in comparison to untreated PA14 biofilms. These results are consistent with QRT-PCR results overall (Table 8, Figure 10). Inconsistencies between RT-PCR and QRT-PCR measured changes in *mgtE* transcription occur with ceftazidime and aztreonam treatments. The QRT-PCR results demonstrated downregulation of *mgtE* transcription following ceftazidime treatment at a concentration of 50 µg/mL and no change in *mgtE* transcription at 5 and 100 µg/mL treatment levels. The RT-PCR results showed the exact opposite, with obvious upregulation of *mgtE* transcripts at all three treatment concentrations. RT-PCR results for aztreonam treatment

of preformed biofilms showed an upregulation of *mgtE* transcripts at 100 and 250 µg/mL treatment levels, but not at 500 µg/mL. The QRT-PCR results showed no changes in *mgtE* transcription following aztreonam treatment. Even with these variable results, most of the data concerning *mgtE* transcription following antibiotic treatment was consistent between RT-PCR and QRT-PCR. RT-PCR is semi-quantitative and is not as sensitive of an assay as QRT-PCR. Also, the RT-PCR results are a single representation of experiments performed in triplicate without statistical analysis, where QRT-PCR results are representative of all three experiments with statistical analysis. Therefore error in the RT-PCR results is more likely, and the QRT-PCR results are expected to be more accurate.

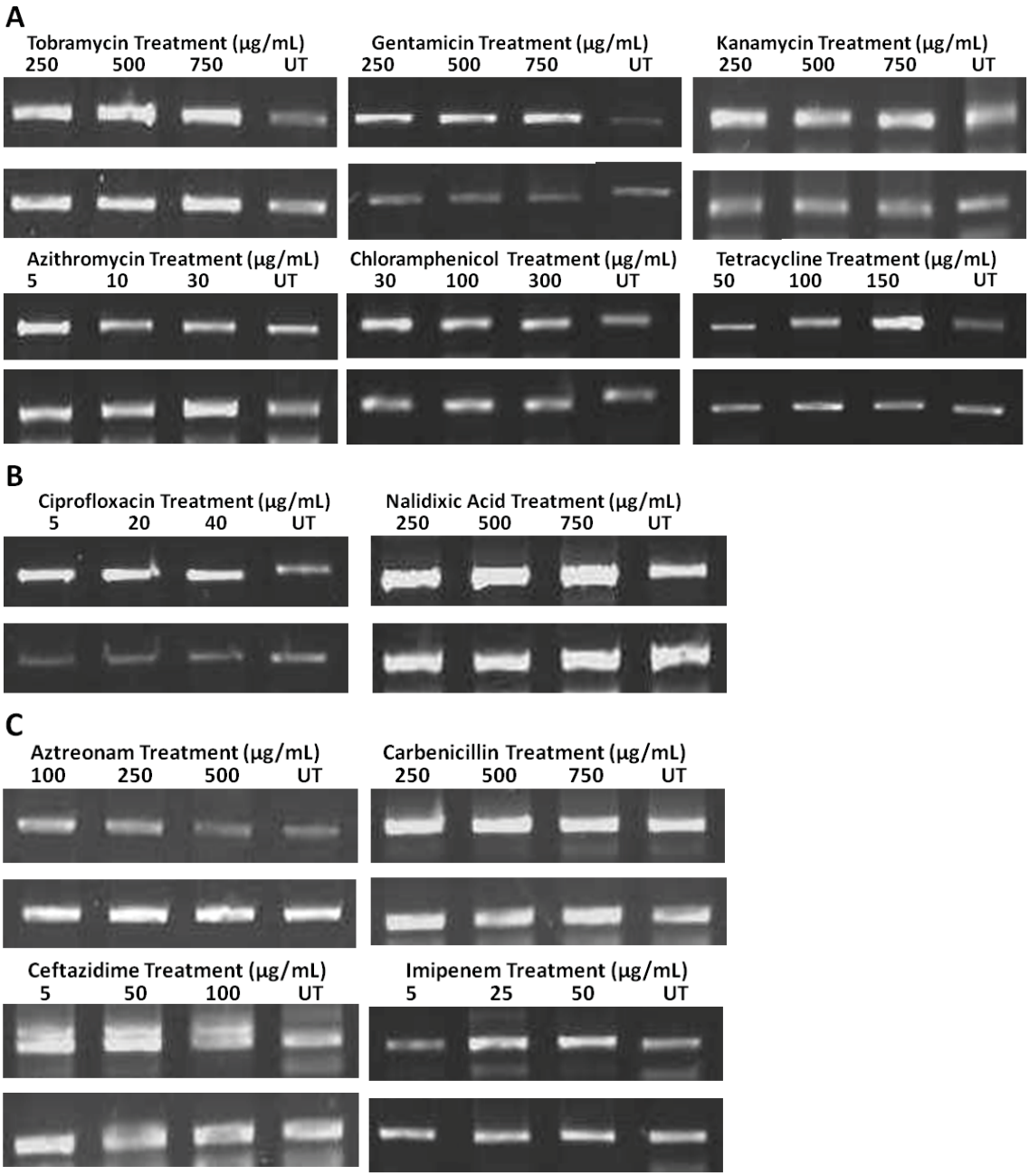

Figure 10. RT-PCR analysis of transcription of *mgtE* following antibiotic treatment. For every experiment with each individual antibiotic, the top row of bands represents transcription of *mgtE* following antibiotic treatment. The bottom row of bands represents expression the control gene, *fbp*, following antibiotic treatment. Levels for the control gene are consistent throughout each experiment. Results for this figure have been divided into three sections based on general mechanisms of action for the antibiotics tested. Figure 10 A are antibiotics that inhibit protein synthesis. Figure 10 B are the quinolones, which impair the DNA gyrase functionality. Figure 10 C are antibiotics that impair cell wall synthesis. Data is a representative of three experiments (n=3). (UT=Untreated control)

To control for changes in bacterial levels following antibiotic treatments which could explain changes in transcriptional expression of *mgtE*, CFU/mL analyses were acquired for all twelve antibiotics in triplicate. Antibiotic treatment affected pre-formed PA14 biofilm levels in different ways depending on the antibiotics used. If antibiotic treatment did not cause a significant reduction in bacterial levels compared to untreated controls, then the treatment levels of these antibiotics are considered subinhibitory. The antibiotics that were administered at subinhibitory levels were kanamycin, carbenicillin, ceftazidime, aztreonam, ciprofloxacin, nalidixic acid, and imipenem ($p>0.05$) (Figure 11).

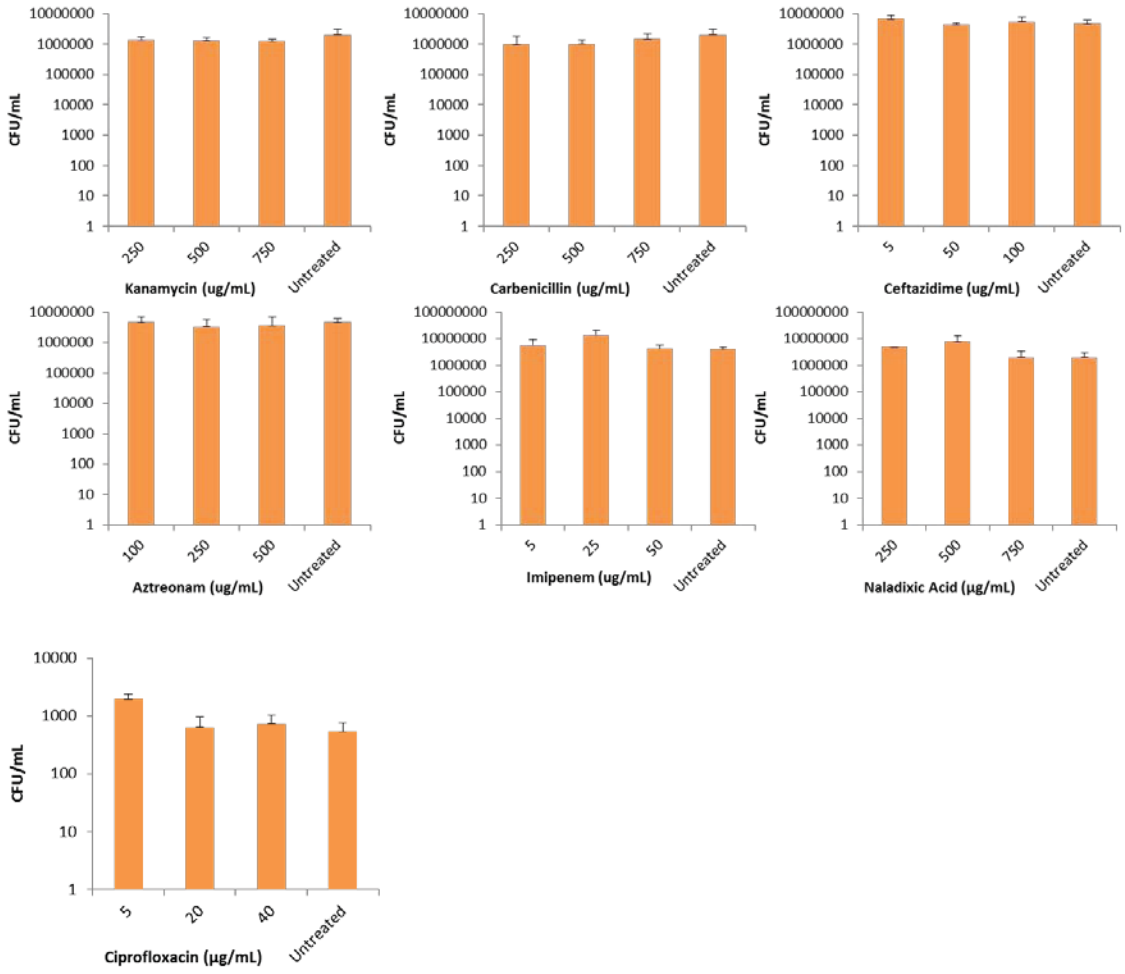

Figure 11. CFU/mL analysis of antibiotic treatment at subinhibitory levels. Kanamycin, carbenicillin, ceftazidime, aztreonam, imipenem, nalidixic acid, and ciprofloxacin were all administered at treatment levels considered subinhibitory because the CFU/mL counts were not significantly reduced in comparison to untreated controls ($p>0.05$). Data presented on a logarithmic scale. Data represents experiments performed in triplicate ($n=3$).

The remaining antibiotic treatments, including tobramycin, gentamicin, azithromycin, tetracycline, and chloramphenicol, were administered at sublethal levels for at least one concentration of the administered antibiotic to the pre-formed biofilm ($p<0.05$) (Figure 12). Sublethal levels of antibiotic treatment were assessed by

significant changes in bacteria levels (CFU/mL) between biofilms treated with antibiotics and untreated controls.

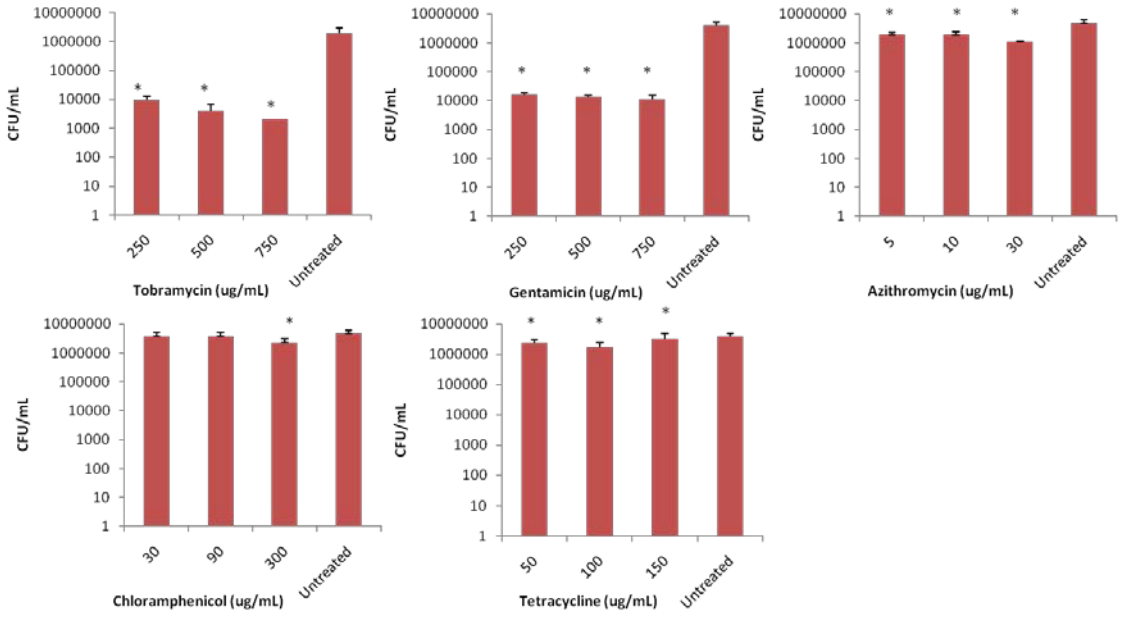

Figure 12. CFU/mL analysis of antibiotic treatment at sublethal levels. At least one treatment concentration of the following antibiotics, tobramycin, gentamicin, azithromycin, and chloramphenicol, resulted in significantly reduced numbers of viable bacterial cells ($p<0.05$) in comparison to untreated controls. These concentrations are considered sublethal. Data presented on a logarithmic scale. Data represents experiments performed in triplicate (n=3).

3.2 Quantitative RT-PCR Analysis of Antibiotic Treated Biofilms

cDNA samples previously analyzed via RT-PCR were used for quantitative RT-PCR (QRT-PCR) analysis to measure transcriptional regulation of *mgtE* following antibiotic treatment. QRT-PCR results, like RT-PCR results, were normalized to the constitutively expressed gene, *fbp*. Statistical analysis confirmed that most antibiotic treatments resulted in the transcriptional upregulation of *mgtE*. Aminoglycosides, macrolides, tetracyclines and chloramphenicol inhibit protein synthesis. Tobramycin,

gentamicin, and kanamycin (the aminoglycosides tested) all resulted in significant transcriptional upregulation of *mgtE* (Figure 13). As previously reported, tobramycin treatment of preformed PA14 biofilms at a concentration of 500 μg/mL resulted in upregulation of *mgtE* transcripts in comparison to untreated PA14 controls; furthermore, treatment with 750 μg/mL of tobramycin also resulted in significant upregulation of *mgtE* transcripts ($p<0.001$), but 250 μg/mL treatment of tobramycin did not ($p>0.05$). Both gentamicin and kanamycin treatment at all three concentration levels tested (250, 500, and 750 μg/mL) resulted in significant upregulation of *mgtE* transcripts ($p<0.01$). Treatment of pre-formed PA14 biofilms with azithromycin, a macrolide, resulted in significant upregulation of *mgtE* transcripts at concentrations of 10 μg/mL and 30 μg/mL ($p<0.05$, not two fold increase), but not at a concentration of 5 μg/mL ($p>0.05$). Treatment of pre-formed PA14 biofilms with chloramphenicol resulted in significant upregulation of *mgtE* transcripts at all three antibiotic concentrations, 30, 90, and 300 μg/mL ($p<0.001$). Tetracycline treatment also resulted in significant upregulation of *mgtE* transcripts at all three treatment concentrations, 50, 100, and 150 μg/mL ($p<0.001$) (Figure 13).

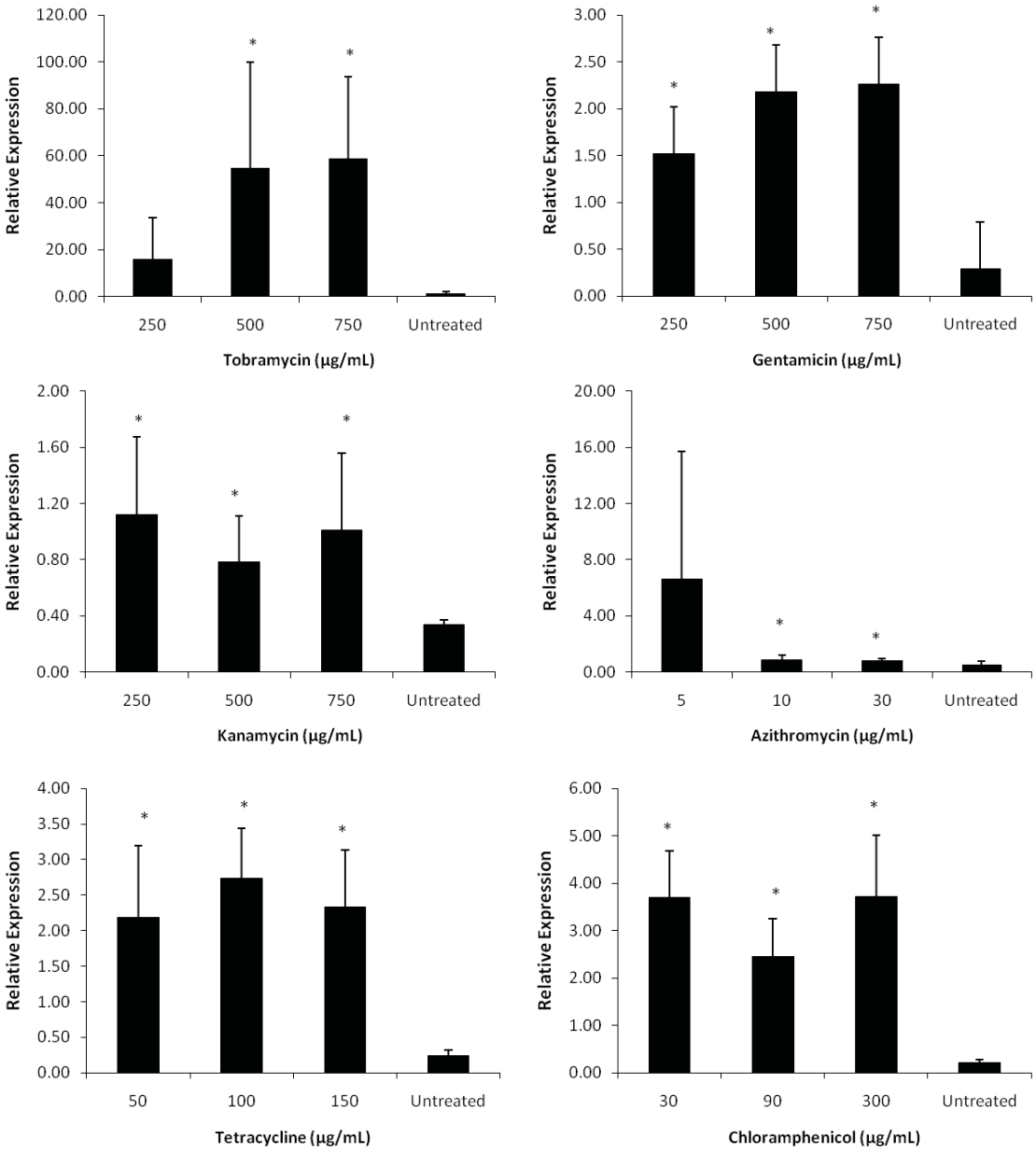

Figure 13. QRT-PCR analysis of transcriptional changes in *mgtE* following treatment with antibiotics that inhibit protein synthesis. All antibiotics tested resulted in significant upregulation of *mgtE* for most concentrations tested ($p<0.05$, two fold increases in transcription for significant results except azithromycin). Data represents experiments performed in triplicate (n=3).

Quinolones inhibit DNA gyrase (topoisomerase II) to block DNA replication. Both ciprofloxacin and nalidixic acid are quinolones. Nalidixic acid is an artificially synthesized quinolone. Treatment with both ciprofloxacin and nalidixic acid led to

significant upregulation of *mgtE* transcripts at all three antibiotic levels tested, 5, 20, and 40 µg/mL ($p<0.001$) and 250, 500, 750 µg/mL ($p<0.01$), respectively (Figure 14).

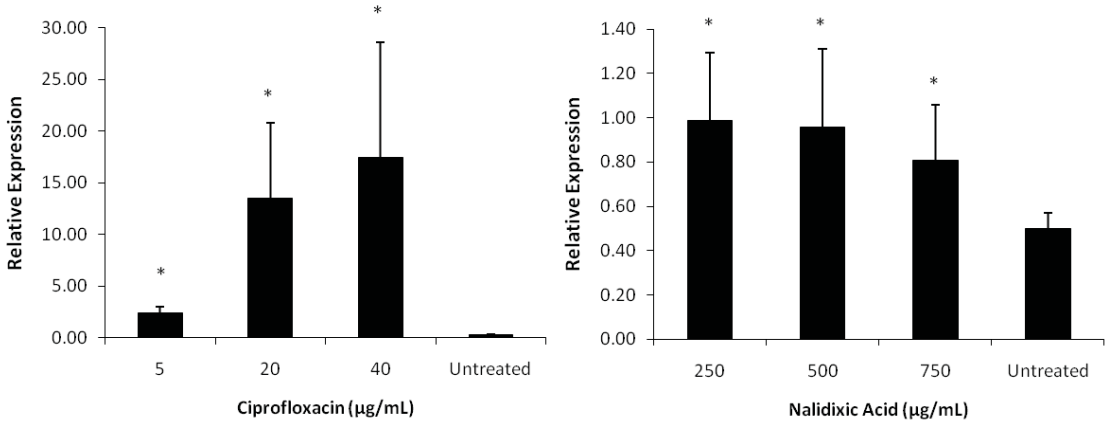

Figure 14. QRT-PCR analysis of transcriptional upregulation of *mgtE* following quinolone treatment. Quinolone treatment resulted in significant upregulation of *mgtE* transcripts at all antibiotic concentrations tested (ciprofloxacin, $p<0.001$ and nalidixic acid, $p<0.01$, two fold increases in transcription for significant results). Data represents experiments performed in triplicate (n=3).

Inhibiting cell wall synthesis is another general mechanism of action of antibiotics. The antibiotics tested that fall under this category are carbapenems, cephalosporins, monobactams, and penicillins. This group is the only group of antibiotics that, following treatment, did not induce significant increases in *mgtE* transcriptional levels. Imipenem, a carbapenem, treatment of pre-formed PA14 biofilms resulted in significant upregulation of *mgtE* transcription at two of the three administered antibiotic levels, 5 µg/mL and 25 µg/mL ($p<0.01$) but not at 50 µg/mL ($p>0.05$) (Figure 15). Carbenicillin, a penicillin, and aztreonam, a monobactam, treatments resulted in unchanged *mgtE* transcriptional levels at all concentrations tested, 5, 50, and 100 µg/mL ($p>0.05$), and 250, 500, and 750 µg/mL ($p>0.05$), respectively (Figure 16). Treatment with ceftazidime, a cephalosporin, also demonstrated varied effects on transcription of *mgtE*, but in a unique way that was not observed with any other antibiotics treatments

tested in this study. At the concentrations 5 and 100 µg/mL, no change in *mgtE* transcripts was observed. Interestingly, when biofilms were treated with 50 µg/mL of ceftazidime, a significant downregulation of *mgtE* transcription was observed compared to untreated controls ($p<0.005$) (Figure 17). This downregulation effect cannot be explained by a significant reduction in viable bacteria because all three ceftazidime treatment levels were subinhibitory (Figure 11).

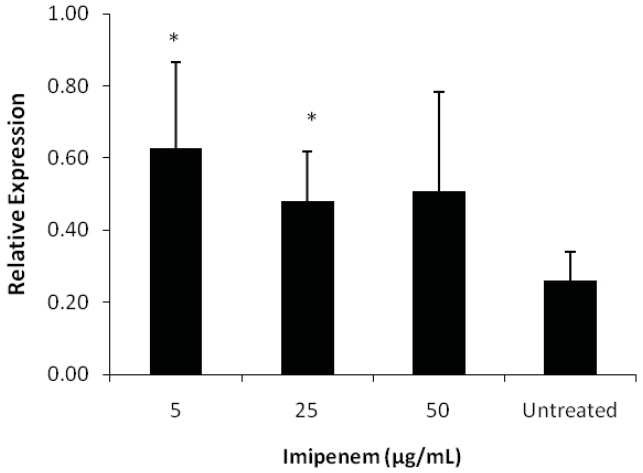

Figure 15. QRT-PCR analysis of transcriptional changes in *mgtE* following imipenem treatment. Treatment of pre-formed biofilms with imipenem resulted in significant upregulation of *mgtE* transcription for two of the three treatment levels ($p<0.01$, two fold increases in transcription for significant results). Data represents experiments performed in triplicate (n=3).

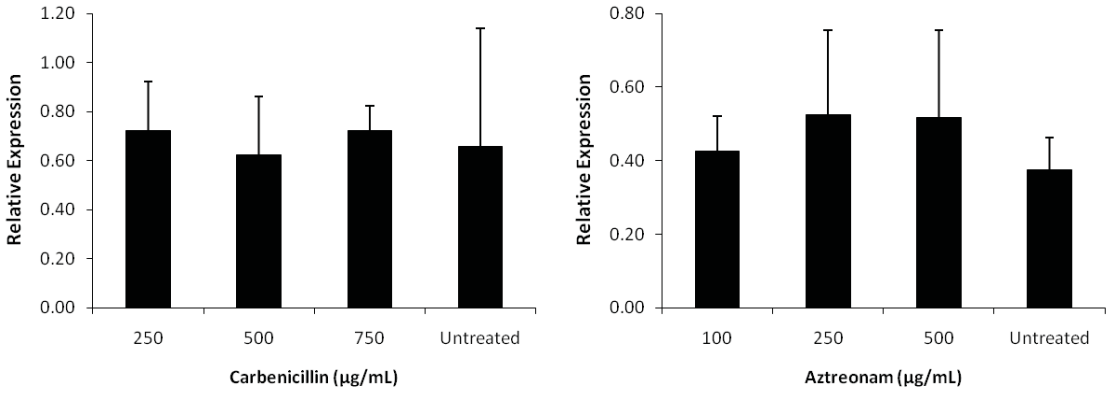

Figure 16. Antibiotic treatment resulting in unchanged *mgtE* transcription. Both carbenicillin and aztreonam treatments result in unchanged levels of *mgtE* transcripts ($p>0.05$). Data represents experiments performed in triplicate (n=3).

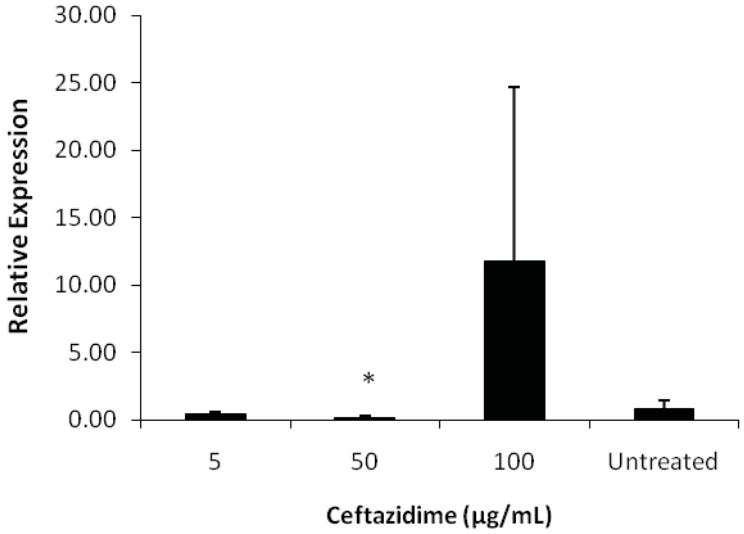

Figure 17. Antibiotic treatment resulting in decrease of *mgtE* transcripts. Ceftazidime treatment resulted in a unique finding. At 50 µg/mL, the antibiotic induced a significant downregulation of *mgtE* transcription compared to untreated PA14 biofilms ($p<0.005$). The two other concentration levels, 5 µg/mL and 100 µg/mL, resulted in no significant change in *mgtE* transcription following treatment ($p>0.05$, not a two fold decrease). Data represents experiments performed in triplicate (n=3).

3.3 Analysis of *Staphylococcus aureus* MgtE

As demonstrated, an isogenic mutation of *mgtE* in WT PA14 results in increased cytotoxicity compared to WT PA14. Placing *mgtE* on the multi-copy plasmid, pMQ72,

to create pMQ177 and transforming this vector into the isogenic Δ*mgtE* strain, SMC3604, results in rescuing of the WT PA14 phenotype by decreasing cytotoxicity often lower than WT levels[34]. Based on these findings, I set out to analyze if *mgtE* homologues expressed *S. aureus* strains will also rescue the WT PA14 phenotype by decreasing cytotoxicity. *S. aureus* is a Gram-positive bacterium that forms biofilms and is a common human pathogen. *mgtE* homologues of two laboratory *S. aureus* strains, Newman and RN3990, were isolated and placed on the vector, pMQ72, individually to create pCR9 and pCR10. Then, both plasmids, pCR9 and pCR10, were transformed individually into SMC3604. Therefore, *S. aureus mgtE* homologues were expressed by multicopy plasmids in a PA14 isogenic Δ*mgtE* mutant background so the *S. aureus* MgtE homologue is the only MgtE protein being produced. Cytotoxicity assays were performed to ascertain the cytotoxic effects of both MgtE homologues against our CFBE cell line. We found a reduction in cytotoxicity by the presence of both plasmids, in comparison to PA14 pMQ72 and SMC3604 pMQ72, therefore demonstrating the *S. aureus mgtE* homologues ability to rescue the highly cytotoxic phenotype of strain SMC3604 with a non-PA14 MgtE protein (Figure 18).

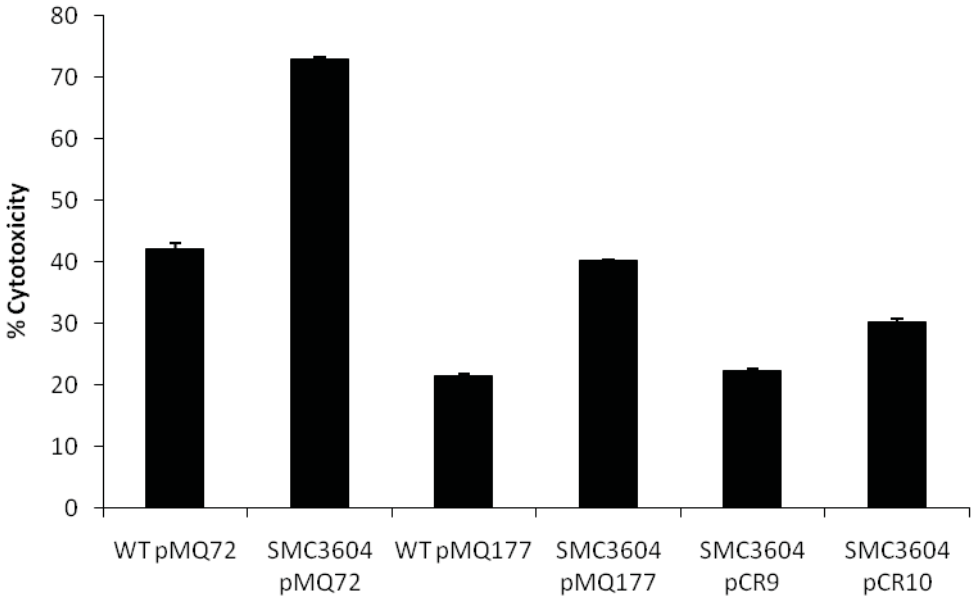

Figure 18. *S. aureus mgtE* homologue effects on cytotoxicity. The *S. aureus mgtE* homologues are able to rescue the wild type phenotype as demonstrated with the plasmids, pCR9 and pCR10. The empty vector, pMQ72, and pMQ177 (expressing PA14 m*gtE*) were placed in both WT PA14 and SMC3604 and used as controls for this experiment. Data is a representative of three experiments individually performed in triplicate (n=3).

3.4 Western Blotting to Elucidate the Translational Regulation of MgtE

To further elucidate the impact of antibiotic treatment on *mgtE* regulation, translational effects were assessed. A histidine-tagged MgtE strain was designed, CVR3, and this strain was utilized throughout all of the Western blot studies. Both a histidine specific primary antibody and a nickel conjugate system were employed. The nickel conjugate will bind a histidine-tagged protein and has an enzyme that will chemiluminesce following the addition of the Western Lightning reagent.

The co-culture biofilm model was utilized for all three assays, RT-PCR, QRT-PCR, and Western blotting. The BCA protein assay kit was used to control for protein loading in the SDS-page. Both eukaryotic and prokaryotic protein was present, as the

nature of the co-culture model system involves mixing of the cells. This resulted in varied and unreliable results. Although the primary His-tag antibody was able to detect the planktonically grown bacteria expressing the histidine-tagged MgtE (Figure 19), this antibody was not sensitive enough to detect the histidine-tagged bacteria once the bacteria were in a biofilm state, and non-specific binding was also a concern (Figure 20).

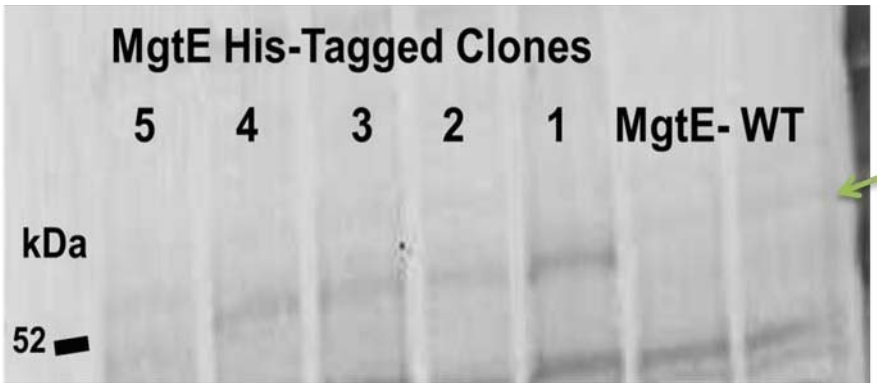

Figure 19. Primary Histidine-tag antibody recognizes Histidine-tagged MgtE in the CVR3 strain. The histidine-tagged MgtE expresssed by strain, CVR3 represented in five clones above, is recognized by the primary antibody, as indicated by the green arrow (MgtE approximate weight, 54 kDa). Both strains not containing a histidine tag, SMC3604 (MgtE-) and WT PA14 are not recognized. This blot represents planktonic growth of all bacterial strains.

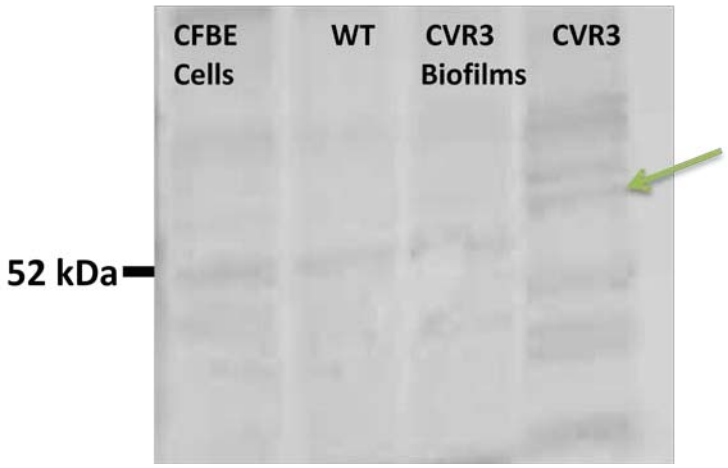

Figure 20. Histidine-tagged MgtE is not detected during biofilm growth by primary Histidine-tag antibody. Histidine-tagged MgtE expressed in strain CVR3 is detected only during planktonic growth. When this strain is allowed to form a biofilm, the his-tagged MgtE is no longer detected. Controls are both the CFBE cells, which the biofilms are formed on and WT PA14 biofilms grown on CFBE cells. Furthermore, non-specific binding is an issue with this antibody and the non-specific banding pattern is different between biofilm growth and planktonic growth, which demonstrates changes in protein expression or protein modification.

The nickel conjugate system was more sensitive and gave promising results initially (Figure 21), but those results did not replicate. Quickly, it became evident that the nickel conjugate was binding non-specifically to a eukaryotic protein at the same approximate molecular weight as MgtE (54 kDa) (Figure 22).

I have been unable to demonstrate, by utilizing the Western blot technique, the translation of *mgtE* following antibiotic treatment. Future directions should include raising a specific antibody to MgtE, hopefully eliminating many of the problems encountered with the histidine-tag sensitivity and detection and eukaryotic protein expression. Furthermore for the purpose of this study, a functional assay could be utilized to indirectly test for increased MgtE levels, as described below (section 3.5).

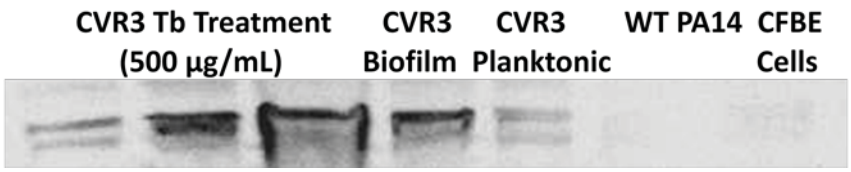

Figure 21. Nickel conjugate detection of Histidine-tagged MgtE. His-tagged MgtE is detected in biofilm growth (strain CVR3) on CFBE cells following tobramycin treatment (500 µg/mL) and in planktonically grown CVR3 in comparison to controls, WT PA14 and CFBE cells only.

Figure 22. Nickel conjugate detection of unknown eukaryotic protein. CFBE cells are expressing a protein at approximately 54 kDa that is strongly recognized by the nickel conjugate. This representative finding was replicated, and places into question the findings in Figure 21 and the viability of this system in detecting the histidine-tagged MgtE.

3.5 Functional Assay to Detect Changes in MgtE Translation

Since detection of a histidine-tagged MgtE protein expressed by the CVR3 biofilm via Western blotting has proven impossible with the methods available at this time, a functional assay based on cytotoxicity changes should be performed. This assay would indirectly indicate if MgtE is being translated following antibiotic treatment. Using the laboratory WT PA14 strain, biofilms are formed on the CFBE cell line as already explained in the sections discussing the RT-PCR and QRT-PCR experiments (sections 3.1 and 3.2). Biofilms were treated with antibiotics at the same concentrations as in prior experiments but for one hour, instead of thirty minutes. Cytotoxicity assays were performed to determine if the regulation of *mgtE* transcripts, demonstrated by RT-PCR and QRT-PCR, are being translated in the same fashion. If they are, then cytotoxicity toward the CFBE cell line will change. If the antibiotics resulted in increased transcription of *mgtE* then a decrease in cytotoxicity toward the CFBE cells

should be observed as overexpression of the MgtE protein compared to WT PA14 expression of the MgtE protein results in decreased cytotoxicity toward the CFBE cells. This assay should also indicate if the antibiotic treatment causing no change or a decrease in *mgtE* transcripts induces the same trend during translation of MgtE. Preliminary studies indicate that *mgtE* transcripts are being translated following antibiotic treatment. This has been demonstrated following kanamycin treatment of preformed PA14 biofilms (Figure 23).

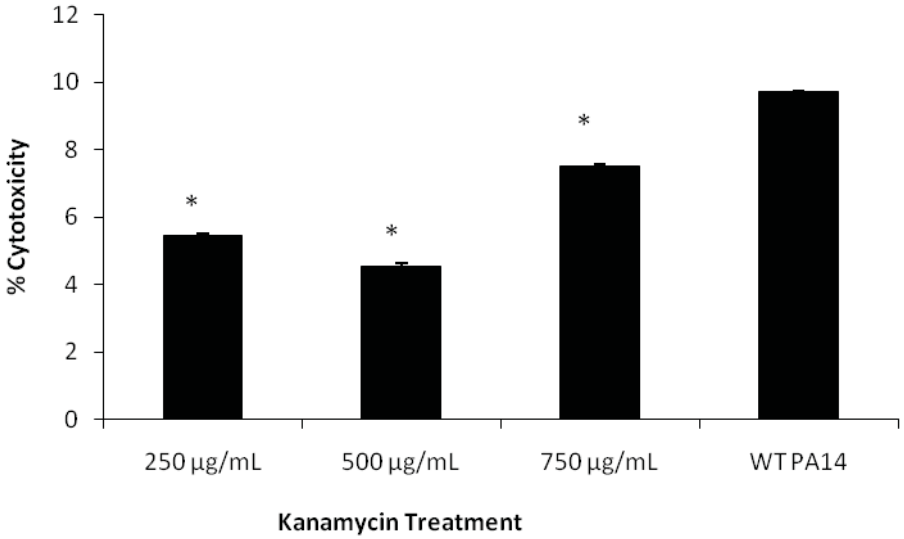

Figure 23. Translation of MgtE following kanamycin treatment. Percent cytotoxicity significantly decreased ($p<0.05$, n=3) following kanamycin treatment at three concentrations compared to the untreated control (WT PA14). These treatment concentrations were the same concentrations used to ascertain transcriptional regulation of *mgtE*, and these results support previous findings (n=2).

3.6 Connecting *mgtE* with a Known Regulatory Pathway

As changes in transcriptional upregulation of *mgtE*, for the most part, are universal among the antibiotics tested for this study and it has been proven that *mgtE* plays a role in virulence modulation, I hypothesized the *mgtE* is interacting with a

regulatory pathway involved in virulence modulation. To further elucidate a possible interaction, a transposon mutant library[115] was employed. This library is a sequenced library of PA14 genes that have been randomly transposon mutated. Following a selection process, which involved performing defined search of the transposon mutants on an excel spreadsheet, to narrow down the screen to just regulatory proteins, I screened this library, for qualitative changes in cytotoxicity, either hypercytotoxic or hypocytotoxic, toward our CFBE cell line in comparison to WT PA14. For the transposon mutations that had a hypercytotoxic effect on the CFBE cell line, I transformed these mutants with the *mgtE* complement plasmid, pSMC21. This plasmid is a multi-copy plasmid expressing *mgtE*. The plasmid, pMQ177, was used as an the overexpressed *mgtE* control in WT PA14. Both plasmids, pMQ177 and pSMC21, are the same plasmid backbone with the selection cassette changed. Gentamicin is the selective cassette on pMQ177, and carbenicillin is the selective cassette on pSMC21. The transposon mutations are under a gentamicin selection, therefore rendering pMQ177 unusable.

If MgtE is not interacting with an individual transposon mutant expressing a hypercytotoxic phenotype, cytotoxicity should decrease as MgtE does not depend on the mutated gene to express its phenotype. But, if MgtE does depend on the mutated gene to express its phenotype, there will no change in the hypercytotoxic effect of mutant on the cell line. The same concept is used to examine a possible MgtE interaction with the hypocytotoxic transposon mutants. A *mgtE* deletion was performed in these mutants. If MgtE does not interact with the hypocytotoxic transposon mutant, then cytotoxicity should increase. If cytotoxicity does not increase or decreases, then these results indicate

a possible interaction between MgtE and the transposon mutated gene. Of the 17 hypercytotoxic transposon mutated genes identified in the initial screen, I was able to transform pSMC21 into 11 transposon mutants and test them for cytotoxicity changes. I found two genes, *nasT* and *narX*, that maintained increased cytotoxicity with the plasmid overexpressing *mgtE* masking the expected decreased cytotoxic effect (Figure 24). I identified 25 hypocytotoxic transposon mutant strains in which to perform an isogenic deletion of *mgtE* using pSMC233-Amp and accomplished this task with 14 of these strains. Of the 14 strains tested, the two genes *argR* and *pII* after triplicate testing, demonstrated possible interactions with *mgtE* by inhibiting the expected increased cytotoxic effect of an *mgtE* deletion (Figure 25).

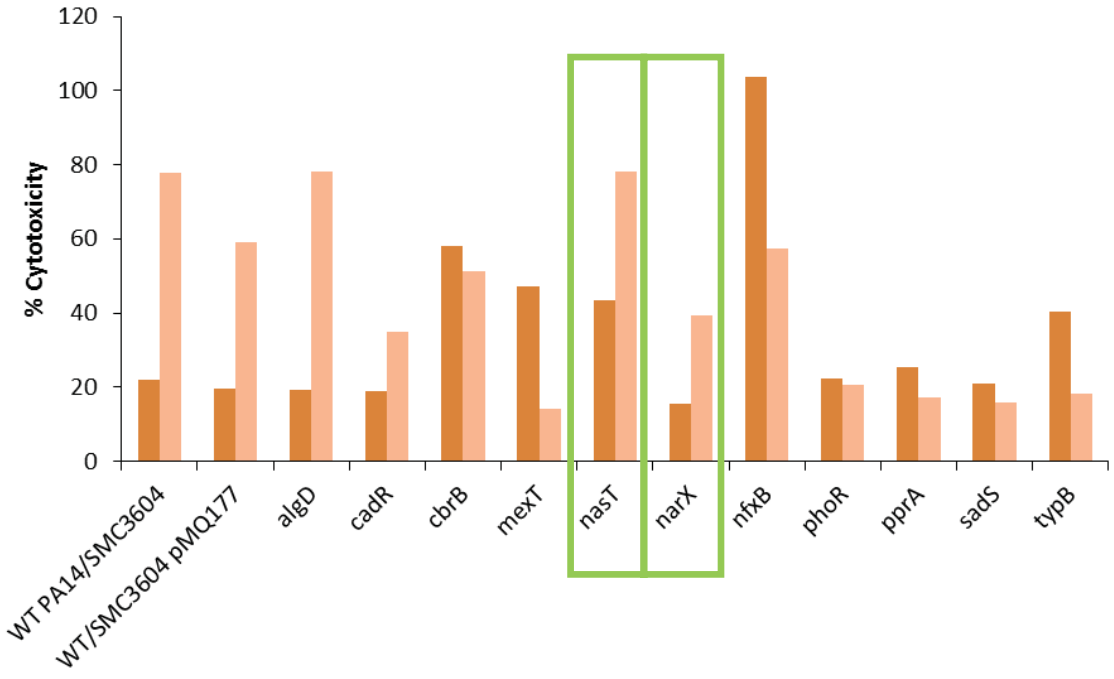

Figure 24. Cytotoxicity results of effect of *mgtE* complementation in conjunction with transposon mutated genes. This bar graph represents the transposon mutant (left bar, dark orange) and the transposon mutant/*mgtE* complement(right bar, light orange) for eleven different transposon mutated strains representing eleven different mutated genes. Transposon mutations in both *nasT* and *narX* resulted in increased cytotoxicity during initial screens. The multi-copy complement plasmid expressing *mgtE* should reduce the cytotoxicity of the mutants. In both mutants, cytotoxicity increased instead of decreasing representing a possible interaction. Data is a representative of three experiments individually performed in triplicate. Results for *algD*/*mgtE* complementation shown above has not replicated (n=3).

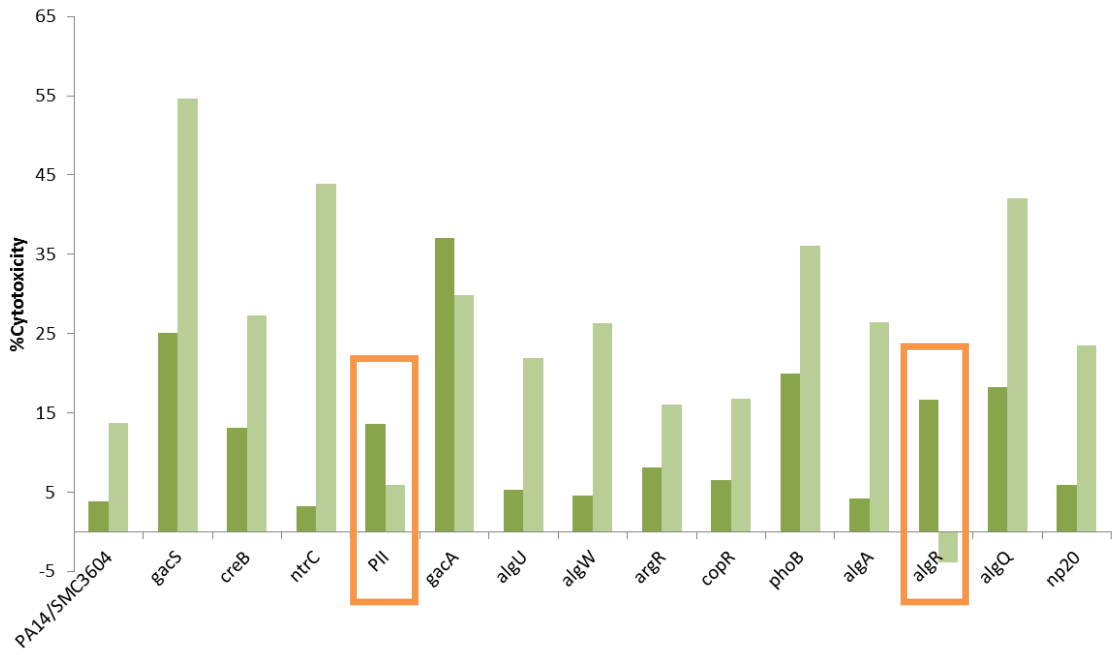

Figure 25. Cytotoxicity results of effect of *mgtE* deletion in conjunction with transposon mutated genes. This bar graph represents the transposon mutant (left bar, dark green) and the transposon mutant/Δ*mgtE* deletion (right bar, light green) for fourteen different transposon mutated strains representing fourteen different mutated genes. Transposon mutations in both *pII* and *algR* resulted in decreased cytotoxicity during initial screens. A isogeneic deletion of *mgtE* was performed and cytotoxicity decreased instead of increasing representing a possible interaction. Deletion of *mgtE* in transposon mutant *gacA* background also led to a slight decrease in cytotoxicity in this particular experiment but was not replicated. Data is a representative of three experiments individually performed in triplicate (n=3).

3.7 $MgSO_4$ Concentrations and Biofilm Formation of Magnesium Transporters and MgtE Mutants

Using the static biofilm assay, biofilms were formed in varying levels of $MgSO^4$, both millimolar and micromolar concentrations. Different mutant strains with deletions of different magnesium transporters were tested. The purpose of these studies was an attempt to link magnesium transport and biofilm formation. The normal magnesium concentration used in this type of asssay is 1 mM. The strains tested were SMC3604,

SMC3640, SMC3643, SMC3644, SMC3646, SMC3647, and WT PA14. Variable results were observed, as no distinct theme in changes in biofilm formation could be ascertained following changes in $MgSO^4$ availability between the mutant strains and WT PA14 (Figures 26 and 27). Concentrations of magnesium in the CF airway (1.9 µM) do not vary significantly from the normal (non-CF) airway (2.3 µM). Altering $MgSO^4$ at the concentrations below did not significantly alter biofilm formation between mutants and WT PA14.

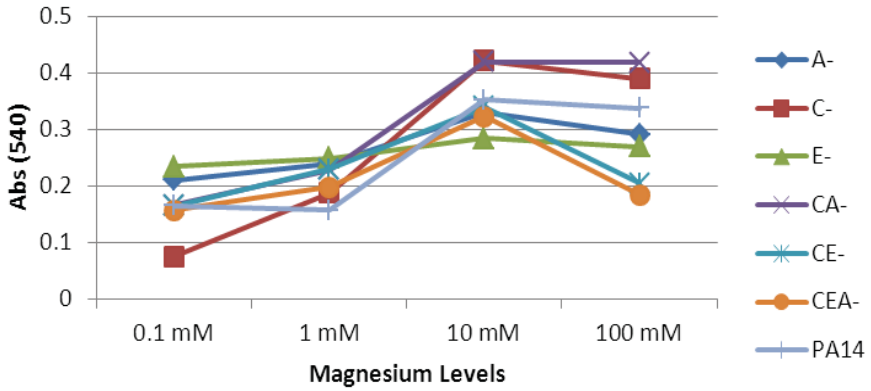

Figure 26. The effect of magnesium at millimolar concentrations on biofilm formation. Biofilm formation follows the same trend between all mutants indicating that changes in magnesium, although overall may induce slight increases in biofilm formation, no changes between mutants and WT PA14 biofilm formation is evident. SMC3604, Δ*mgtE*, shows the lowest response via change in biofilm formation in comparison to the other mutants. Data is a representative of three experiments individually performed in triplicate (n=3).

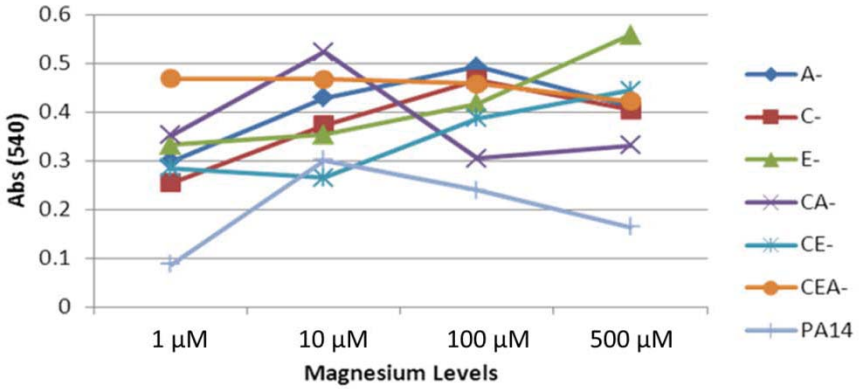

Figure 27. The effect of magnesium at micromolar concentrations on biofilm formation. Overall biofilm formation is slightly increased for all mutants in comparison to WT PA14. Specific trending does seem to change between mutant biofilm formation at different concentrations of magnesium. At micromolar concentrations, SMC3647, Δ*mgtE,* Δ*mgtC,* Δ*mgtA*, presents with the most unchanging biofilm phenotype compared to other mutants, similarly to the SMC3640 biofilm phenotype in Figure 25. The CF airway magnesium levels fall to the left of this graph, approximately a 2 µM concentration. At this level of magnesium, biofilm formation is increased in comparison to WT PA14 levels, but very little change in biofilm formation between the mutant strains is observed. Data is a representative of three experiments individually performed in triplicate (n=3).

CHAPTER FOUR DISCUSSION

4.1 Regulation of *mgt*E via Antibiotics

Although the hypothesis that all antibiotics tested would induce upregulation of *mgtE* transcription was not proven completely true, most antibiotics tested did lead to increased transcription of *mgtE*. Antibiotic interaction with *P. aeruginosa* biofilms is rather complex, as different antibiotics have different demonstrated effects on biofilm induction, and architecture, by inducing biofilm formation, inhibiting quorum sensing, and upregulating virulence factors and modulators. As previously demonstrated, *mgtE* has been shown to have virulence modulatory effects via inhibition of the T3SS[34]. When the T3SS is inhibited, biofilm architecture is altered. Therefore, there seems to be an indirect relationship between increased expression of *mgtE* by antibiotic treatment and changes in biofilm architecture. Furthermore, the antibiotic treatments in this study varied, whether subinhibitory or sublethal, which is an important distinction as studies have shown that antibiotic levels can induce different genetic and phenotypic changes in the *P. aeruginosa* biofilm.

Many antibiotics used to treat bacterial diseases are derivatives of naturally occurring microbial products which are produced by microorganisms. These microorganisms produce antibiotic compounds as a result of competitive environments where different microbes are competing for the same environmental niche[116].

Aminoglycosides are an example of a clinically relevant group of antibiotics, and three different aminoglycoside antibiotics were tested in this study: tobramycin, gentamicin, and kanamycin. Specifically, tobramycin is produced by *Streptomyces tenebrarius* specifically in response to the presence of *P. aeruginosa* in its environmental niche, soil. *P. aeruginosa* possesses an inducible resistance to tobramycin[117] suggesting an adaptive response. Another selected adaptive response may be biofilm formation by *P. aeruginosa* following interaction with tobramycin in these specific niches, and this response translates into the clinical setting[118]. Subinhibitory concentrations of tobramycin have been shown to increase *P. aeruginosa* biofilm formation[119]. Ciprofloxacin treatment, below the minimal inhibitory concentrations (MIC) or at subinhibitory levels, also induces an increase in biofilm formation. Carbenicillin and ceftazidime treatment did not[78], so not all antibiotic treatments lead to increased biofilm formation.

The macrolide azithromycin has been shown to inhibit guanosine diphosphomannose dehydrogenate in the alginate biosynthetic pathway, which is a pathway that contributes to mucoidy phenotypic changes during chronic *P. aeruginosa* infection. In the CF airway, the production of alginate induces local inflammatory responses and increased sputum viscosity; therefore, for treatment purposes in the clinical setting, reduction of alginate production is very beneficial[120]. Furthermore, azithromycin is able to inhibit quorum sensing in *P. aeruginosa*[121], and quorum sensing regulates certain virulence genes, including repression of the T3SS[122]. Ciprofloxacin and ceftazidime also exhibit strong quorum sensing inhibitory effects[121], although the specific genes that the antibiotics interact with to cause this effect varies. Interestingly, treatment

with ceftazidime, as the predominant anti-pseudomonal antibiotic, in treatment of CF has promoted the epidemic spread of a multi-resistant nonmucoid strain of *P. aeruginosa*[123], which is not surprising considering this antibiotic is able to inhibit quorum sensing and has no role in stimulating biofilm formation.

Treatment with certain antibiotics, such as ciprofloxacin at sublethal levels (MIC), may induce production of certain virulence factors by the *P. aeruginosa* biofilm. Specifically, subinhibitory ciprofloxacin treatment will induce protease secretion. Secretion of proteases damages mammalian matrix proteins. Considering biofilms are nearly impossible to eradicate with antibiotic treatment, induction of virulence factors following antibiotic treatment is a serious concern[124]. Treatment of *P .aeruginosa* biofilms with imipenem at the subinhibitory level, 1 μg/mL, causes increased alginate production by inducing increased expression of many important regulatory proteins in the alginate biosynthesis pathway, including AlgR[125].

Both RT-PCR and QRT-PCR analysis of *mgtE* transcription by preformed biofilms following antibiotic treatment showed an upregulation of *mgtE* expression by most antibiotics tested. Analysis via QRT-PCR demonstrated that treatment by three antibiotics did not lead to *mgtE* upregulation. Both carbenicillin and aztreonam resulted in unchanged *mgtE* transcription levels at all three antibiotic concentrations tested, and ceftazidime treatment resulted in a downregulation of *mgtE* transcription at one concentration, 50 μg/mL, following treatment. These three antibiotics all function as inhibitors of cell wall synthesis, but are not the only antibiotics tested with this mechanism of action. Other antibiotics that inhibit cell wall synthesis resulted in increased transcription of *mgtE* consistent with the stated hypothesis, such as imipenem.

Carbenicillin and aztreonam were tested at subinhibitory concentrations, although, once again, other antibiotics were also tested at subinhibitory concentrations and exhibited the expected effect, an upregulation of *mgtE* transcripts. Therefore, a simple explanation for the observed effects of these two antibiotics on *mgtE* transcriptional regulation is not possible. Postulation of possible explanations can lead to future directions for this research.

Overall, the antibiotics that resulted in increased expression of *mgtE* likely lead to reduced expression of the T3SS complex, which is correlated with changes in biofilm architecture. Elicitation of changes in biofilm architecture could be explained by demonstrating a role for *mgtE* in induction of pathways that lead to the mucoid and biofilm forming state of *P. aeruginosa*; one such pathway would be the alginate biosynthesis pathway. Carbenicillin, an anti-pseudomonal penicillin, and aztreonam, also an anti-pseudomonal antibiotic, should have had the same effect on *mgtE* transcription as the aminoglycosides, imipenem, and the quinolones. But, as stated earlier, treatment with both carbenicillin and aztreonam at subinhibitory levels show no change in *mgtE* transcription compared to an untreated control. Treatment at subinhibitory levels could, in conjunction with the fact that these antibiotics are a bactericidal antibiotic that kill only rapidly growing cells, could explain why *mgtE* transcripts were not increased or decreased. Bactericidal antibiotics treated at subinhibitory levels might not stress the biofilm cells enough to induce regulatory changes, therefore, not increasing expression of *mgtE*[126]. Although imipenem and kanamycin are also bactericidal antibiotics, and treatment with these antibiotics resulted in upregulation of *mgtE* transcription, this response is more complex than a basic stress response according to levels of antibiotic

treatment and mechanism of action of the antibiotic. As mentioned earlier in this discussion, previous studies demonstrate that treatment with carbenicillin at subinhibitory levels did not increase biofilm formation, therefore *mgtE* transcriptional upregulation with this particular antibiotic treatment regimen is not plausible considering the correlative evidence between *mgtE* expression and biofilm architecture changes. Evidence for impact on virulence by aztreonam has not been studied, so this type of correlation cannot be made.

Treatment with ceftazidime, at the concentration of 50 μg/mL, resulted in decreased expression of *mgtE* without changes to CFU/ml counts, therefore, this decrease in transcription following treatment is not explained with a reduction of viable cells. As mentioned earlier in this discussion, treatment with this antibiotic as the primary agent in response to chronic *P. aeruginosa* CF airway infections resulted in highly resistant non-mucoid strains of *P. aeruginosa*. This data implies that ceftazidime does not promote biofilm formation and, therefore, should not result in an upregulation of *mgtE* but, in fact, should actually downregulate expression of *mgtE* to promote a nonmucoidy phenotype. Overall, these results further implicate *mgtE* in biofilm architectural changes and virulence modulation.

Lastly, it is important to note that all antibiotics that deviated from the transcriptional upregulation trend of *mgtE* inhibit cell wall synthesis and were tested at subinhibitory concentrations only. This is most likely not a coincidence. Further work should explore this aspect by testing these three antibiotics, carbenicillin, aztreonam, and ceftazidime, at sublethal doses, as well as testing other antibiotics in the same classes, including anti-pseudomonal penicillins, cephalosporins, and monobactams, to confirm

the effect found with these three antibiotics. The other nine antibiotics tested that resulted in upregulation of *mgtE* expression should be tested at either sublethal or subinhibitory levels, depending on prior testing, to further elucidate this upregulation effect. I would suspect that changing between subinhibitory and sublethal concentrations of the antibiotic treatments that resulted in transcriptional upregulation of *mgtE* may not cause significant changes in the results to this study.

4.2 Translation of *mgtE* following antibiotic treatment

In order to examine whether the transcriptional effects on *mgtE* following antibiotic treatment could also be demonstrated on a translational level, Western blotting was employed. A specific antibody was not available for *P. aeruginosa* MgtE, so a construct was designed and resulted in a strain of *P. aeruginosa* that expressed a histidine-tagged MgtE protein only. This strain has a histidine-tagged *mgtE* inserted on the chromosome in the isogenic, Δ*mgtE* mutant background. This histidine-tag is expressed right after the start codon on the protein, therefore expressing on the N-terminus side of the protein. Both a primary antibody specific for histidine-tagged proteins and a nickel-conjugate system were used. Neither system was able to definitively show translation of *mgtE* following antibiotic treatment, for different reasons. The primary antibody was not sensitive enough to detect histidine-tagged MgtE produced by PA14 biofilms, and non-specific binding was an issue. Interestingly, non-specific binding patterns changed between planktonically grown bacteria and biofilm forming bacteria of the same strain, implying changes in protein expression and/or changes in protein modification as the bacteria enter a biofilm forming state of growth. The nickel-conjugate system was

detecting a eukaryotic protein at the same molecular weight as MgtE, therefore making this system not useful in demonstrating MgtE translation. Evidence has been presented in this thesis that implicate the protein, MgtE, in regulatory pathway involvement following biofilm formation on CFBE cells, and this is strong evidence that MgtE is getting translated during the changes from planktonic growth to biofilm growth, implying that since certain antibiotics have been shown to promote biofilm growth and transcription of *mgtE* is increased under these particular antibiotic treatments, then translation of MgtE is most likely occurring. To continue using Western blotting as a technique to identify translational effects of antibiotic treatment on MgtE, a specific antibody needs to be raised. Furthermore, a future study should be performed for the purpose of correlating antibiotic treatment at subinhibitory concentrations to changes in cytotoxicity. If antibiotic treated biofilms result in cytotoxicity decreases in comparison to untreated biofilms, then translation of the increased transcripts of *mgtE* is most likely occurring. This has been demonstrated through functional assays meant to use changes in cytotoxicity to ascertain translation of *mgtE* transcripts. Preliminary tests have been performed using functional assays and kanamycin treatment. Kanamycin was chosen because it is the only aminoglycoside treated at subinhibitory concentrations for all RT-PCR and QRT-PCR analysis. Kanamycin treatment resulted in a signficant decrease of cytotoxicity toward the CFBE cells compared to an untreated control (Figure 23). These findings indirectly demonstrate that the increased *mgtE* transcripts that are produced following kanamycin treatment are being translated at a higher rate in comparison the the untreated control. Furture studies should involve more functional assays being performed with the other antibiotics tested in this study.

4.3 Implicating MgtE in Regulatory Pathway Involvement

To explain the demonstrated MgtE effects in virulence modulation, the hypothesis that MgtE in acting in a regulatory pathway was examined. A *P. aeruginosa* transposon mutated and sequenced library was employed[115]. Findings, based on cytotoxicity changes, associate *mgtE* interaction with several different genes including *pII*, *narX*, *nasT*, and *algR*.

PII, NasT, and NarX are proteins that are involved in nitrogen sensing and nitrogen metabolic pathways. Both PII and NasT activate pathways involved in nitrogen metabolism. As discussed earlier, NarX is part of a two component regulatory system that has a demonstrated ability to inhibit arginine fermentation by binding to the *arcDABC* operon and preventing stimulus of the operon through ArgR[77]. As the addition of arginine to bacterial assays has been shown to stimulate biofilm growth, exploring the interaction of MgtE with the NarX-NarL pathway would be interesting. MgtE could be indirectly promoting arginine synthesis and, therefore, stimulating biofilm growth by interacting with NarX and preventing the activation of NarL. If NarL activation is inhibited, then NarL could not bind to the arginine fermentation operon and the operon could be stimulated by ArgR, leading to an increase in biofilm formation. This thought is reinforced with the data presented in this thesis. Overexpression of *mgtE* in the transposon mutant background of *narX* resulted in increased cytotoxicity. The effect of overexpression of *mgtE* is blocked by the *narX* mutant, and NarL could be activated by another source or still be activated by the *narX* mutant. When NarL is activated, inhibition of arginine synthesis is expected and biofilm architecture may change, leading to increased cytotoxicity. Furthermore, more than one gene implicated in nitrogen

metabolism was found which seems to further suggests possible interactions of *mgtE* with nitrogen regulatory proteins.

Evidence presented in this thesis implicates an interaction between *mgtE* and *algR* genes. AlgR is a regulatory protein that functions in many different pathways, most notably the alginate biosynthesis pathway. Induction of this pathway results in *P. aeruginosa* expressing a mucoidy phenotype. The mucoidy phenotype is employed during chronic infection of the CF airway and results in biofilm formation. AlgR is part of the two component regulatory system, AlgR-AlgZ pathway, interacting with AlgU[127]. AlgU is the key regulatory protein that encodes sigma factor, σ^{22}, which activates many genes essential for induction of the alginate pathway[128]. Furthermore, *algR* is an essential gene for *P. aeruginosa* pathogenesis, as an *algR* mutant is less virulent than a wild-type strain in an acute septicemia mouse modeled infection[129]. AlgR is also required for twitching motility, which is associated with increased virulence[130]. This evidence, as well as proteomic analysis of an *algR* mutant, suggests that AlgR is a global regulator impacting the expression of many different genes[129], including mediating the repression of the T3SS genes. When AlgR is mutated, an increased expression of certain T3SS genes is observed, including *exsA* and *exoS*. ExsA is the master regulator for the T3SS in *P. aeruginosa*[131]. These findings imply important but complex interactions of AlgR with other virulence pathways in *P. aeruginosa*.

The findings in this study show a strain with transposon mutated *algR* resulted in decreased cytotoxicity, which is an unexpected finding if the transposon mutation results in a loss of function. Transposon mutations can also result in increased function of the protein. If this is the case, then the findings presented here, of decreased cytotoxicity,

would be expected. Arbitrary-primed PCR followed by sequencing from the transposon in this particular mutant would undeniably provide a better explanation of where the transposon is inserted in the gene. Also, a gene deletion of *algR*, a double mutation of *algR* and *mgtE*, and overexpression of *algR* should be performed. In the transposon mutated *algR* background, a deletion mutation of *mgtE* was performed, and results show a probable interaction between these two proteins. The expected results of increased cytotoxicity of the *mgtE* deletion was masked by the *algR* mutation maintaining a decreased cytotoxic effect on the CFBE cell line, even below wild type levels. This would imply that MgtE is upstream of AlgR in an unknown pathway, where the increased cytotoxic effect expected with the *mgtE* deletion is blocked by the mutation in *algR*. AlgR and MgtE exhibit similar cytotoxic phenotypes. Both, when overexpressed, result in decreased cytotoxicity, and, when deleted from the chromosome, result in increased cytotoxicity. Therefore, it is plausible these two proteins could be connected in some way as they exert similar effects in virulence modulation in *P. aeruginosa*.

4.4 Transcription, Translation, and Regulatory Pathway Interactions of MgtE

These findings come together to tell an important story about the impact that *mgtE* has on virulence modulation in *P. aeruginosa*. When a pre-formed *P. aeruginosa* biofilm is treated with a multitude of different antibiotics, they induce a similar response by the biofilm. They induce a stress response. The biofilm is able to sense danger, most likely through many different signaling pathways. The biofilm's bacterial constituents can respond to this stressor in two main ways. They can choose to leave the biofilm as individual planktonic cells. Although, this is a good strategy with certain environmental

stressors, such as lack of nutrients, antibiotic treatment would surely kill any cells that decide to leave the biofilm. The other choice is to stay in the biofilm and try to shield themselves from the "attack". Therefore, following antibiotic treatment, I postulate that biofilm constituents sense the antibiotics and change regulation of key genes with the goal of surviving this "attack", and, to that end, they universally upregulate certain genes. One gene that is upregulated is *mgtE*. The transcriptional upregulation of *mgtE* and ensuing translation of MgtE interacts with AlgR, sending a signal through AlgR inducing the activation of the *algD* operon resulting in transcription and translation of alginate. Alginate is secreted and contributes to a further induced mucoidy phenotype, by contributing to the exopolysaccharide matrix of the biofilm. This matrix functions as a protective shield from outside stressors, and increasing this matrix would result in survival of bacterial constituents inside of the biofilm (Figure 28).

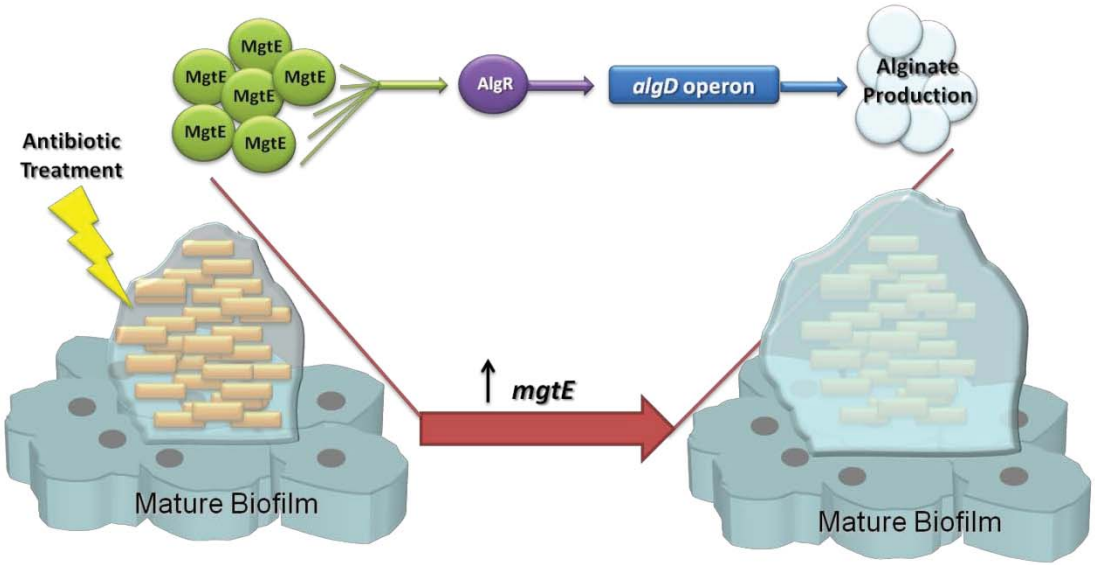

Figure 28. Postulated schematic antibiotic treatment on virulence modulation through *mgtE*. This diagram is a summary of my interpreted results based on data presented in the thesis. Antibiotic treatment induces a stress response from the biofilm resulting in increased transcription and translation of *mgtE*. Increased expression of MgtE results in activation of the *algD* operon through AlgR leading to increased alginate production contributing to increased secretion of the exopolysaccharide matrix resulting in protection of the biofilm from the antibiotics.

4.5 Exploring *S. aureus* MgtE function

S. aureus is an initial colonizer of the CF airway, although, eventually *P. aeruginosa* becomes the primary colonizer as a person with CF ages and bacterial infections become more complex[110]. *S. aureus* is a gram positive bacterium that is able to form biofilms and expresses a homologue of the MgtE protein expressed by *P. aeruginosa*. For these reasons, *S. aureus mgtE* homologues were analyzed for the ability to complement the decrease of cytotoxicity that *P. aeruginosa* naturally expressing MgtE initiates. By placing the *mgtE* homologue from two strains of *S. aureus*, Newman and RN6390, on the multi-copy plasmid, pMQ72, and transforming the plasmid into the PA14 isogenic mutant strain SMC3604, direct effects of the MgtE homologues on

cytotoxicity were observed. The homologues were able to complement innate MgtE function in *P. aeruginosa*. These findings further elucidate the functionality of MgtE. These proteins, *S. aureus* MgtE homologues and WT PA14 MgtE, share similar structures, but are not identical. Therefore, by comparing amino acid similarities, one could deduce important conserved structural components of both molecules that make the apparent effects on cytotoxicity possible, most likely by communicating with regulatory proteins. Furthermore, these results could implicate the demonstrated function of MgtE in virulence modulation in other pathogenic microorganisms, as these results imply a mechanism for MgtE functionality in virulence modulation in *S. aureus*. As there are many different microorganisms that can cause significant infection in the CF airway, analysis of MgtE homologues in these organisms would prove highly informative. As MgtE functionality is further elucidated, MgtE may become recognized as a universal virulence modulator in biofilm forming bacteria, and because of this, may be utilized as a target for therapy for patients who are infected with biofilm diseases, and not just biofilm diseases of the CF airway.

4.6 Does Changing Magnesium Levels Impact Biofilm Growth?

Biofilm assays were performed with six different magnesium transporter mutants under varying levels of magnesium (both millimolar and micromolar). These experiments represent an attempt to link magnesium transporters and biofilm formation. MgtE has been linked to virulence modulation through interactions with the T3SS, which leads to changes in biofilm architecture. The findings of this study support the idea that magnesium transport and biofilm formation are not directly linked, but indirectly through

interactions with regulatory pathways that induce biofilm formation. Mutations of biofilm transporters and changes in magnesium levels did not cause significant changes in biofilm formation, where the biofilm was prevented from forming or forming at extremely high rates. These findings support the idea presented in this discussion that biofilm formation and architecture changes are linked with MgtE through interactions with the alginate biosynthesis pathway, and this interaction is not thought to be dependent on magnesium availability.

CHAPTER FIVE GK-12 FELLOWSHIP

5.1 Introduction: Bringing Biofilms into the High School Classroom

Inquiry based science education has consistently been proposed as an essential mechanism to employ when educating K-12 students in the sciences. In the classroom, inquiry based lessons may include identifying and posing questions, designing and conducting investigations, analyzing data, using models, and communicating findings[132]. Science teachers have differing views of science which influence their incorporation of inquiry based instruction when they design their curriculum. Educators that view science as an objective body of knowledge created by an "inflexible scientific method" often are impeded in accurately employing inquiry in their classroom. In contrast, successful integration of inquiry based education is often utilized by teachers that have a more contemporary understanding of the nature of science[133]. When students are faced with inquiry based activities, studies have indicated that students are positively engaged with inquiry tasks and are able to understand and design meaningful experiments[134]. Other impacts on the success or lack of success in teaching science to K-12 students involve parental influences and the mindset of each individual student[135].

Studies show that students decline in attitude, interest, and motivation toward science throughout primary and secondary education levels[136]. Such declines have been linked to a more "traditional" learning atmosphere as defined by less student-centered

instruction, fewer classroom discussions and debates, an increase in lecturing, and a close adherence to textbooks[137]. Introducing students to hands-on science activities, rather than just mastering science content, nurtures an interest in science as scientific investigation through an active endeavor. All of these factors are important to understanding how to communicate science in the K-12 classroom. However, current research demonstrates that inquiry based education is a productive way to overcome such obstacles[138], which implicates inquiry based learning as a vital tool when teaching science.

To perpetuate this idea, the National Science Foundation (NSF) funds a national program designed to bring STEM (science, technology, engineering, and math) graduate student researchers into urban middle and high school classrooms to assist in education of students while employing inquiry based education and, simultaneously, introducing students to cutting edge graduate research. The program is called the GK-12 program. The competitive fellowship charges graduate student participants with developing an inquiry based project that can be performed by the students at their K-12 school on current science topics, science methodology, and laboratory technique.

Our laboratory investigates virulence modulation of *Pseudomonas aeruginosa* in the CF airway and particularly the ability of *P. aeruginosa* to form biofilms, which are innately resistant to antibiotics. Biofilms are traditionally defined as bacteria adhering to a solid surface and undergoing global regulatory changes to form a protective structure that is innately antibiotic resistant[6]. Biofilms are implicated in many chronic disease states and represent a cutting edge area of research. Importantly, biofilms are found everywhere, as bacteria utilize biofilms as a protective mechanism. Therefore, bacteria that do not cause human disease form biofilms as a part of their normal lifestyle.

Due to the fact that *P. aeruginosa* is an opportunistic pathogen and, therefore, may not be safe to bring into a high school classroom, I developed a way to introduce high school students to both biofilm forming bacteria and resistance of biofilms to antimicrobial agents by using environmental bacteria and household antimicrobials. This lesson was developed for urban Indianapolis high schools, Pike High School and Arsenal Tech High School. The biology text used in the particular classrooms that this activity was developed for did not mention biofilms, although Indiana curriculum requirements do include microbiology. Specifically, standard B.1.12 requires students to compare and contrast form and function of eukaryotic and prokaryotic organisms. Therefore, the ability of microbes to form biofilms is an excellent way to introduce microbiology to the high school classroom and make the discussion relevant and appealing to the student body by discussing human health and biofilms. Indiana curriculum requirements also include Ecological concepts, specifically standards B.1.40 and B.1.45 which require students to understand how communities form and are maintained through cooperation and competition. These concepts are beautifully introduced using biofilms. The students at these Indianapolis high schools, in both biology and environmental science classes, were exposed to biofilms, a topic that they would not have been exposed to otherwise.

This paper will demonstrate that utilizing inquiry based educational techniques allowed for successful introduction of a complex topic, biofilms, to entry and advanced level high school biology students. In conjunction, these students also learned about experimental design and formulation of a hypothesis. Furthermore and most importantly, researchers report that this study initiated an excitement for science expressed by the students who participated.

5.2 Materials and Methods: Biofilms From Pond Water

5.2.1 Preparing Pond Water Samples

A pond, as part of an outdoor laboratory located at Pike High School in Indianapolis, IN, was the source of the water for this experiment. The pond water was acquired by taking direct samples from the pond by the participating students. The researchers found that the pond water samples should be well shaken before sub-sampling, as the water contained a heterogeneous mixture of microorganisms and plant material. The pond water samples were then sub-sampled at dilution factor of 1:100 into test tubes containing 5 mL of LB (non-selective nutrient broth, composed of 10% tryptone, 5% yeast extract, and 5% sodium chloride). These test tubes were placed on a shaker at 37°C overnight (approximately 16 hours). Studies at Arsenal Tech High School utilized nonselective agar (LB agar) to isolate individual bacteria from a pond water sample based on colony morphology. The sub-isolates were further characterized via gram staining.

5.2.2 Static Biofilm Assay

A biofilm assay utilizing polyvinyl chloride 96 well plates was employed for this study (modified from Hinsa *et al.* 2006[139]). Prepared pond water samples were diluted into LB at a dilution factor of 1:100 and then aliquotted into the 96 well plates at a volume of 100 μL per well or fill each well approximately half way, including a negative control of LB in a designated portion of the plate. The 96 well plates were covered with the corresponding lid and placed at 37°C overnight (approximately 16 hours). Biofilms

will form in the 96 well plates at the base of the well or at the air-liquid interface depending on the bacteria present in the sample. For this laboratory exercise, preliminary testing was performed to ensure that the bacteria in this particular pond were able to form biofilms. Most bacteria are, but the pond contained different organisms including strains of bacteria and eukaryotes that could hinder the formation of biofilms through competition or predatory interactions. We found that if the pond water bacteria was overgrown (>16 hours), biofilm formation significantly declined. Studies at Arsenal Tech High School utilized both the pond water bacteria and *Staphylococcus aureus* laboratory strains for biofilm assays.

5.2.3 Preparing Antimicrobial Agents

The antimicrobial agents used in experiments at both high schools were Clorox bleach, Dawn liquid dish soap, Kroger brand liquid bathroom cleaner, Kroger brand mouthwash containing alcohol, and 70% isopropanol (representing the active ingredient in hand sanitizer). All the antimicrobials were prepared to mimic their use in everyday life. A 13:7 solution of bleach and water was made for this experiment and added directly the well containing the biofilm without further dilution or inclusion of a nutrient broth. This solution of bleach was indicated as the proper solution to use for sanitation on the back of the bleach container. The dish soap was mixed with sterile deionized water until a qualitative quantity of sudsing was acquired, approximately 2-3 mL of soap to 500 mL of water. This was added in the same fashion as the bleach. The liquid bathroom cleaner, mouthwash, and 70% isopropanol were not diluted and added in the same fashion as the bleach. Studies at Arsenal Tech High School utilized five different

antimicrobials agents: isopropanol (100%), methanol (100%), ethanol (95%), n-butanol (100%), and isoamyl alcohol (100%). These agents were added directly to preformed biofilms without diluting, therefore requiring no preparation.

5.2.4 Antimicrobial Experiment

Growth medium was removed from all the wells of the inoculated biofilm assay following overnight incubation via a sterile micropipette. Once removed, the students added the prepared antimicrobial agents at a concentration of 125 μL per well or filling approximately two-thirds of the well. This ensured that the antimicrobials had come in contact with the pre-formed biofilms in each well. The students were responsible for designing their experiments to include positive and negative controls. The negative control should be maintained on the plate as growth media only wells and the positive control was maintained as untreated wells containing biofilms in growth media. Following addition of antimicrobials in the designated experimental wells, the students placed the corresponding lid on the 96 well plates and incubated the plates at 37°C overnight (approximately 16 hours).

5.2.5 Crystal Violet Staining

To qualitatively measure the impact of the antimicrobials on the pre-formed biofilms in the 96 well plate, the plate was stained with crystal violet. Crystal violet stains all bacteria present in each well of the plate, whether Gram positive or Gram negative. A crystal violet solution should be made at a concentration of 0.1% crystal violet in deionized water (sterile water is not necessary). The researcher demonstrated

how to perform the following procedure, and then the students were tasked with staining their biofilm assays. First, they emptied each well of the 96 well plate to be stained and immersed the plate in a container filled with water. Then, they removed the water by hitting the inverted plate on a solid surface covered with a paper towel several times. Next, they filled each well with 150 µL or approximately three-fourths of the way full with 0.1% crystal violet and let the plates incubate at room temperature for 12 minutes. In an empty container, they turned the plate upside down over a container allowing the crystal violet stain to fall out of each well of the 96 well plate and into the container. With two consecutive water containers, they immersed the plate into the first container so that each well is filled with water and hit the inverted plate onto a solid surface covered with a paper towel as described earlier and repeated this step. Then, they did the same with the second container of water. The students left the plate upside down on a paper towel to dry overnight and, the following day, they qualitatively analyzed their plate for the presence of biofilms.

5.2.6 Spectrophotometry

For quantitative analysis of biofilm growth, students at Arsenal Tech High School added a solution of 30% acetic acid to their pre-stained and dried biofilm assay plate so that the acetic acid solution contacts that stain (150 µL in a 96 well plate). The stain was dissolved in the acetic acid following a ten minute incubation at room temperature. Then, they transferred the acetic acid to a flat bottom plate that is compatible with the spectrophotometer. Changes in stain intensity can be quantified by measuring OD_{550}

(optical density at 550 nm) in the spectrophotometer. The students then graphed their results and performed statistical analysis (Student's *t* test assuming equal variances).

5.2.7 Student Assessment

Students from two urban high schools, Pike High School and Arsenal Tech High School, in Indianapolis, Indiana participated in this study. Ethnicity breakdowns demonstrate the large percentage of minority students at both schools (Figure 29).

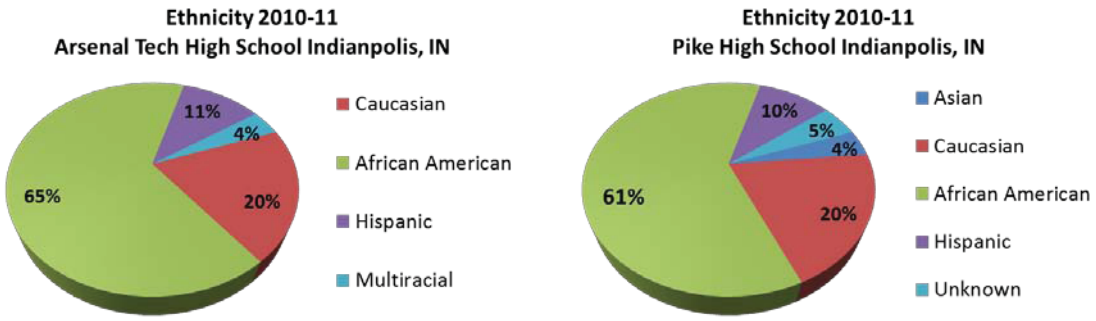

Figure 29. Representation of ethnicity for Arsenal Tech High School and Pike High School. Eighty percent of students at both schools are minorities.

Both introductory biology and advanced biology students were asked to participate in our study. Students at Pike High School were asked to complete a pre-test survey and a post-test survey comprising eight questions measuring two subscales, biofilm knowledge gained and experimental design knowledge gained. Students (n=10) were informed that these surveys would not count toward their grade in the class, but rather will be utilized as a tool for the researchers to assess learning. IRB approval (IRB approval #1102-50) was acquired, participating students signed an assent form, and parents of minors signed a consent form (Appendix A, Appendix B, and Appendix C). Student information was de-identified before analysis.

5.3 Results: Experimentation and Assessment

5.3.1 The Laboratory Activity at Pike High School: Day 1

The laboratory exercise developed for Pike High School biology students was comprised of four sections spanning four days (Appendix D). This lab was incorporated into the course curriculum and, therefore, a required activity for all students. The study was performed with a researcher and a single licensed teaching professional spanning 6 class periods of both advanced and introductory biology students. Prior to starting this activity, the students were given a pre-test to ascertain their knowledge of biofilms and experimental design (Appendix E). The laboratory activity began with an introduction of biofilms, including the ecology, phenotype, and human health implications, followed by a discussion on experimental design, including hypothesis determination, controls and variables, and data collection. Following this introduction, the students were asked to break up into teams of 2-3 students. These teams were then led outside to the outdoor lab at Pike High School and acquired a water sample from the pond on site (Figure 30). The students aliquoted their pond water sample into 5 mL of nutrient broth at a dilution factor of 1:100 in a test tube and placed their tubes in an incubator overnight.

Figure 30. Pike High School outdoor lab. This pond was located right outside the IPS school where this study took place. Students had access to the pond to gather water samples and were able to grow bacteria by inoculating LB nutrient broth with pond water.

5.3.2 The Laboratory Activity at Pike High School: Day 2

Following the initial step of adding the pond water to nutrient agar to enhance the growth of the bacteria already present in the pond water, the students began to design their biofilm assay experiment, assigning positive and negative controls to various wells of their 96 well plate. The researcher explained the purpose of the biofilm assay to the students. Essentially, a 96 well biofilm assay allows the scientist to grow biofilms in a variety of wells, while maintaining some wells as controls, and to test the biofilms with different variables. The first step to the biofilm assay is allowing biofilms to form in the 96 well plate. After the students have designed their experiment using a worksheet provided to them and identifying controls and variable wells appropriately (Appendix F), the students were given a 96 well plate and their pond water bacteria. The students performed a 1:100 dilution of their dense pond water bacteria sample into nutrient broth and aliquoted 100 μL of this solution into the appropriate wells of the 96 well plate. The students used sterile nutrient broth as a negative control. Following inoculation of all the

wells, the students placed their biofilm assay plates in the incubator. Biofilms will form at either the air-liquid interface of the well or at the bottom of the well. This depends on the type of bacteria forming the biofilm (Figure 31).

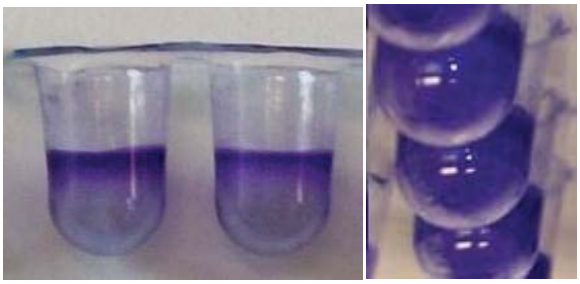

Figure 31. Biofilm growth varies between bacteria. Biofilms can form at the air-liquid interface forming a ring motif around the plastic well. Or, biofilms will form at the bottom of the well, coating the entire bottom of the plastic well. These differences in adherence and growth depend on the bacteria forming the biofilm.

5.3.3 The Laboratory Activity at Pike High School: Day 3

At this stage in the laboratory activity, the students were asked to build upon their initial experimental design and formulate a hypothesis on antimicrobial treatment effects on their biofilms. Students were allowed to choose two of five different common household antimicrobials provided to them. They were to record which antimicrobials they used and in which well they would place the antimicrobials to test their effect on the pre-formed biofilms on their experiment worksheet (Appendix F). The antimicrobials were added to the biofilms in the 96 well plate as described in the materials and methods.

5.3.4 The Laboratory Activity at Pike High School: Day 4

The students analyzed their results following staining of their 96 well biofilm assay plate. The staining of the biofilms allowed the students to make qualitative observations. These observations were then recorded in detail by the students on specific

provided worksheets (Appendix F). The results demonstratd that both the mouthwash and the 70% isopropanol treatments were unsuccessful in eradicating the biofilm when compared to the positive untreated control. Furthermore, the observed effect for the 70% isopropanol treatment was increased biofilm formation. The students were asked to prepare a five minute presentation for their class to explain their experimental design and results followed by a two minute question and answer session. Also, a post-test (exactly the same as the pre-test) was given to all of the students participating in this laboratory activity (Appendix E).

5.3.5 The Laboratory Activity at Arsenal Tech High School

Two students at Arsenal Tech High School further characterized the effects of antimicrobial treatment on pond water biofilm formation as part of a senior capstone project for a biology class. They identified two isolates of bacteria from the pondwater based on colony morphology and gram staining (Figure 32).

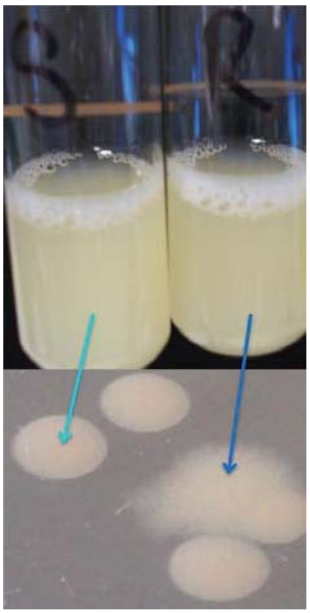

Figure 32. Colony morphology of bacterial isolates from pond water. A gram negative, smooth colony was isolated and a gram positive, rough colony was isolated. Most likely the smooth colony is a pseudomonad, and the rough colony is a bacillus species of bacteria.

First, the students confirmed that the isolates could form biofilms and looked for differences in biofilm levels between sub-isolates (Figure 33). The isolates formed biofilms at similar rates but formed at a significantly lower rate than biofilms formed from the total pond water bacteria and an equal mixture of the two bacterial isolates ($p<0.05$).

Further testing of the bacterial isolates individually and an equal mixture of the isolates using the same antimicrobial treatments as performed at Pike High School ensued were performed. Similar results were demonstrated. We found that biofilm formation was significantly increased following 70% isopropanol treatment of pre-formed biofilms composed of a combination of rough and smooth isolates ($p<0.001$). This effect was not demonstrated across samples, as both pond water and single isolates biofilm levels were significantly decreased. Although this observed decrease was significant, the decrease in

biofilm levels was not as drastic in comparison to other antimicrobial treatment (Figure 34). Next, we tested the same bacterial samples with five different alcohols, methanol (100%), ethanol (95%), n-butanol (100%), isoamyl alcohol (100%), and isopropanol (100%), chosen because of their different chemical structures and practical uses. Biofilm assay results demonstrated a significant increase in biofilm formation of all bacterial samples following treatment of all five alcohols ($p<0.05$), except for biofilms formed from a combination of smooth and rough isolates and treated with n-butanol. This particular treatment resulted in statistically unchanged biofilm levels ($p>0.05$). These tests were performed in triplicate, and the 100% isopropanol treatment resulted in significant increases in biofilm levels in comparison to prior tests where the biofilms were treated with 70% isopropanol (Figure 35). In order to relate this effect to medically relevant bacteria, we performed the same experiment using two laboratory strains of *S. aureus* and had the same effect, with significant increases in biofilm formation following treatment with all five alcohols ($p<0.05$) (Figure 36).

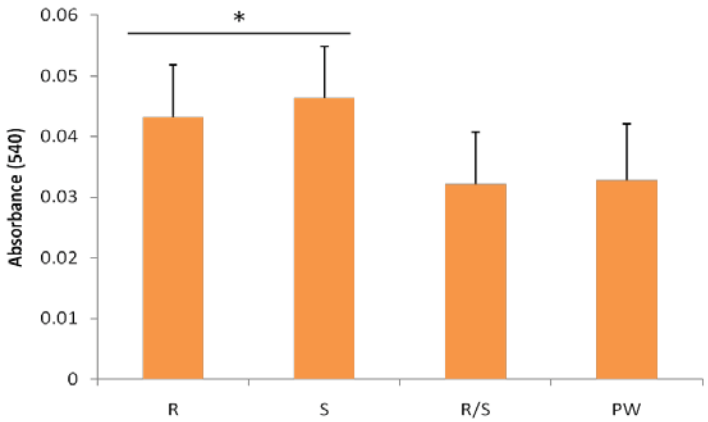

Figure 33. Biofilm formation of pond water isolates, mixture of the isolates, and the pond water. The isolates (R, rough and S, smooth) formed biofilms at a significantly higher rate ($p<0.05$) than biofilm formation of the mixture of the isolates and the pond water sample. Data represents three experiments individually performed in triplicate ($n=3$).

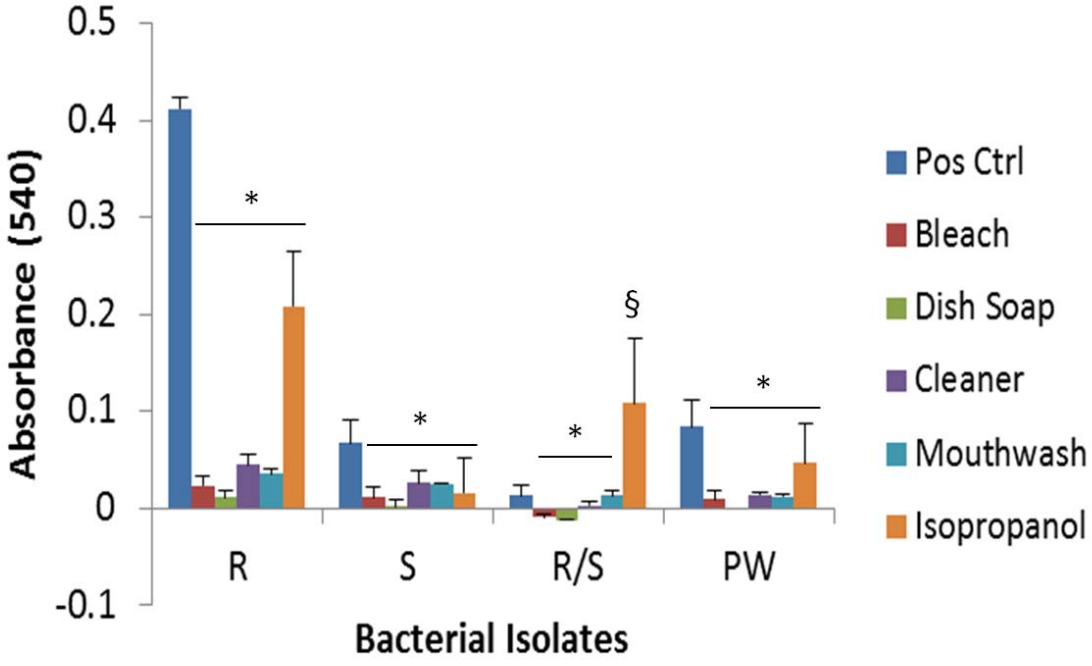

Figure 34. Household antimicrobial treatment effect on pond water and isolates. Significant reduction in biofilm levels compared to positive controls for all bacterial isolates and combinations tested, although the 70% isopropanol treatment resulted in reduced killing of the biofilms in comparison to other treatments (*,$p<0.05$). Furthermore, biofilms formed from a combination of rough and smooth isolates resulted in a significant increase of biofilm levels following treatment with 70% isopropanol (§,$p<0.001$). Data represents three experiments individually performed in triplicate (n=3).

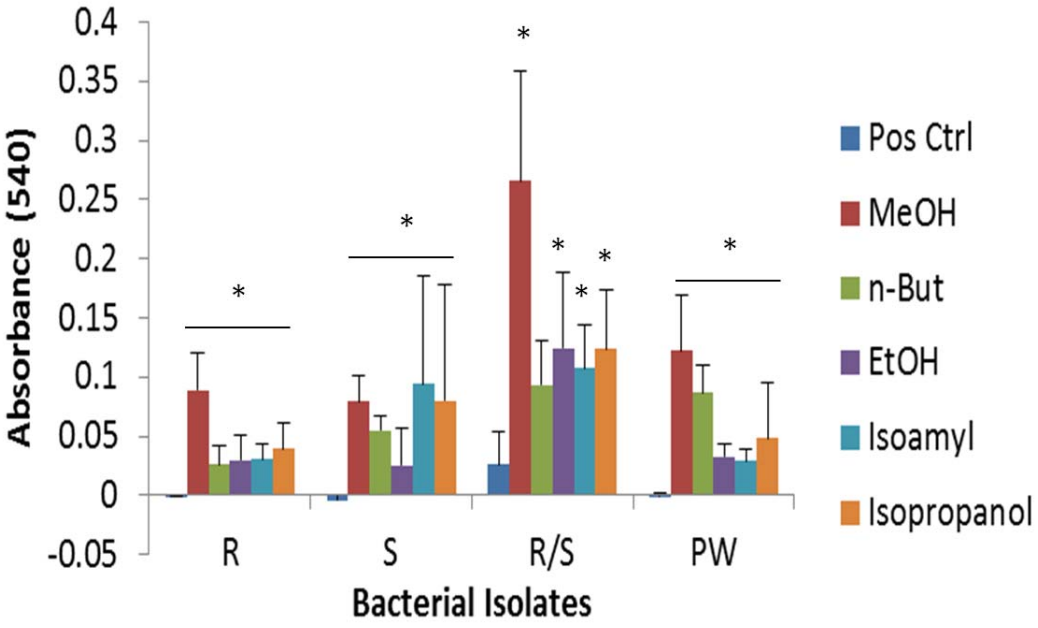

Figure 35. Alcohol treatment effect on pond water and isolates. Biofilm formation of bacterial isolates and combinations of isolates increased in comparison to positive, untreated controls for most alcohol treatments. Alcohol treatment of rough isolates, smooth isolates, and pond water pre-formed biofilms resulted in a significant upregulation of biofilm levels ($p<0.01$, $p<0.05$, $p<0.001$, respectively). Alcohol treatment of biofilms formed from the rough and smooth bacterial isolates combination resulted in significant upregulation of biofilm levels following all alcohol treatments ($p<0.01$) except for n-butanol ($p>0.05$). Data represents three experiments individually performed in triplicate ($n=3$).

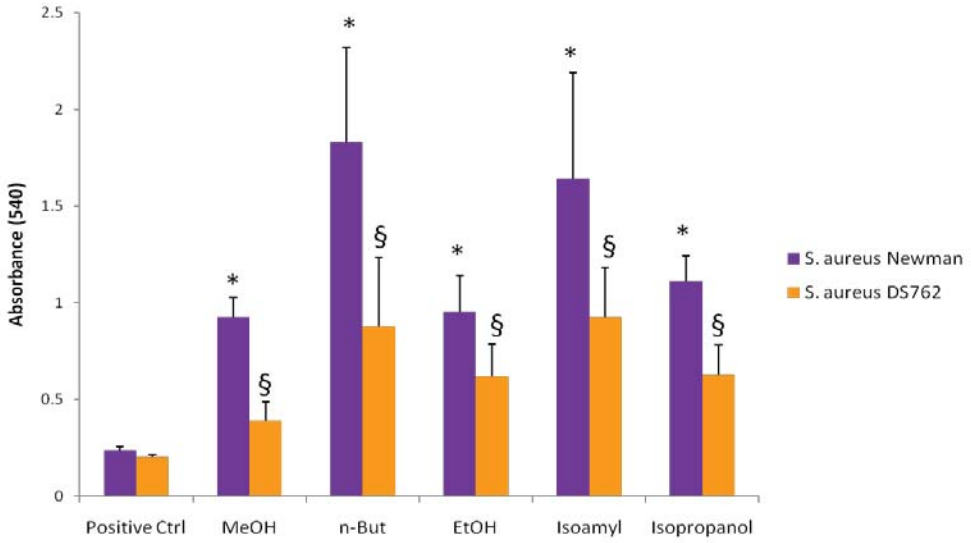

Figure 36. Alcohol treatment effect on *S. aureus* strains. Biofilm formation increased in comparison to positive, untreated controls following all alcohol treatments ($p<0.05$, * and § indicates significance for *S. aureus* Newman and DS762 respectively, n=3). Data represents three experiments individually performed in triplicate.

5.3.6 Learning Assessment of Inquiry Based Laboratory Activity

Student pre-test and post-test scores were scored and analyzed via a *t* test controlling for unequal variances. Students' total pretest scores ($M = 2.62$, $SD = 1.75$) were compared to their total posttest scores ($M = 5.36$, $SD = 1.75$). Students' total scores were significantly higher after completing the lab activity, $t(10) = 6.37$, $p < .001$, $\eta^2 = .81$. Similar results were found when examining the two subscales. Knowledge of biofilms, $t(10) = 2.80$, $p < .05$, $\eta^2 = .47$, and knowledge of experimental design, $t(10) = 4.03$, $p < .01$, $\eta^2 = .64$, were both significantly higher after completing of the activity (Figure 37).

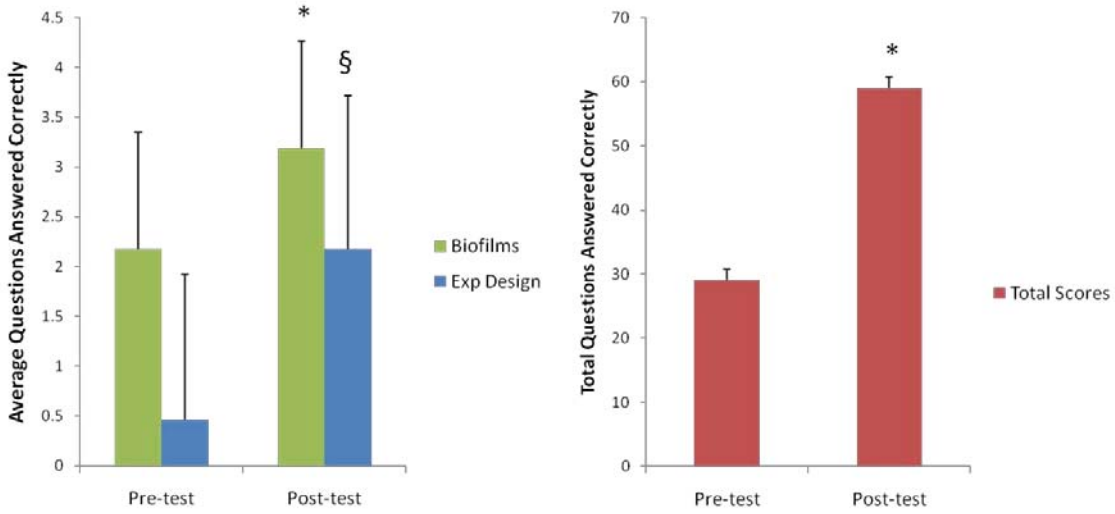

Figure 37. Inquiry based learning activity statistics. A significant increase in post-test scores was achieved in comparison to pre-test scores for both subscales (biofilm knowledge (*$p<0.05$), experimental design knowledge (§$p<0.01$), and total scores ($p<0.001$), n=10).

5.4 Discussion: Real Science Taught Through Inquiry Based Instruction

This laboratory activity was designed for several important reasons. First, this activity has further demonstrated the importance of inquiry based instruction for the sciences. This activity also elicited an excitement for science in the students that participated in this study making science more accessible to urban student populations. Urban school systems struggle with many different challenges that impair the learning environment. Students often grapple with home lives that are less than desirable, substance abuse issues, and gang mentality. High school graduation rates for Indianapolis Public Schools (IPS) in 2008-2009 was 48.6%. Ethnicity breakdowns for IPS results in 77% of students are minorities. The percentage of students granted free lunches is also 77%.

The NSF funded GK-12 Urban Educators program targets these schools, because these schools are in need of assistance in reaching their student body and promoting education, especially in the STEM areas. This program tasks the graduate student fellows with bringing their research into the high school classroom to elicit a positive response to science. The laboratory activity that was developed as part of this initiative not only met these standards but exceeded them. The laboratory activity was accessible to the students, and was a representation of "real" science being performed in collegiate academic institutions. It allowed the students to formulate a hypothesis and analyze scientific data. Students responded positively to the activity, and they learned about experimental design, the ecology of communities, and health implications of chronic biofilm-forming bacterial infections. Moreover, the students at Pike High School discovered a very interesting effect of alcohol treatment on environmental biofilms. Mouthwash, containing alcohol, and isopropanol was not just ineffective at killing the pre-formed biofilm, but biofilm levels increased following treatment. The students at Arsenal Tech High School analyzed these results, and asked more questions, performed more experiments, and demonstrated the effect of alcohol induced increases in biofilm formation not only in the environmental biofilms but in *S. aureus* biofilms following treatment of not only isopropanol, but four other alcohols, including methanol, ethanol, n-butanol, and isoamyl alcohol. These students participated in the process of scientific discovery, much like college trained scientists do every day.

The student population that was examined via statistical analysis following completion of pre-test and post-test surveys, examining knowledge gained following participation in this laboratory activity, showed a significant increase in knowledge on

two subscales measured, biofilm knowledge and experimental design knowledge. Although biofilm knowledge gained may not be extremely important to their further success in the sciences, this study demonstrates that complex science concepts are successfully taught employing inquiry based learning activities. The experimental design knowledge gained from this activity is supremely important, as these students will need to utilize this way of thinking not only in other science classes, but in life. Understanding how to design an experiment based on a hypothesis makes use of one's reasoning abilities, which translates to all aspects of life.

Often urban students are “left behind” in the educational system due to many reasons. The school systems struggle with capturing these students’ attention long enough to teach them basic science concepts. The traditional method of teaching STEM subjects, through lecturing and homework assignments with periodic testing, is not working. Properly designed inquiry based instruction provides a way to capture urban student's attention and maintain an interest in the subjects. This is the direction in which primary and secondary education should be moving.

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APPENDICES

Appendix A. IRB Approval Paperwork and Study Protocol

Appendix A contains IRB approval paperwork documenting IRB approval for the study and study protocol outlined in Chapter Five of this thesis.

INTERDEPARTMENTAL COMMUNICATION
IU Human Subjects Office - Indianapolis

DATE: April 22, 2011

TO: Kathleen Marrs
Biology
SL 330
IUPUI

FROM: Pete King
IU Human Subjects Office - Indianapolis

SUBJECT: Final Approval

Study Number: 1102-50
Study Title: Bringing Health Psychology and Microbiology into the High School Curriculum to Study Human Diseases: An Investigation of Inquiry-Based Learning - Sponsor: N/A

The study listed above has received final approval from the Institutional Review Board (IRB-02). Please note that subjects must be provided with and sign a current informed consent document containing the IRB approval stamp.

Special requirements for the inclusion of prisoners: Please note that unless your study has received approval for the inclusion of prisoners, you may not enroll and/or otherwise involve a prisoner in your study. Special requirements apply if an individual enrolled on the study either is a prisoner or has become a prisoner during the course of his/her study participation (and the study has not been previously granted approval for the enrollment of prisoners as a subject population). If the investigator becomes aware that a subject is a prisoner, all research interactions and interventions with the prisoner-participant must cease. If the investigator wishes to have the prisoner-participant continue to participate in the research, the IU Human Subjects Office - Indianapolis must be notified immediately (317-274-8289). In most cases, the IRB will be required to review the protocol at a convened meeting before any further research interaction or intervention may continue with the prisoner-participant. Refer to the IU Standard Operating Procedure (SOP) on *Vulnerable Populations* for further information. The SOP is available at http://researchadmin.iu.edu/Forms/human_subjects/hs_iupui/Standard_Operating_Procedures%20_03%2008.pdf.

As the principal investigator of this study, you assume the responsibilities as outlined in the SOP on *Responsibilities of Principal Investigators*, some of which include (but are not limited to):

1. **CONTINUING REVIEW** - A status report must be filed with the IRB at least annually. IU Human Subjects Office staff will generate these reports for your completion. This study is approved from **April 13, 2011** to **April 12, 2012**. **If your study is not re-approved by this date, the study will automatically expire, which means that all research activities, including enrollment of new subjects, interaction and intervention with current participants, and analysis of identified data, must cease.**
2. **STUDY AMENDMENTS** - You are required to receive prospective approval from the IRB for ANY changes to the research study, including changes to protocol design, dosages, timing or type of test performed, population of the study, and informed consent statement, prior to implementation. This request is made via an amendment form, which can be obtained at: http://researchadmin.iu.edu/HumanSubjects/IUPUI/hs_forms.html.
3. **UNANTICIPATED PROBLEMS INVOLVING RISKS TO SUBJECTS OR OTHERS AND NONCOMPLIANCE** - You must promptly report to the IRB any event that appears on the **List of Events that Require Prompt Reporting to the IRB.** Refer to the SOP on *Unanticipated Problems Involving Risks to Subjects or Others and Noncompliance* for more information and other reporting requirements. The SOP can be found at: http://researchadmin.iu.edu/Forms/human_subjects/hs_iupui/Standard_Operating_Procedures%20_03%2008.pdf. NOTE: If the study involves gene therapy and an event occurs which requires prompt reporting to the IRB, it must also be reported to the Institutional Biosafety Committee (IBC).
4. **UPDATED INVESTIGATIONAL BROCHURES, PROGRESS REPORTS and FINAL REPORTS** - If this is an investigational drug or device study, updated clinical investigational brochures must be submitted as they occur. These are submitted with an amendment form. Progress or final reports must be provided to the IRB with your written assessment of the report, briefly summarizing any changes and their significance to the study.
5. **ADVERTISEMENTS** – You can only use IRB-approved advertisements to recruit participants for your study. If you will be advertising to recruit study participants and the advertisement was not submitted to the IRB at the time your study was reviewed and approved, a copy of the information contained in the advertisement and the mode of its communication must be submitted to the IRB as an amendment to the study. These advertisements must be reviewed and approved by the IRB PRIOR to their use.
6. **STUDY COMPLETION** – You are responsible for promptly notifying the IRB when the study has been completed (i.e. there is no further subject enrollment, not further interaction or intervention with current participants, including follow-up, and no .further analysis of identified data). To notify the IRB of study completion, please obtain a Continuing Review – Closeout Report form at http://researchadmin.iu.edu/HumanSubjects/IUPUI/hs_forms.html and submit it to the IU Human Subjects Office - Indianapolis.
7. **LEAVING THE INSTITUTION** - If the principal investigator leaves the Institution, the IRB must be notified as to the disposition of EACH study.

PLEASE REFER TO THE ASSIGNED STUDY NUMBER AND THE EXACT TITLE IN ANY FUTURE CORRESPONDENCE WITH OUR OFFICE. In addition, SOPs exist which cover a variety of topics that may be relevant to the conduct of your research. Please visit http://researchadmin.iu.edu/Forms/human_subjects/hs_iupui/Standard_Operating_Procedures%20_03%2008.pdf for a current copy of the IU SOPs for Research Involving Human Subjects. All documentation related to this study must be neatly typed and must also be maintained in your files for audit purposes for at least three years after closure of the research; however, please note that research studies subject to HIPAA may have different requirements regarding file storage after closure. If you have any questions, please call the IU Human Subjects Office - Indianapolis at 317-274-8289.

Please see the IRB approval email attached to this document, as well as the Documentation of Review and Approval, for a list of all documents approved with this submission.

Appendix B. Informed Consent Forms for Inquiry Study

Appendix B contains the two forms used to obtain informed consent from parents of minor participants in study and from participants of the study that are over eighteen.

INDIANA UNIVERSITY INFORMED CONSENT STATEMENT FOR

Bringing Health Psychology and Molecular Biology to the High School Curriculum Using Inquiry-Based Learning

Your child is invited to participate in a research study of how science can be taught using inquiry. Your child was selected as a possible participant because he or she is a student in a science class at either Arsenal Technical High School or Pike High School. We ask that you read this form and ask any questions you may have before agreeing for your child to be in the study.

The study is being conducted by Misty Hawkins (Department of Psychology), Carly Redelman (Department of Biology) and Kathleen Marrs (Department of Biology) at IUPUI on behalf of the GK-12 National Science Foundation Fellowship.

STUDY PURPOSE

The purpose of this study is to determine how students learn through inquiry. Inquiry is a type of learning that encourages students to actively explore problems by asking questions and doing hands-on learning activities alone or with other students. Past research has shown that inquiry-based teaching is a successful technique for helping students learn. Using inquiry to learn about health psychology and microbiology is a way to help high school students learn about these particular subjects and also learn more about how to do scientific research.

We want to know if using inquiry to teach this material will help high school students increase their content knowledge of health psychology and molecular biology concepts and encourage them to want to learn more about science.

NUMBER OF PEOPLE TAKING PART IN THE STUDY:

If your child agrees to participate, your child will be one of 200 subjects who will be participating in this research.

PROCEDURES FOR THE STUDY:

Your child will be taught lessons about health psychology and microbiology regardless of your child's participation in this study. Lessons will be taught by the child's regular classroom teacher and by research scientists who study health psychology or molecular biology. The lessons may ask students to complete the following tasks to learn about health psychology: (1) create and engage in mental stress tasks (such as doing hard arithmetic problems), (2) report his or her feelings, thoughts, or behaviors, (3) measure other people's feelings, thoughts, or behaviors, (4) measure his or her own physical symptoms or others' physical symptoms, such as heart rate, blood pressure, and body fat, and (5) complete homework or quizzes assessing his or her knowledge of the activities. To learn about microbiology, students will complete lessons that ask them to complete the following tasks: (1) go to sites that contain water samples, (2) collect and prepare water samples, (3) analyze water samples for bacteria growth, (4) observe and report features of bacteria growth, and (5) complete homework and quizzes assessing his or her knowledge of the activities. Not all students will complete all activities listed above.

RISKS OF TAKING PART IN THE STUDY:

While in the study, loss of confidentiality is a potential risk; however, your child's name and any identifying information will be removed before publication. Therefore, your child's information will be anonymous. Given that we will be working with water in the natural environment, the risks associated with sampling and analysis are very minimal since appropriate laboratory precautions will be taken.

BENEFITS OF TAKING PART IN THE STUDY:

The benefits to participation that are reasonable to expect are that your child will help researchers to understand how inquiry can be used to help students explore science and give them a way to understand scientific inquiry. Since your child will take part in the unit as part of your child's normal classroom requirements, taking part in the study will require no more or less work from your child.

ALTERNATIVES TO TAKING PART IN THE STUDY:

Instead of being in the study, your child has the option to only participate in the classroom activities without being part of the research study. Your child will have all the same learning experiences and requirements but his or her data will not be evaluated and used in the research report.

CONFIDENTIALITY

Efforts will be made to keep your child's personal information confidential. We cannot guarantee absolute confidentiality. Your child's personal information may be disclosed if required by law. Your child's identity will be held in confidence in those reports that may be published and/or databases in which results may be stored. The following organizations may review your child's records: Office of Human Research Protections, Indiana University Institutional Review Board, or the Indiana University Human Subjects Office.

COSTS

There are no costs involved for the students involved in this research project.

PAYMENT

Your child will not receive payment for taking part in this study.

COMPENSATION FOR INJURY

In the event of physical injury resulting from your child's participation in this research, necessary medical treatment will be provided to your child and billed as part of your child's medical expenses. Costs not covered by your child's health care insurer will be your responsibility. Also, it is your responsibility to determine the extent of your child's health care coverage. There is no program in place for other monetary compensation for such injuries. However, your child is not giving up any legal rights or benefits to which your child is otherwise entitled.

FINANCIAL INTEREST DISCLOSURE

The researchers have no financial interest in this research.

CONTACTS FOR QUESTIONS OR PROBLEMS

For questions about the study contact the lead researcher Kathleen Marrs at (317) 278-4551. If you cannot reach the researcher during regular business hours (i.e. 8:00AM-5:00PM), please contact the Indiana University Human Subjects Office at (317) 278-3458 or (800) 696-2949.

For questions about your child's rights as a research participant or to discuss problems, complaints or concerns about a research study, or to obtain information, or offer input, contact the Indiana University Human Subjects Office at (317) 278-3458 or (800) 696-2949.

VOLUNTARY NATURE OF STUDY

Taking part in this study is voluntary. You may choose not to allow your child to take part or your child may leave the study at any time. Leaving the study means that your child's information will not be used in our research. Leaving the study will not result in any penalty or loss of benefits to which your child is entitled. Your decision whether or not to allow your child to participate in this study will not affect your child's current or future relations with his or her teachers and it will not affect your child's course grade.

USE OF SPECIMENS

No specimens will be taken as part of this research.

SUBJECT'S CONSENT

In consideration of all of the above, I give my consent for my child to participate in this research study.

I will be given a copy of this informed consent document to keep for my records. I agree to allow my child, _____ ,to take part in this study.
(name of child)

Parent's Printed Name:_____

Parent's Signature:_____**Date**:_____

(must be dated by the parent)

Printed Name of Child:_____

Printed Name of Person Obtaining Consent:_____

Signature of Person Obtaining Consent:_____**Date**:_____

**NOTE: Printed name lines are optional.

INDIANA UNIVERSITY INFORMED CONSENT STATEMENT FOR

Bringing Health Psychology and Molecular Biology to the High School Curriculum Using Inquiry-Based Learning

You are invited to participate in a research study of how science can be taught using inquiry. You have been selected as a possible participant because you are enrolled in honors microbiology laboratory courses at IUPUI. We ask that you read this form and ask any questions you may have before agreeing to be in this study.

The study is being conducted by Misty Hawkins (Department of Psychology), Carly Redelman (Department of Biology) and Kathleen Marrs (Department of Biology) at IUPUI on behalf of the GK-12 National Science Foundation Fellowship.

STUDY PURPOSE

The purpose of this study is to determine how students learn through inquiry. Inquiry is a type of learning that encourages students to actively explore problems by asking questions and doing hands-on learning activities alone or with other students. Past research has shown that inquiry-based teaching is a successful technique for helping students learn. Using inquiry to learn about health psychology and microbiology is a way to help high school students learn about these particular subjects and also learn more about how to do scientific research.

We want to know if using inquiry to teach this material will help students increase their content knowledge of microbiology and chemical biology concepts and encourage future interest in biology.

NUMBER OF PEOPLE TAKING PART IN THE STUDY:

You will be one of 200 subjects who will be participating in this research.

PROCEDURES FOR THE STUDY:

You will be taught about microbiology regardless of your participation in this study. Lessons will be taught by your professor and by research scientists who study microbiology and chemical biology. The lessons will ask you to complete the following tasks to learn about microbiology and chemical biology: (1) perform biofilm assays using common bacterial strains, including *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus* (2) create/utilize altered amino acids in biofilm assay (3) analyze biofilm assay for bacteria growth, (4) observe and report features of bacteria growth, and (5) demonstrate knowledge gained from activity via surveys administered by researcher.

RISKS OF TAKING PART IN THE STUDY:

While in the study, loss of confidentiality is a potential risk; however, your name and any identifying information will be removed before publication. Therefore, your information will be anonymous. Given that we will be working with pathogenic bacteria, proper protocol and aseptic techniques will be taught and monitored to minimize risks associated with sampling and analysis.

BENEFITS OF TAKING PART IN THE STUDY:

The benefits to participation that are reasonable to expect are that you will help researchers to understand how inquiry can be used to help students explore science and give them a way to understand scientific inquiry. Since you will take part in the unit as part of your normal classroom requirements, taking part in the study will require no more or less work from you.

ALTERNATIVES TO TAKING PART IN THE STUDY:

Instead of being in the study, you have the option to only participate in the classroom activities without being part of the research study. Your will have all the same learning experiences and requirements but your data will not be evaluated and used in the research report.

CONFIDENTIALITY

Efforts will be made to keep your personal information confidential. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. Your identity will be held in confidence in those reports that may be published and/or databases in which results may be stored. The following organizations may review your child's records: Office of Human Research Protections, Indiana University Institutional Review Board, or the Indiana University Human Subjects Office.

COSTS

There are no costs involved for the students involved in this research project.

PAYMENT

You will not receive payment for taking part in this study.

COMPENSATION FOR INJURY

In the event of physical injury resulting from your participation in this research, necessary medical treatment will be provided to youand billed as part of your medical expenses. Costs not covered by your health care insurer will be your responsibility. Also, it is your responsibility to determine the extent of your health care coverage. There is no program in place for other monetary compensation for such injuries. However, you are not giving up any legal rights or benefits to which you are otherwise entitled.

FINANCIAL INTEREST DISCLOSURE

The researchers have no financial interest in this research.

CONTACTS FOR QUESTIONS OR PROBLEMS

For questions about the study contact the lead researcher Kathleen Marrs at (317) 278-4551. If you cannot reach the researcher during regular business hours (i.e. 8:00AM-5:00PM), please contact the Indiana University Human Subjects Office at (317) 278-3458 or (800) 696-2949.

For questions about your rights as a research participant or to discuss problems, complaints or concerns about a research study, or to obtain information, or offer input, contact the Indiana University Human Subjects Office at (317) 278-3458 or (800) 696-2949.

VOLUNTARY NATURE OF STUDY

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study means that your information will not be used in our research. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. Your decision whether or not to participate in this study will not affect your current or future relations with your professors and it will not affect your course grade.

USE OF SPECIMENS

No specimens will be taken as part of this research.

SUBJECT'S CONSENT

In consideration of all of the above, I consent to participate in this research study.

I will be given a copy of this informed consent document to keep for my records.

Printed Name:_____

Signature:_____**Date**:_____

(must be dated by the parent)

Printed Name of Person Obtaining Consent:_____

Signature of Person Obtaining Consent:_____**Date**:_____

**NOTE: Printed name lines are optional.

Appendix C. Informed Assent Forms for Inquiry Study

Appendix C contains the informed assent forms specifically for participants that are under eighteen. In order to participate in the study, students under eighteen must complete an informed assent and their parents must complete an informed consent.

Indiana University Assent to Participate in Research

Bringing Health Psychology and Microbiology into the High School Curriculum to Study Human Diseases: An Investigation of Inquiry-Based Learning

We would like to ask you to be in our research study. A research study is a way of finding out information. We are trying to find out more about using inquiry , a teaching method in which students learn through exploration, active thinking, and interpretation of data (e.g., "hands-on" laboratory assignments) to teach high school students about science and scientific methodology by bringing modified collegiate level research into a high school classroom.

Why am I being asked to be in this research study?

You are asked to participate in our study because you are a student in a science class at Pike High School or Arsenal Tech High School. Your participation will help teachers incorporate new, current, and important research projects into high school curriculum.

What will happen during this research study?

As part of your coursework, you were asked to participate in certain labs. You may have been asked to take surveys to assess your learning as part of the lab. You received a grade for participating in this lab, and this is the only expectation from you to be part of this study. Nothing more will be required.

We would like to take the data that you analyzed, the data from the surveys, and the lab procedure that you followed and make the information available to other teachers. These teachers could teach the lab to their students.

Are there any bad things that might happen during a research study?

Sometimes bad things happen to people in research studies. These bad things are called "risks". The risks of participating in this study are minimal. Because this study was a required part of your coursework, the risks were discussed at the time of the lab, and the lab was found to be nearly risk free.

One of the risks in this study is that your information may be seen by other people. To protect you from this risk, all materials with your name and information will be "de-identified". This means that the data, whether from the survey or the lab work itself, will be anonymous and no names or identification of any kind will be used. This way, if someone sees your work, they will not know that it belongs to you.

Are there any good things that might happen during the research study?

Sometimes good things happen to people who are in research studies. These good things are called "benefits". The benefits of participating in our study may include a better understanding

of how research is done and a better understanding of important health related topics, such as heart disease and cystic fibrosis. Another important benefit is that you will help researchers and teachers understand how to teach such topics to other high school students. This will also help teachers better understand how students learn and how to make science interesting and fun.

Will I get money or payment for being in this research study?

You will not get any type of payment, whether money or goods, for participating in our study.

Who can I ask if I have any questions?

If you have any questions about this study, you can ask your parents or guardians or the researcher (your GK-12 fellow). Also, if you have any questions that you didn't think of now, you can ask later. Feel free to contact Carly Redelman at cvasher@iupui.edu or Misty Hawkins at miahawki@iupui.edu.

What if I don't want to be in this study?

If you don't want to be in this study, you don't have to. It is up to you. If you say you want to be in it and then change your mind that is okay too. All you have to do is tell us. No one will be mad at you or upset with you.

My choice:

_____ _____
Subject's Signature Date

_____
Subject's Name

_____ _____
Signature of person obtaining assent Date

_____
Name of person obtaining assent

Appendix D. The Laboratory Exercise

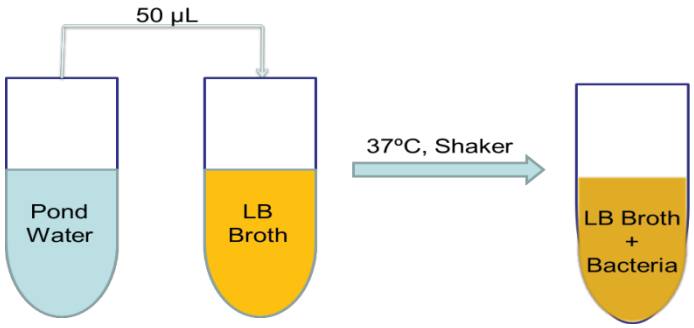
Appendix D contains the actual laboratory exercise followed by the students at Pike High School and Arsenal Tech High School.

Biofilm Lab

Goal: To grow bacterial biofilms using a biofilm assay model system and treat the biofilms with anti-microbial agents (compounds that can kill bacteria).

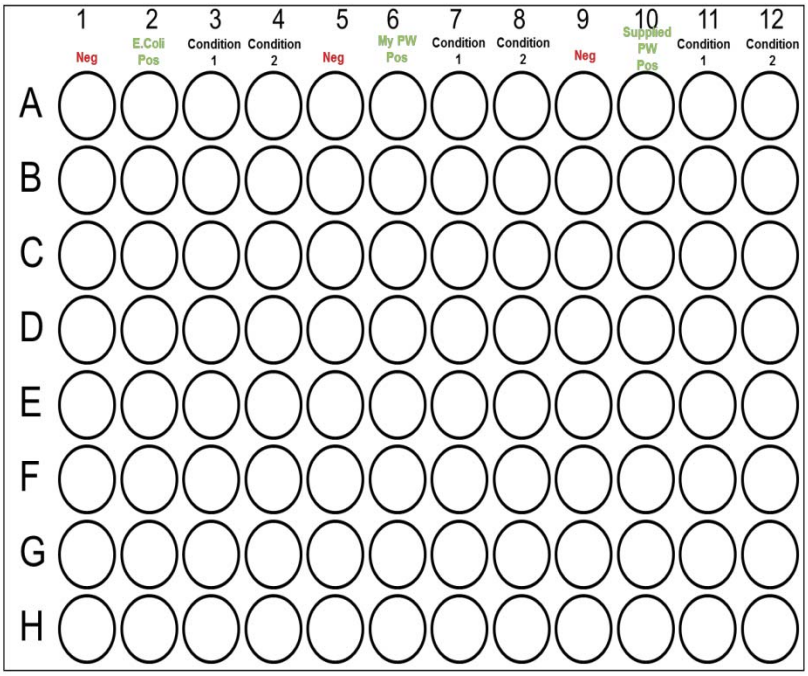
Method: This lab will take four days to complete.

1. **Day 1: Acquire your pond water sample.**
    a. All students should divide into groups of 3-4 people.
    b. Each group will acquire a test tube and go outside to the outdoor lab.
        i. The test tube should be filled half way with a sample of water from the pond.
    c. Samples will be grown up overnight:
        i. This is performed by diluting 50 uL of each sample into a sterile culture tube that contains 5 mL of LB nutrient broth (1/100 dilution).



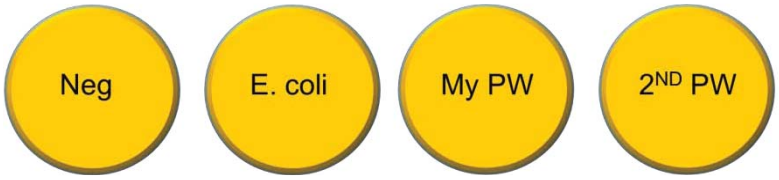
2. **Day 2: Grow biofilms from pond water samples (your sample and another sample) and S17 (a strain of *E. coli*)**
    a. Using the example below, fill out your attached 96 well plate diagram to match the example. This will assist you when you are gathering your

data at the end of the lab

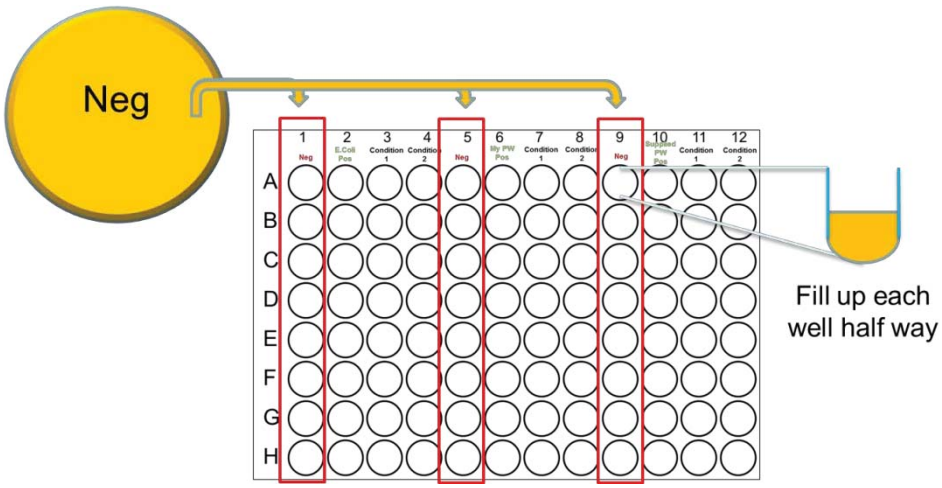


i. **UNDER CONDITIONS, PLEASE FILL IN YOUR SPECIFIC CONDITIONS.**

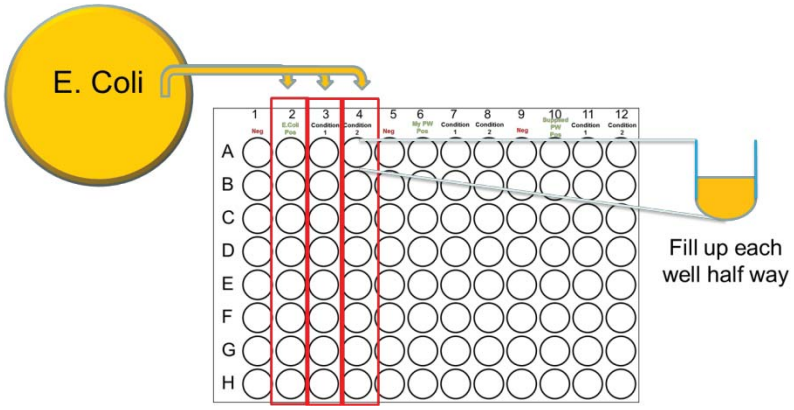
b. Using the supplies at each lab table, follow these instructions to set up your biofilm assay:
   i. You will have four petri dishes at your lab table labeled: Negative control, E. coli, My pond water, Secondary pond water. The negative control is just LB (liquid broth)



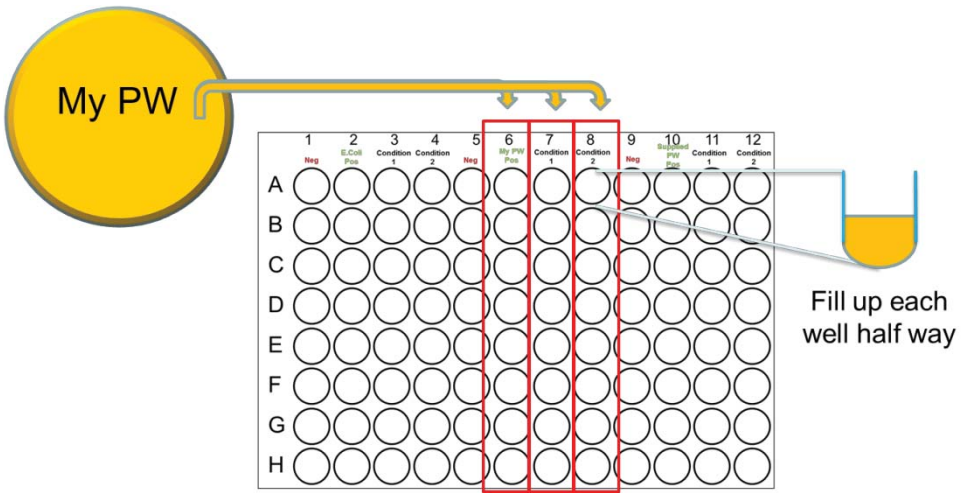
   ii. Sitting on top of each of these dishes is a disposable pipet. **DO NOT MIX THESE UP. EACH PIPET WILL BE USED FOR THE ASSIGNED DISH ONLY!**
   iii. Start with the negative control pipet and petri dish and fill the assigned wells **approximately half way.**



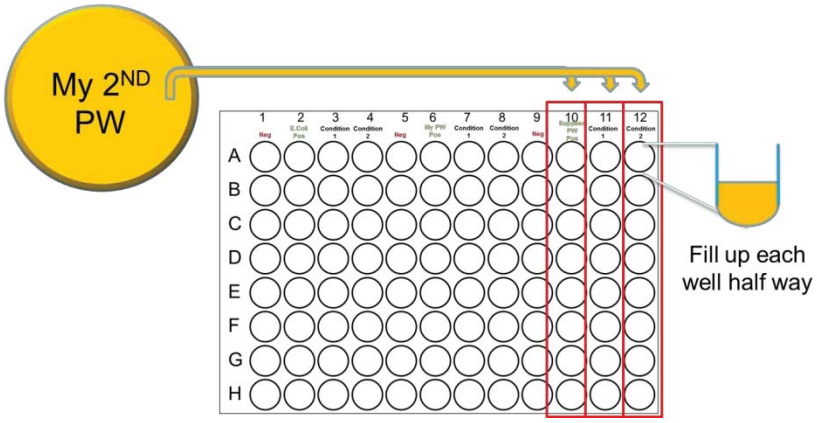
iv. Next fill the assigned wells with E.coli using the assigned E. coli pipet and E. coli petri dish.



v. Next fill the assigned wells with My PW using the assigned pipet and petri dish



vi. Lastly, fill the assigned wells with 2nd PW using the assigned pipet and petri dish



c. Place your 96 well lid on your 96 well plate. It is directional. Then, place your plate in the supplied Tupperware containers. Finally, clean up your station by placing your petri dishes and pipets in the biohazard bag supplied.

d. As a class, we will place your assays in the incubator. They will incubate overnight at 37°C.

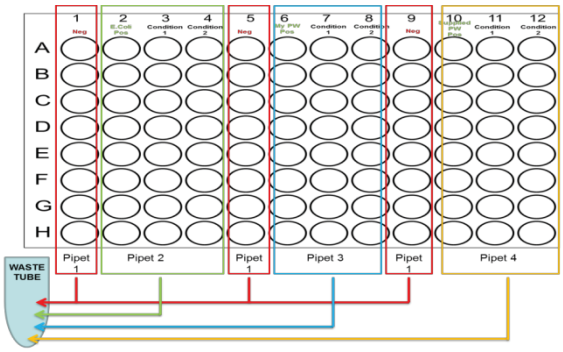
3. **<u>Day 3: Remove old media and add fresh media with antimicrobials to preformed biofilms.</u>**

a. Remove the old media from all of the wells using the assigned pipets and placing the liquid into the waste tube on your bench top.

i. Just stick the pipet straight down to the bottom of the plate and suck up the liquid in each well squeezing the liquid out of the pipet in between each well into the waste tube. **Do not move**

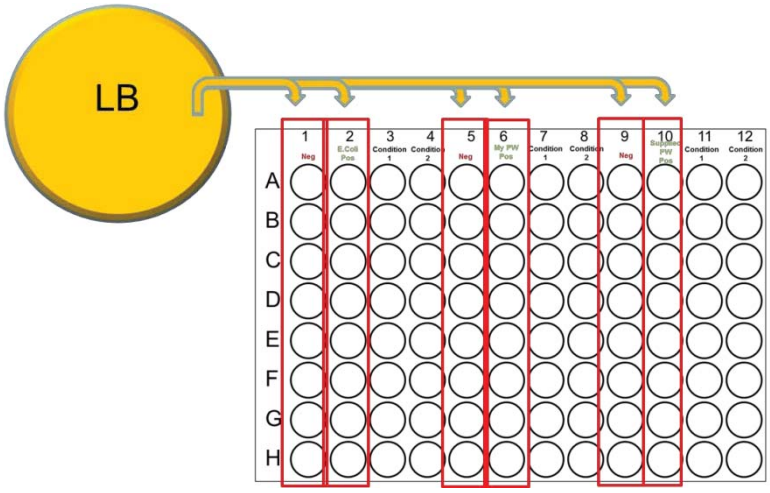
the pipet around in the well or along the sides of the wells. It doesn't have to be perfect, just get most of the liquid out of the wells.

1. **Do not cross contaminate. Use the assigned pipets for the labeled wells.**

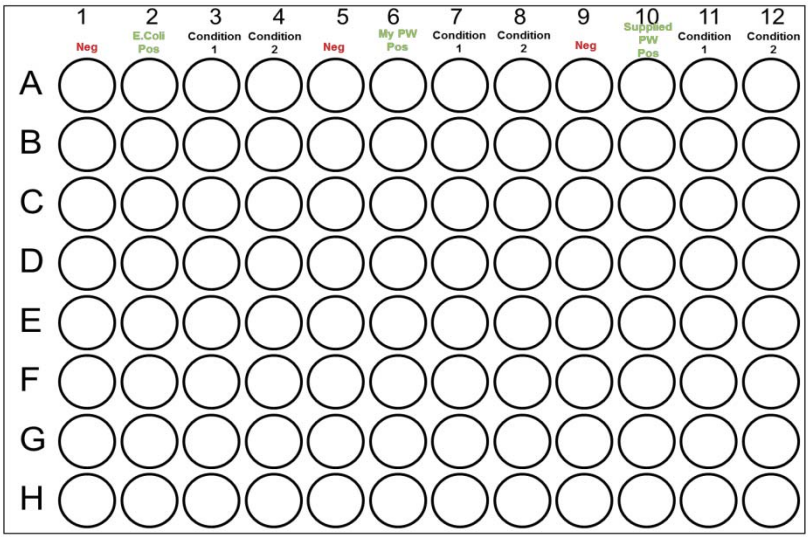


b. Apply labeled media (just like last time) according to the following diagrams.

   i. First, put fresh LB from the labeled petri dish and labeled pipet into the negative and positive control wells.

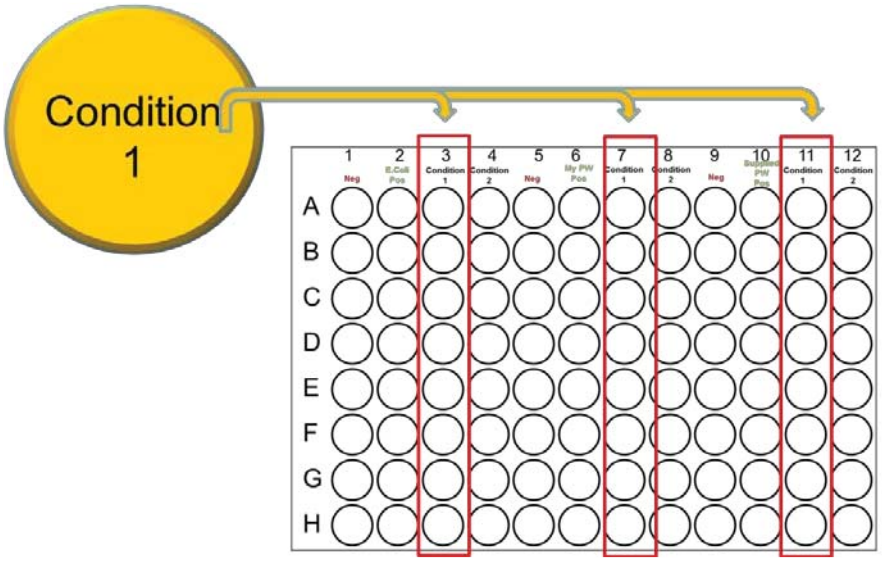


   ii. Next, come and choose your antimicrobial agents for your experimental wells. You will choose only two out of the four antimicrobial agents to use for all three different microbial biofilms: Bleach, Dish Soap, Bathroom Cleaner, Hand Soap. Make note on your original diagram which antimicrobial agents you used.

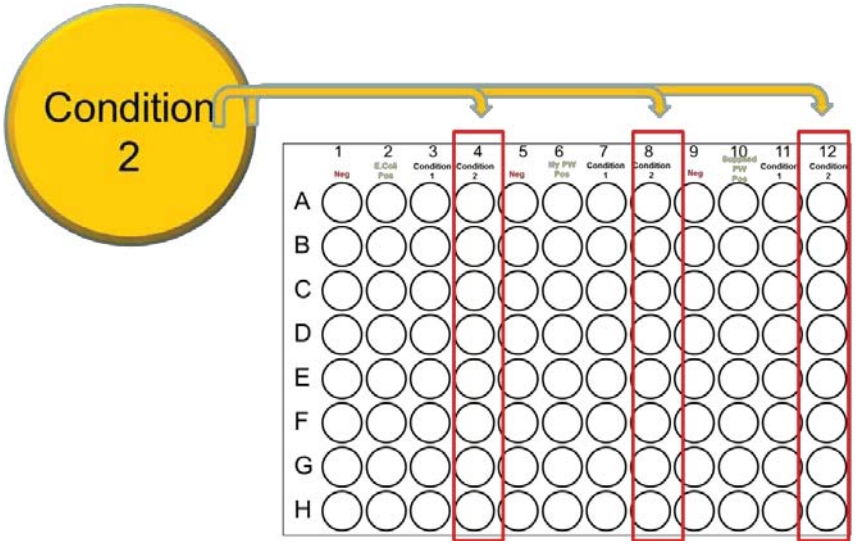


UNDER CONDITIONS, PLEASE FILL IN YOUR SPECIFIC CONDITIONS.

iii. Next, apply antimicrobial condition 1 liquid broth into the appropriate wells with the assigned pipet.



iv. Then, apply antimicrobial condition 2 liquid broth into the appropriate wells with the assigned pipet.



v. Place 96 well lid back on 96 well plate and place plate in the Tupperware container supplied. These plates will be incubated again at 37°C overnight.

Appendix E. Pre-test and Post-test Assessment for Study

Appendix E contains the pre-test and post-test assessments (assessments were the same) utilized at Pike High School to analyze the effectiveness of the inquiry based activity.

Biofilm Lab Pre-Test

Name:_____ Pd:_____

Date:_____

Please answer the following questions to the best of your ability. It is normal for you not to know the answer to some of these questions, yet, but please try to take an educated guess.

1. What is a biofilm?

2. How would a biofilm form?

3. What organisms would you find in a biofilm?

4. How are bacteria grown in a lab?

5. What is the purpose of antimicrobial agents?

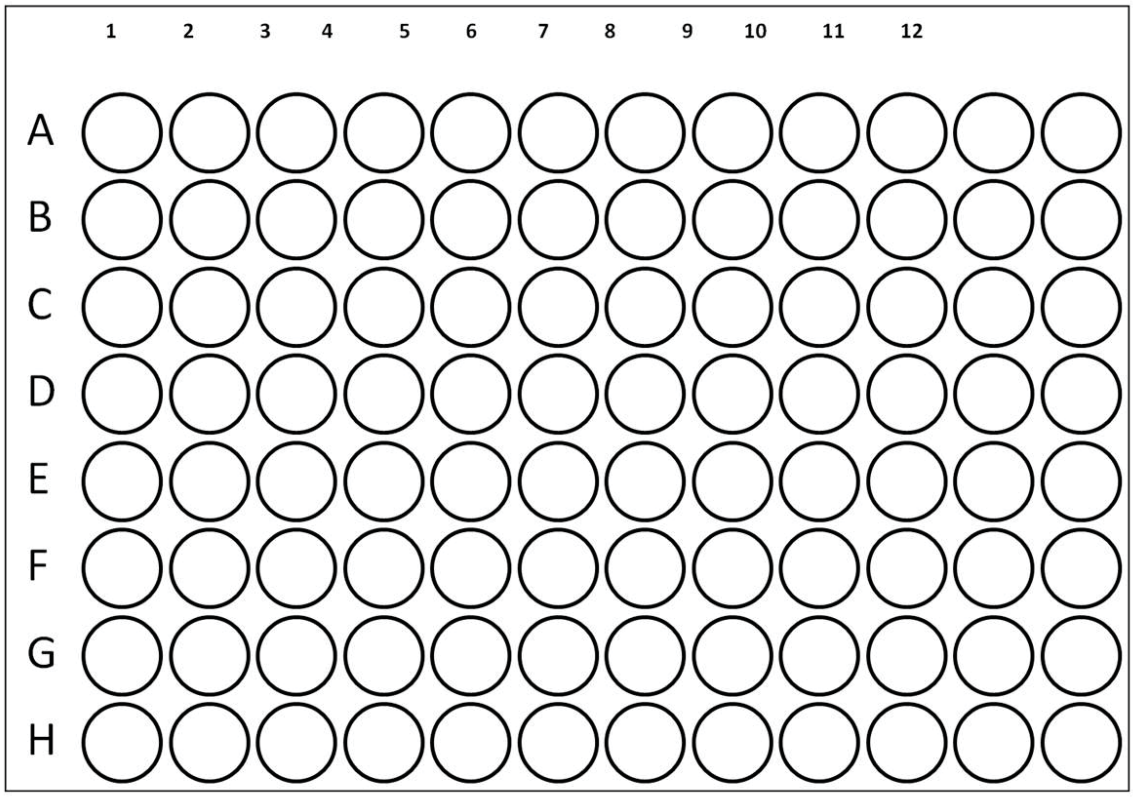
6. What is a positive control in an experiment? What is a negative control in an experiment?

7. What are the necessary components to a good experiment?

8. What is a biofilm assay? What could be the benefits of using a biofilm assay?

Appendix F. Experimental Design Worksheet

Appendix F contains the worksheet utilized for experimental design and recording qualitative data. It is a drawing of a 96 well plate.



Use to above diagram to mark exactly what condition is in each well.
An example will be provided for you to follow .

VITA

VITA

As a child, I enjoyed creating slides and looking at them under the microscope. By the age of twelve, I knew that I wanted to be a Biologist. Following my high school graduation, I had to work for three years in order to save up enough money to go to college. I attended Ball State University at 21 years old, and completed my degree four years later in Biology with a minor in Psychology. I worked extremely hard to pay my way through college. Following graduation, I worked for a year in industry as an Organic Chemist for an environmental company. Then, I started pursuing my MS in Biology at IUPUI. I have enjoyed my time at IUPUI and participated in many different clubs and organizations. I started a club for Biology graduate students at IUPUI called Bio PUGS. I also served as the School of Science Graduate Student Council Secretary for one year. Furthermore, I was a part of the Technology Committee as the graduate student representative. I also competed successfully for many different travel grants and a fellowship. I have been awarded four travel grants, both internal and external, in my time at IUPUI. I also was awarded the NSF funded GK-12 Fellowship. Furthermore, I have presented 10 posters at conferences and meetings. I have already earned authorship on one publication, and I have submitted a first author publication to the journal, American Biology Teacher. I intend on submitting three more articles for publication in various journals.

Following graduation, I will work in industry for one year in Indianapolis. Then, my husband and I are moving to Durham, North Carolina. I will work for another year in industry while my husband completes his fellowship in MSK Radiology. Following the initial year of working in industry, I intend on applying to graduate programs at Duke University, University of North Carolina, and North Carolina State. I would like to get my PhD, complete a post-doc, and pursue a career as a PI at a major university.