Molecular Characterization of the Interactions of the Resistance-Nodulation-Division Pump MexJK of *Pseudomonas aeruginosa* with Outer Membrane Proteins OprM and OpmH

by

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ABSTRACT

Pseudomonas aeruginosa is an important Gram-negative opportunistic pathogen, which has become a significant threat due to wide-spread antimicrobial resistance. Resistance Nodulation Division efflux pumps are important contributers to antimicrobial resistance in *P. aeruginosa* and other organisms. Resistance nodulation division efflux pumps are composed of three proteins; however, the mechanism by which the three proteins interact to form a functional complex remains, for the most part, unknown. The goal of this project is to further our understanding of how these proteins interact, to better understand how these efflux pumps operate in hopes of identifying novel therapeutic targets. We constructed a single copy expression system to study MexJK in the presence of either OprM or OpmH and confirmed these constructs using qRT, immunoblot and MIC. Chimeric OprM proteins were constructed by swapping α-helical domains from the OpmH protein. Using these chimeric proteins, we were able to identify regions within OprM/OpmH that may be responsible for substrate specificity by generating chimeric proteins. Taken together this data broadens our understanding of how this complex interacts.

Key Words: Multidrug resistance, Resistance nodulation division, Efflux, Gram-Negative, Chimera

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LIST OF ABBREVIATIONS

A_{600nm}	Absorbance at 600nm
aacC1	Acetyltransferase 3-1 encoding gene conferring Gm-resistance
AHL	Acylated homoserine lactones
AI	Autoinducer
Amp	Ampicillin
bp(s)	Base pair(s)
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
Cb	Carbenicillin
CBB	Coomassie brilliant blue
cDNA	Complementary deoxyribonucleic acid
CF	Cystic fibrosis
CLSI	Clinical and Laboratory Standards Institute
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate(s)
DSP	Dithiobis [succinimidyl propionate]
DTT	Dithiothreitol
Flp	*Saccharomyces cerevisiae* recombinase enzyme
Gm	Gentamicin
GSNO	*s*-nitrosoglutathione
H6	Hexa-histidine tag
HAP	Hospital acquired pneumonia
HCAP	Healthcare associated pneumonia
HRP	Horseradish peroxidase
ICU	Intensive care unit

IPTG	Isopropyl-β-D-thiogalactoside
Kb(s)	Kilobase(s)
kDa	Kilodalton
LB	Lysogeny broth
LPL	Lipoprotein lipase
MATE	Multidrug and toxic compound extrusion family
MDR	Multidrug resistant
MES	2-(*N*-morpholino)ethanesulfonic acid
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration
MFP	Membrane fusion protein
MFS	Major facilitator superfamily
mQH_2O	MilliQ H_2O
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NO	Nitric oxide
NRT	No reverse transcriptase control
NTC	No template control
OMP	Outer membrane protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with 0.1% Tween-20
PCR	Polymerase chain reaction
PHYRE2	Protein Homology/AnalogY Recognition Engine v2.0
RND	Resistance nodulation division
RT	Room temperature
sacB	*Bacillus subtilis* levan sucrase-encoding

	gene
SB	Sample buffer
SDS	Sodium dodecyl sulphate
sec	Second(s)
SMR	Small multidrug resistance family
T7 Pol	T7 polymerase
TEMED	Tetramethylethylenediamine
TFBI	Transformation buffer I
TFBII	Transformation buffer II
v/v	Volume / volume
VAP	Ventilator associated pneumonia
w/v	Weight / volume
x *g*	Gravitational force

I.

INTRODUCTION

A. Antibiotic Resistance in Pseudomonas aeruginosa

i. P. aeruginosa and Clinical Relevance

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen, prevalent in nosocomial infections. *Pseudomonas spp.* are commonly found as part of the normal flora on the skin but are capable of being highly infectious in immunocompromised individuals causing chronic pulmonary infections, particularly in cystic fibrosis (CF) patients (Gillis, *et al.*, 2005) in addition to being able to cause urinary tract infections, infection of burn wounds and displaying high rates of infection in cancer patients (Kumari, *et al.*, 2009).

The success of *P. aeruginosa* as a nosocomial pathogen can be attributed to its intrinsic resistance to several antimicrobial agents including antibiotics, biocides and heavy metals (Li, 1995). As a result, *P. aeruginosa* infections are difficult to treat and often life threatening. *P. aeruginosa* has been identified as a primary cause of pneumonia in hospitals. *P. aeruginosa* is the second leading cause of hospital-acquired pneumonia (HAP), healthcare-associated pneumonia (HCAP) and ventilator-associated pneumonia (VAP) with rates of infection rising in individuals that remain in the intensive care unit (ICU) for longer than 4 days (Driscoll, *et al.*, 2007).

A study conducted in 2007 compared rates of nosocomial infections in European public hospitals and found that *P. aeruginosa* accounted for 11-13.8% of all nosocomial infections when a microbiological isolate was successfully identified, with a higher percentage of rates of *P. aeruginosa* infections being found in the ICU, (13.2-22.6%) (Driscoll, *et al.*, 2007). Multidrug resistant (MDR) *P. aeruginosa* is common in Canadian hospitals as well. For example, a CANWARD study collected 1549 *P. aeruginosa* isolates from 15 different Canadian hospitals, of which 136 were MDR (Walkty, *et al.* 2012). It was found that the majority of MDR isolates were resistant to ceftazidime, piperacillin-tazobactam, meropenem and ciprofloxacin, while remaining susceptible to colistin (Walkty, *et al.* 2012).

ii. Mechanisms of Resistance:

Antimicrobial resistance of Gram-negative bacteria like *P. aeruginosa* may be attributed to a combination of several mechanisms. These mechanisms include low outer

membrane permeability, inactivation of antibiotic by enzymes (ex. β-lactamases, aminoglycoside modifying enzymes), alteration of the molecular target and active efflux of the antimicrobial agent (Figure 1). Several clinically relevant antibiotics used to treat *P. aeruginosa* infection must traverse the cellular membrane to reach their molecular targets within the cell. The presence of an additional outer membrane in Gram-negative bacteria limits permeability of antibiotic molecules. Penetrating molecules may be removed from the cell by an assortment of efflux pumps. The synergistic relationship between the outer membrane permeability and the efflux pumps is the most important factor influencing intrinsic antibiotic resistance in Gram-negative bacteria like *P. aeruginosa* (Liu, *et al.*, 1996).

B Efflux Pumps

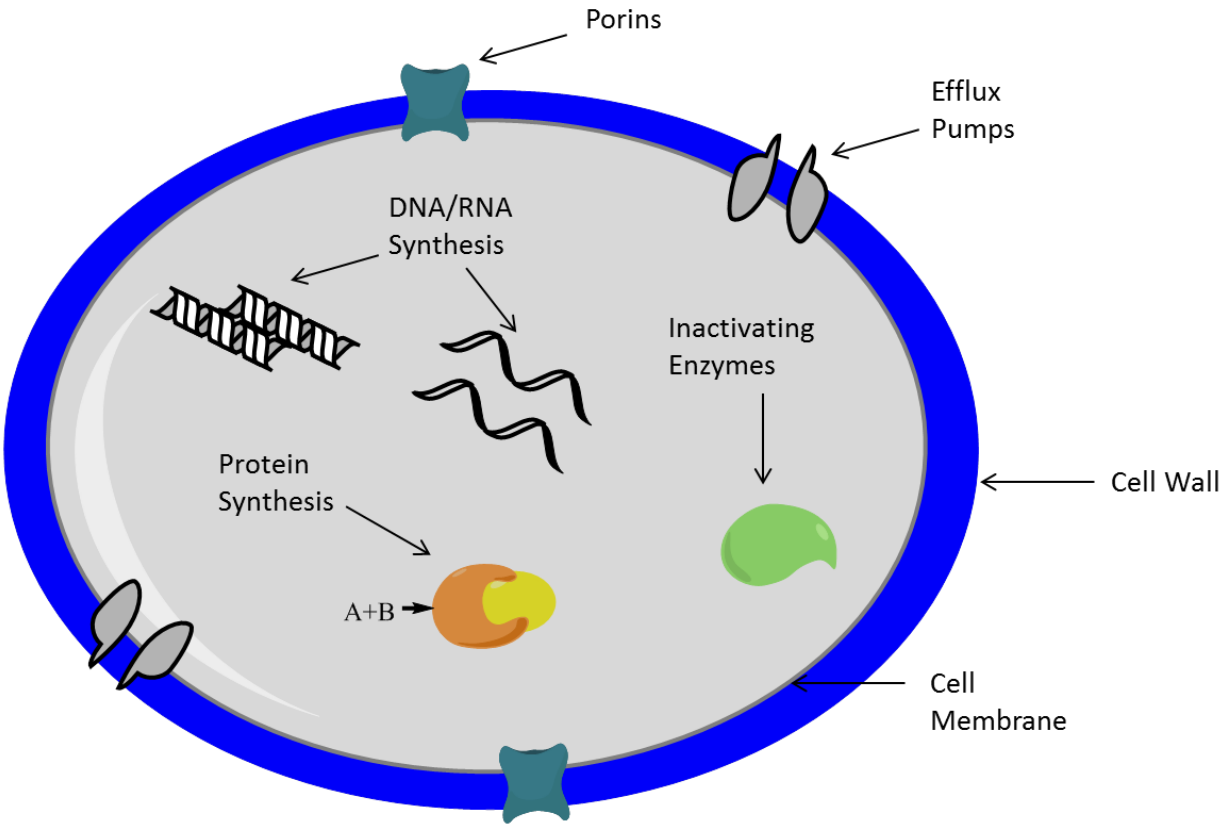
i. Resistance Nodulation Division Efflux Pump Structure

There are five families of multidrug resistance efflux pumps: the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multidrug and toxic-compound extrusion (MATE) family, the small multidrug resistance (SMR) family and the resistance nodulation division (RND) family (Piddock, 2006).

Efflux of antibiotics from the periplasm is a critical mechanism of antibiotic resistance utilized by Gram-negative cells. *P. aeruginosa* is capable of actively effluxing antibiotics from the periplasmic space using efflux pumps primarily from the RND family. The RND complex is tripartite in nature, composed of an outer membrane channel protein (OMP), inner membrane RND transporter, and a membrane fusion protein (MFP) (Kumar and Schweizer, 2005). The RND transporter provides the energy for the molecular transport and is often referred to as the RND "pump" protein (Misra and Bavro, 2009). The OMP interacts with the RND protein in the periplasm producing a channel capable of transporting antibiotics into the extracellular space. The MFP is believed to stabilize the interactions between the RND transporter and the OMP (Misra and Bavro, 2009). All three of these components are essential for efflux function.

The RND transporters are comprised of a homotrimer folded into an α-helical transmembrane domain, with a large, soluble periplasmic domain. The top of the

Figure 1. Different mechanisms of antimicrobial resistance in Gram-negative bacteria. Bacteria have semi-permeable membranes which may limit the passage of some types of antibiotics into the cell. Multidrug resistance efflux pumps allow the bacterium to pump antimicrobials out of the cell. Porins maintain osmotic pressure by allowing the entrance/exit of hydrophilic small molecules but do not permit the passage of larger molecules. Enzymes can modify the antibiotic so that it is no longer recognized by the target, or modify them so that they are no longer functional. This is particularly prevelant with antibiotics that alter the ribosome or interfere with ribosome binding to inhibit protein synthesis. Inhibition of mRNA synthesis occurs by binding to DNA-dependent RNA polymerase inhibiting initiation. Inhibition of DNA synthesis occurs by inhibiting either DNA gyrases or topoisomerases or binding to the alpha subunit. A represents mRNA and B represents tRNA. (Fluit *et al.*, 2001)


Porins
Efflux
Pumps
DNA/RNA
Synthesis
Inactivating
Enzymes
Protein
Synthesis
A+B
Cell Wall
Cell
Membrane

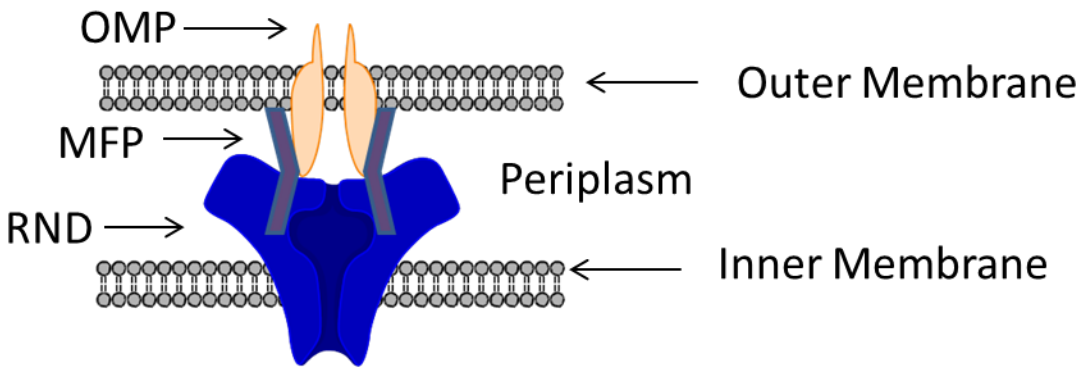
periplasmic domain interacts with the OMP (Misra and Bavro, 2009). The crystal structure of AcrB, a RND transporter found in *E. coli* revealed that the protein contained three domains: the membrane-spanning domain, the pore domain and the TolC-docking domain (Murakami, *et al.*, 2002).

The crystal structures of TolC (Koronakis, *et al.* 2000) and OprM (Akama, *et al.*, 2004) the OMPs from *E. coli* and *P. aeruginosa* respectively have been solved, and while there is little amino acid homology between the two proteins they fold in a similar homotrimeric nature both with an outer membrane-embedded β-barrel and an α-helical domain which extends into the periplasm (Misra and Bavro, 2009).

MFPs are lipoproteins which have a lipidated N-terminal end anchored to the inner membrane, exposing the majority of their structure to the periplasm. Crystal structures of AcrA (Mikolosko, *et al.*, 2006) and MexA (Akama, *et al.*, 2004) from *E. coli* and *P. aeruginosa* respectively contain three linearly-arranged domains: a β-barrel domain containing six anti-parallel β-strands and a shorter α-helix, and a central lipoyl domain made up of 4 β-strands separated by an α-helical hairpin domain (Misra and Bavro, 2009). It has been shown that the hairpin domain of the MFP is what is interacting with the OMPs, while the β-barrel is interacting with the RND transporter (Misra and Bavro, 2009).

These proteins are usually genetically encoded as part of a single operon with the MFP encoded first, followed by the RND transporter and finally the OMP. Often a regulator is found which is encoded in the opposite direction located upstream of the MFP. The MexAB-OprM RND efflux system in *P. aeruginosa* is an example of this (Li, *et al.* 1995) where *mexA*, *mexB*, and *oprM* are the MFP, RND transporter and OMP, respectively, and *mexR* is the repressor, encoded upstream of *mexA* (Srikumar, *et al.* 2000). However, there are instances when the operon may consist of only the genes encoding for the MFP and RND proteins only, and the gene encoding for the OMP is encoded elsewhere in the genome. The MexJK-OpmH efflux complex in *P. aeruginosa*, would be an example of this, where *mexJ* and *mexK* are encoded in an operon, with their

Figure 2. Structure of a Resistance Nodulation Division efflux pump. The RND pump (blue), traverses the inner membrane and is a drug-proton antiporter. The membrane fusion protein (purple) acts as an adaptor to stabilize the interaction between the RND and outer membrane protein (pink). The outer membrane protein, acts as a channel, through which substrates can pass (Misra and Bavro, 2009).


OMP
Outer Membrane
MFP
Periplasm
RND
Inner Membrane

local repressor *mexL*, while the OMP, OpmH is found as a standalone gene elsewhere in the *P. aeruginosa* genome (Chuanchuen, *et al.* 2003).

ii. Clinical Relevance of RND Efflux Pumps

RND pumps are widespread throughout Gram-negative organisms, and as a group exist as the most clinically relevant efflux system (Morita, *et al.* 2012). To date twelve RND efflux pumps have been described in *P. aeruginosa*, these include MexAB-OprM (Li, et al., 1995), MexCD-OprJ (Poole, *et al.*, 1996), MexEF-OprN (Kohler, *et al.*, 1997), MexGHI-OpmD (Aendeker, *et al.*, 2002), MexMN (Mima, *et al.*, 2005), MexPQ-OpmE (Mima *et al*, 2005), MexVW-OprM (Li, *et al*., 2003), MexXY-OprM (Mine, *et al.*, 1999), TriABC-OpmH (Mima, *et al.*, 2007), MuxABC-OpmB (Mima, *et al.*,2009), and MexJK-OprM/OpmH (Chuanchuen, *et al.*, 2002) (Table 1). Although MexAB-OprM and AcrAB-TolC of *P. aeruginosa* and *E. coli* respectively, are used as models to understand the organization and mechanism of these RND efflux complexes, there exist many more examples. Specifically in *P. aeruginosa*, the MexXY system is known for its high levels of intrinsic aminoglycoside resistance, with upregulation of the complex being the most common form of increased resistance (Armstrong and Miller, 2010). While it is widely accepted that the efflux of antibiotics is not the primary function of RND pumps (Table 1) some recent findings have shed light on their natural functions. For example, it was demonstrated that the expression of the MexEF-OprN pump in *P. aeruginosa*, when in the presence of a nitrosative stressor, is induced (Fetar, *et al.*, 2011). This is particularly important as it is known that epithelial cells in the lungs produce nitric oxide (NO) when stimulated by bacteria or bacterial products. This could lead to selective pressure for MexEF-OprN antimicrobial resistant mutants (Fetar, *et al.*, 2011). *s*- nitrosoglutathione (GSNO) a source of NO was shown to induce MexEF-OprN; however, when mutants either overexpressing or lacking these genes were tested, there was no difference in susceptibility to GSNO seen (Fetar, *et al.*, 2011). This supported the hypothesis that, nitrosative stress may be a selective pressure for MexEF-OprN (Fetar, *et al.* 2011). MexEF-OprN is regulated by MexT, a positive regulator, which was shown to be induced in response to airway epithelial cells. This led to the hypothesis that MexT is related to pathogen-host interactions (Fargier, *et al.*, 2012).

Table 1. Substrate profiles of RND multidrug efflux pumps characterized in *P. aeruginosa.*

Efflux Pump Name	Substrate	Reference
MexAB-OprM	β-lactams, β-lactamase inhibitors, chloramphenicol, fluoroquinolones, macrolides, novobiocin, tetracycline, triclosan, trimethoprim, ethidium bromide, SDS, thiolactomycin, cerulenin, acylated homoserine lactones	Poole, 2001
MexCD-OprJ	β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, tetracycline, triclosan, trimethoprim, ethidium bromide, SDS, crystal violet, acriflavine	Poole, 2001
MexEF-OprN	chloramphenicol, fluoroquinolones, triclosan, trimethoprim, Pseudomonas quinolone signal	Kohler, *et al.*, 2001; Poole, 2001
MexXY-OprM	fluoroquinolones, aminoglycosides, tetracycline, erythromycin	Li *et al.*, 2002
MexJK-OprM/OpmH	erythromycin, tetracycline/triclosan	Chuanchuen, *et al.*, 2002
MexGHI-OpmD	vanadium, precursors to acylated homoserine lactones	Aendekerk, *et al.*, 2002
MexVW-OprM	chloramphenicol, fluoroquinolones, erythromycin, trimethoprim, ethidium bromide, acriflavine	Li, *et al.*, 2003
MexMN	chloramphenicol, thiamphenicol	Mima, *et al.*, 2005
MexPQ-OpmE	fluoroquinolones, tetracycline, chloramphenicol, macrolides	Mima, *et al.*, 2005
TriABC-OpmH	triclosan	Mima, *et al.*, 2007
MuxABC-OpmB	novobiocin, aztreonam, macrolides, tetracycline	Mima, *et al.*, 2009

iii. Mechanism of RND Efflux Pump

Multiple studies have been performed to elucidate the domains responsible for pump function and specificity. In 2002, Elkins and Nikaido replaced both of the large periplasmic loops of AcrD a RND transporter from *E. coli*, with those from AcrB, also from *E. coli*, which effectively modified the substrate specificity of the AcrAD-TolC complex to that of AcrAB-TolC (Elkins and Nikaido, 2002). Alternatively, when they replaced the transmembrane regions of AcrD with those sequences of AcrB there was no effect on substrate specificity. This showed that the substrate specificity is determined primarily by the periplasmic domain and that the critical binding of substrates likely occurs here.

In 2002 Zgurskaya *et al.* used chimeras of the AcrB pump of *E. coli* and the MexB pump from *P. aeruginosa* to show that substrate specificity was largely determined by the second external loop of these proteins (residues 612-849) (Tikhonova, *et al.,* 2002). It was also shown that the region spanning the extracytoplasmic domain between helices 2-7 defined the specificity of interaction with the periplasmic components (Tikhonova, *et al.,* 2002). Finally they showed that the N-terminal periplasmic loop and the first part of the C-terminal loop are what determine the interaction of the RND pumps with their associated periplasmic adaptor proteins.

The conformational changes in RND protein structure associated with the efflux of substrates remains poorly defined. Several models proposing the steps required from substrate recognition to expulsion have been proposed (Murakami, *et al.* 2006; Fernandez-Reico, *et al.*, 2004; Pietras, *et al.*, 2009). Currently, the model proposed by Weeks *et al.* (Weeks, *et al.* 2010) is considered the strongest. This model describes a 5-step substrate binding mechanism whereby binding of the substrate leads to opening of the OMP via conformational changes communicated through the MFP. These are, (i) an initial interaction between the AcrB hairpin loop and the TolC turns (ii) trigger a partial opening of TolC (iii) binding of the substrate induces conformational changes in AcrB which are transduced to the β-barrel and lipoyl domains (iv) direct interactions between the α-helical hairpin domain of AcrA and intra-protomer grooves allow for TolC helices to extend (v) full dilation of TolC (Weeks, *et al.*, 2011). More recently, a 2012 study

performed by Lu and Zgurskaya (2012) showed that while the RND transporter is not directly interacting with the OMP, a lipoprotein lipase (LPL) is controlling the interaction between the MFP and the OMP and is linking conformational changes in the transporter to transport of substrate through the OMP. Therefore, it is necessary to have interaction of the MFP to the RND transporter in order to stimulate the activities of the transporter.

C. Knowledge Gaps

Currently, very little data is available on the mechanisms of interaction between the three components. It is also not known what role a given substrate plays in the interaction of the three components, if any.

D. Virulence and RND Pumps

It has been shown that in addition to antimicrobials, efflux pumps are also able to pump out host-derived products as well, such as virulence determinants. *P. aeruginosa* virulence relies on the ability to produce several autoinducer molecules; these molecules accumulate in a cell density-dependent manner and induce the expression of multiple targets, specifically of virulence factors. In addition, *P. aeruginosa* contains several multidrig efflux pumps which confer adaptive resistance to antibiotics. These pumps are also able to influence quorum sensing.

i. Quorum Sensing

Acylated homoserine lactones (AHLs) are found in a large number of Gram-negative bacteria; their accumulation in the growth medium reflects cell density and triggers the expression of target genes when a critical level of cell concentration is reached. Quorum sensing (QS) involves an autoinducer synthase, which is responsible for the production of autoinducer (AI) that is released into the growth medium and a transcriptional activator that acts together with the autoinducer to activate the target genes in response to the increase in cell density (Fuqua, *et al.* 1996). *P. aeruginosa* has three quorum sensing systems the Las, Rhl and PQS systems (Pesci, *et al.*, 1999). Each system consists of genes involved in autoinducer synthesis, *lasI, rhlI* and *pqsABCDH,* as well as a transcriptional regulator, *lasR, rhlR* and *pqs,* respectively. These three systems are arranged in a hierarchal order with the Las system positively regulating both the Rhl

(Latifi, *et al.,* 1996) and the PQS (Wade, *et al.,* 2005) systems. It is possible though, for these systems, Rhl and PQS, to be activated in the absence of the Las system under certain environmental conditions, such as growth medium (Medina, *et al.,* 2003). The Rhl system has also been shown to negatively regulate the PQS system (Wade, *et al.,* 2005).

It has been reported that the *P. aeruginosa* RND pump MexAB-OprM is able to efflux out AHLs (Evans, *et al.,* 1998) and that cells of *P. aeruginosa* are not permeable to all QS molecules (Pesci, *et al.,* 1999). The MexAB-OprM pump is able to selectively efflux AHLs, including AHLs from other bacterial species (Minagawa, *et al.,* 2012). Conversely it has been reported that an overexpression of these RND pumps can be detrimental to the cell. For example, in the MexAB-OprM system, where the AHLs are substrates, it has been shown to have reduced virulence for those strains overexpressing this pump as a result of an increase in the efflux of quorum signals (Piddock, L., 2006). This leads to a reduction in the expression of virulence determinants regulated by quorum sensing (Piddock, 2006).

ii. Biofilm Formation

P. aeruginosa forms environmentally and clinically relevant biofilms and is a model organism for their study. *P. aeruginosa* is able to form a mature biofilm in 5-7 days, following three main steps (Rosenberg, *et al.,* 1982). The first step is attachment and involves the planktonic bacteria reversibly attaching to a surface (abiotic or biotic). After adherence to a surface, biofilms begin developing through aggregation characterized by an irreversible attachment to the surface, multiplication of the bacteria and microcolony formation. Once the biofilm is mature, individual cells or aggregates of cells can disperse to allow them to infect other locations (Rosenberg, *et al.,* 1982). The polymeric matrix which surrounds the biofilm cells adds protection against environmental stresses and predation (Donlan and Costerton, 2002).

Several mechanisms have been shown to increase the tolerance of bacterial cells in a biofilm to antimicrobials. These include decreased antimicrobial diffusion, formation of persistor cells, and the induction of specific target genes. Specifically in *P. aeruginosa*,

the intrinsic resistance to several antibiotics has been found to increase when present in a biofilm (Potera, 1999) such as tetracycline, chloramphenicol, quinolones, and β-lactams. These patterns of resistance are similar to those substrates of the MexAB-OprM pump (Soto, 2013).

Several studies have investigated the effects of antibiotics on biofilms using colistin as an antimicrobial and have found that those biofilm cells in the active subpopulation, with a higher metabolic activity, were able to survive, whereas those in the inactive subpopulation located deep within the biofilm were eradicated (Pamp *et al.,* 2008). As well, MexCD-OprJ genes required for colistin tolerance were induced in the active subpopulation under colistin exposure (Chiang, *et al.,* 2012).

iii. Swarming Motility

Bacteria containing flagella may adapt their locomotion machinary when grown on solid surfaces for movement in order to obtain a specialized form of organized movement known as swarming (Calvio, *et al.,* 2005). Swarming motility is characterized by the movement of bacteria in groups of tightly bound cells grown on top of solid media. Swarming cells are longer and more flagellated compared to non-swarm cells (Calvio, *et al.* 2005). There are several environmental signals that have been implicated in affecting the transition from non-swarm to swarm cells, however, cell-density signals seem to be most important (Calvio, *et al.,* 2005).

It has been shown that swarming motility is deficient in mutant strains that overexpress MexEF-OprN (Kohler, *et al.,* 2001). Swarming motility is dependent on rhamnolipid production, and in those strains which overexpress MexEF-OprN, rhamnolipid production is impaired (Kohler, *et al.,* 2001). Rhamnolipid production is under the control of the Rhl system. In those strains overexpressing MexEF-OprN there is a decrease in *rhlI* transcription as well as a decrease in C4-HSL AI production, which may account for the decreases in rhamnolipid production (Kohler, *et al.,* 2001). Rhamnolipid is a a surfactant that is required to reduce surface tension in order for the bacteria to spread across the surface and is controlled by quorum sensing (Kearns, 2010). In addition to MexEF-OprN, several efflux pumps have been shown to pump out quorum

sensing molecules which may reduce the amount of rhamnolipid being produced, and therefore the ability of swarming motility to take place.

iv. Swimming Motility

P. aeruginosa may also contain a single polar flagellum which promotes swimming motility in liquid environments (0.3% agar) (Murray and Kazmierczak, 2006). In swimming motility, similar to twitching motility the cells are moving independently, rather than collectively through quorum sensing. It may be that RND efflux pumps do not affect swimming motility as they do swarming. Since swimming motility is not dependent on quorum sensing molecules which have been recognized as substrates for these pumps, the expression of these pumps likely does not affect the ability of the bacteria to swim.

E. MexJK-OprM/OpmH as a Model for Antimicrobial Resistance Mechanisms

The MexJK pump of *P. aeruginosa* was first described in 2002 after a strain of *P. aeruginosa* lacking MexAB-OprM and MexCD-OprJ (PA0238-1) was exposed to triclosan and the resistant colonies were screened. It was found to overexpress a novel RND pump, MexJK, that was regulated by *mexL* (Chuanchuen, *et al.*, 2002). This pump was found to complex with two different OMP's. MexJK-OpmH effluxes triclosan, while MexJK-OprM effluxes erythromycin (Chuanchuen, *et al.*, 2002) (Figure 3). Triclosan is a commonly used biocide which is effluxed by most RND systems in *P. aeruginosa* (Chuanchuen, *et al.,* 2002). Previous studies have shown that *P. aeruginosa* possess several triclosan resistance mechanisms such as the ability to generate target mutations, produce enzymatic modifications and through active efflux, specifically MexAB-OprM (Chuanchuen, *et al.,* 2001; Chuanchuen, *et al.,* 2003).

It is known that one OMP can interact with several RND-MFP complexes, TolC of *E. coli* is known to interact with several different types of efflux pumps (Koronakis, 2003), while OprM of *P. aeruginosa* interacts with MexAB, MexJK, and MexXY (Poole, *et al.,* 1993; Chuanchuen, *et al.* 2002; Mine, *et al.* 1999). The MexJK pump is unique since it effluxes different substrates depending on whether it forms complex with the OprM or OpmH protein. This asks the question of whether the two complexes,

MexJK-OprM and MexJK-OpmH are formed constitutively or whether their interaction is substrate dependent. The substrate specificity of this complex paired with the uncharacteristic RND-MFP complex promiscuity make MexJK-OprM/OpmH a model system for studying the effect substrate binding has on tripartite pump formation.

Since all three components of this complex are essential for pump activity, by determining if the pump formation is substrate dependent or constitutively expressed, we may be able to develop a method of controlling the formation of the complex.

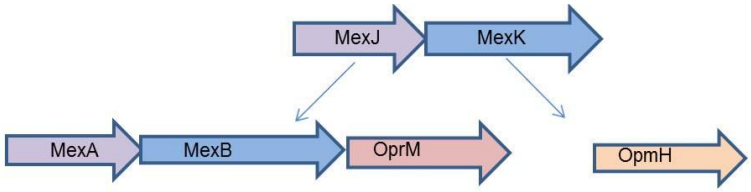
F. Hypothesis:

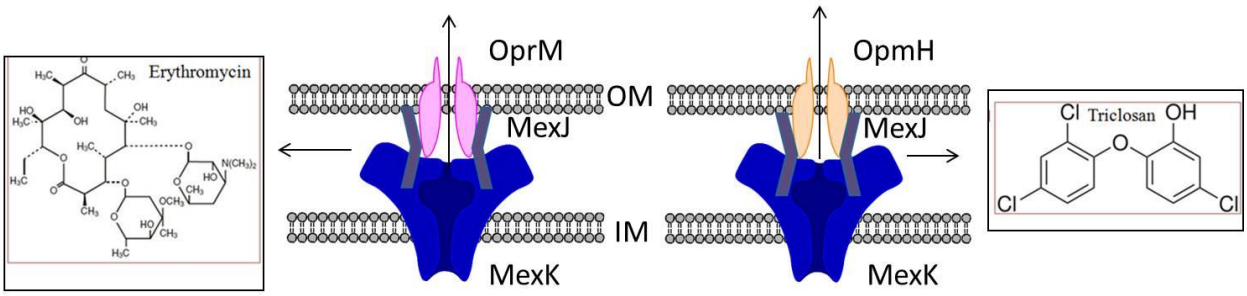
The MexJK RND efflux pump of *P. aeruginosa* forms functional complexes with outer membrane proteins OprM and OpmH, in a substrate dependent manner.

Therefore, the objectives of this thesis were:

- To construct a single copy gene expression system for the OprM protein in the MexJK overexpressing strain of *P. aeruginosa.*
- To understand the molecular mechanisms of interactions of OpmH/OprM with the MexJK complex using chimeric outer membrane proteins and pull-down assays.

Figure 3. Organization of the MexJK-OprM/OpmH Operon. MexJK is encoded as an operon lacking an outer membrane component. It has been shown to form a complex with *oprM* (efflux of erythromycin), which is located as part of the MexAB-OprM operon, in addition to forming a complex with *opmH* (efflux of triclosan) which is not associated with an RND operon (Chuanchuen, *et al.*, 2001).


MexJ
MexK
MexA
MexB
OprM
OpmH


Erythromycin
OprM
OM
MexJ
IM
MexK
OpmH
MexJ
MexK
Triclosan
Cl
OH
O
Cl
Cl

II.

MATERIALS AND METHODS

A. Bacterial Strains, Growth and Culture Conditions

Bacterial strains used in this study are listed in Table 2. Bacteria were routinely cultured in Lysogeny Broth (LB) at 37 ^{o}C (Biobasic Inc., Markham, ON., Canada) and supplemented with the appropriate antibiotic where necessary to maintain plasmids at the following concentrations: 100 µg/mL ampicillin (Bioshop Canada Inc., Burlington, ON., Canada) for maintenance in *E. coli* and 30 µg/mL gentamicin (Bioshop Canada Inc., Burlington, ON., Canada), and 200 µg/mL carbenicillin (Bioshop Canada Inc., Burlington, ON., Canada) for that in *P. aeruginosa*. Counterselection for curing plasmids with the aid of the *sacB* gene was carried out in LB agar medium supplemented with 10% sucrose (Bioshop Canada Inc., Burlington, ON., Canada) and induction of gene expression was achieved by supplementing the growth medium with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Biobasic Inc., Markham, ON, Canada).

B. DNA Manipulations

Plasmids and oligonucleotides used in this study are listed in Table 3 and Table 4 respectively. Plasmid DNA was extracted using EZ-10 Spin Column Plasmid Miniprep Kit (Biobasic Inc., Markham, ON, Canada) according to manufacturer's instructions. The DNA concentration was measured using the Eppendorf BioPhotometer Model AG (Barkhausenweg, Hamburg, Germany) and the samples were stored at -20 ˚C. DNA amplified by polymerase chain reaction (PCR) or digested with restriction enzymes, was resolved by 0.8% agarose (Biobasic Inc., Markham, ON, Canada) gel electrophoresis and purified using the EZ-10 Spin Column DNA Gel Extraction kit (Biobasic Inc., Markham, ON, Canada).

i. E. coli Competent Cell Preparation and Transformation

E. coli DH5α competent cells were prepared according to Inoue and colleagues (Inoue, *et al* 1990). Briefly, *E. coli* DH5α were subcultured with a 1/100 v/v inoculum from an overnight culture and grown at 37 ^{o}C until they reached mid log phase (OD_{600nm}= 0.4-0.6). Cells were harvested in a pre-chilled rotor at 3800 xg and resuspended in 0.4x of original volume of ice cold transformation buffer I (TFBI– 100 mM rubidium chloride (Fisher Scientific, Markham, ON, Canada), 50 mM manganese chloride (Bioshop Canada

Table 2. Bacterial strains used in this study.

Strain	Relevant Characteristics	Reference/ Source
E.coli		
DH5α	F- φ80d *lacZΔ*M15 Δ(*lacZYA-argF) U169 deoR recA1endA1 hsdR17* (rk-, mk+) *phoA supE44 λ- thi-1 gyrA96 relA1*	Taylor, *et al.*, 1993
MT102/pJBA132	Gfp-based-N-Acyl Homoserine-Lactone sensor strain	Anderson, *et al.* 2001
P. aeruginosa		
PA01	*P. aeruginosa* prototroph	Holloway and Zhang, 1990
PA0238	PA01: Δ*mexAB-oprM*, Δ*mexCD-oprJ*	Chuanchuen, *et al.* 2001
PA0702	Δ*mexAB-oprM*,Δ*opmH*,Δ*mexXY*, Δ*mexCD-oprJ*, miniCTx T7	Kumar Lab Collection
PA0200	PA01:Δ*mexAB-oprM*	Schweizer, 1998
PA01172	Δ*mexAB-oprM*, Δ*mexCD-oprJ*, Δ*mexJK*, Δ*mexEF-oprN*, Δ*mexXY*, Δ*opmH*, Δ*triABC*	Kumar Lab Collection
PA050	PA0702:mini-Tn7-Lac	Ganeshanantham, 2011
PA051	PA0702:mini-Tn7-Lac-*opmH*-His	Ganeshanantham, 2011
PA054	PA015:mini-Tn7-Lac-*oprM*-His	This Study
PA058	PA0702:mini-Tn7-Lac-*oprM*-His	This Study
PA067	PA0702:pUC18-Gm-Lac-mini-Tn7-$oprM_{OpmHD1}$	This Study
PA068	PA0702:pUC18-Gm-Lac-mini-Tn7-$oprM_{OpmHD2}$	This Study
PA069	PA0702:pUC18-Gm-Lac-mini-Tn7-$oprM_{opmHWD}$	This Study
PA070	PA0702:pUC18-Gm-Lac-mini-Tn7-$oprM_{OpmHWD}$	This Study
PA071	PA0702:pUC18-mini-Tn7-$oprM_{OpmH\ D1}$	This Study
PA072	PA0702:pUC18-mini-Tn7-$oprM_{OpmH\ D2}$	This Study
PA073	PA0702:pUC18-mini-Tn7-	This Study

	oprM$_{\text{OpmHWD}}$	
PA074	PA0702:pUC18-mini-Tn7-*oprM*$_{\text{OpmH D1:D2}}$	This Study
PA075	PA0702:pUC18-mini-Tn7-*oprM*$_{\text{OpmH D1:D2}}$	This Study

Table 3. Plasmids used in this study.

Plasmid	**Relevant Characteristics**	**Reference/ Source**
pFLP2	Amp^r, source of *Flp* recombinase, *sacB* allows for removal of plasmid by sucrose counterselection	Hoang, *et al* 1998
pTNS2	Amp^r, R6K replicon, helper plasmid encoding site-specific TnsABCD for Tn*7* transposition pathway	Choi and Schweizer, 2005
pJBA132	*gfp*-based N-Acyl Homoserine Lactone sensor plasmid	Anderson, *et al.* 2001
pPLS037	pGEMT-easy-*oprM*-His	Kumar Lab Collection
pPLS038	mini-Tn7T-*opmH*-His , Amp^r, Gm^r	Ganeshanantham, 2011
pPLS040	mini-Tn7T-*oprM*-His, Amp^r, Gm^r	Ganeshanantham, 2011
pPLS078	pUC18-mini-Tn7-Gm-Lac-$oprM_{OpmHD2,}$ Amp^r, Gm^r	This Study
pPLS079	pUC18-mini-Tn7-Gm-Lac-$oprM_{OpmHD1,}$ Amp^r, Gm^r	This Study
pPLS080	pUC18-mini-Tn7-Gm-Lac-$oprM_{OpmHD1,}$ Amp^r, Gm^r	This Study
pPLS081	pUC18-mini-Tn7-Gm-Lac-$oprM_{OpmHD1:D2,}$ Amp^r, Gm^r	This Study
pPLS082	pUC18-mini-Tn7-Gm-Lac-$oprM_{OpmHWD,}$ Amp^r, Gm^r	This Study

Table 4. Oligonucleotides used in this study

Name	**Sequence (5'-3')**	**Target Gene**	**Laboratory Database #**	**Reference**
Tn7R_Fwd	CACAGCATAACTGGACTGATTTC	Tn7R	72	Choi, *et al* 2005
Pa_glmS_Rev	GCACATCGGCGACGTGCTCTC	*glmS* (*P. aeruginosa)*	73	Choi, *et al* 2005
GmFRT-UP	CGAATTAGCTTCAAAAGCGCTCTGA	*aacC1*	48	Choi, *et al.* 2005
GmFRT-Dn	CGAATTGGGGATCTTGAAGTTCCT	*aacC1*	49	Choi, *et al.* 2005
OpmH_Fwd_RT	AGTACCAGAAGGGCGACAAC	*opmH*	142	Ganeshanantham, 2011
OpmH_Rev_RT	ATCGGGATGTTCAGTTCCAG	*opmH*	143	Ganeshanantham, 2011
OprM_F_RT	ATCAACCTGCCGATCTTCAC	*oprM*	144	Ganeshanantham, 2011
OprM_R_RT	GTCTGGATCGCCTTCTCGTA	*oprM*	145	Ganeshanantham, 2011
RpsL_Fwd_RT	GCAACTATCAACCAGCTGGTG	*rpsL*	148	Mima, *et al* 2009
RpsL_Rev_RT	GCTGTGCTCTTGCAGGTTGTG	*rpsL*	149	Mima, *et al* 2009
Pae_ProC_F_RT	GGCGATCCAATCCTTCCAG	*proC*	170	Kumar Lab Database
Pae_ProC_R_RT	TTATTGGCCAACCTGTTCG	*proC*	171	Kumar Lab Database
OpmH_L164F*	CTGACGCTGAAGGCCGACCAGGCGCAGCTGGCCACCAGCAAGGCCGAG	*opmH*	331	This Study
OpmH_A202R*	CGCGCGGGCGCCTTCCACGGCGGTCTGGGCCTCGAGCACGTCGGTCT	*opmH*	330	This Study

OpmH_A379F*	GCCAGCGACGAGTACTACCAGCTCGCCACCGAG ATCGGCTACCAGGTC	*opmH*	329	This Study
OpmH_N409R*	GCTGGTCAGCTGATTGAGGCGGTCGGTGTTGTAG TCGCGCACGGCGGC	*opmH*	328	This Study
OprM_T117F	ACCACCGGCAGTCCGGCGATT	*oprM*	340	This Study
OprM_E481R	CTCGAGTGCGGCCGCATCTTC	*oprM*	341	This Study
OprM_A200R	GTCCAGCTGGCGCTTGAAGGC	*oprM*	342	This Study
OprM_R426F	CGCAACATCGTCGACGTGCTC	*oprM*	343	This Study
OprM_T5222	TCCTTCCTTTCCCTGGCGGTA	*oprM*	332	This Study
MexA_A913	ACGCCACGGATGCGTGTACTG	*mexA*	333	This Study

*nucleotide sequence homologous to *opmH* gene is underlined

Inc., Burlington, ON., Canada), 30 mM potassium acetate (Bioshop Canada Inc., Burlington, ON., Canada), 10 mM calcium chloride (Bioshop Canada Inc., Burlington, ON, Canada), 15% w/v glycerol (Bioshop Canada Inc., Burlington, ON, Canada), pH 5.8). Cells were incubated on ice for exactly 5 minutes, harvested as above and resuspended in 0.04x of original volume of ice cold transformation buffer II (TFBII– 10 mM 3-(N-morpholino) propanesulfonic acid, 10 mM rubidium chloride, 75 mM calcium chloride, 15% w/v glycerol, pH 6.5). Cells were incubated on ice for 30-60 mins and 100 µl aliquots were made and immediately frozen on dry ice to be stored long term at -80 °C.

Transformations of chimeras were performed using the heat shock method (Sambrook and Russell, 2001). Briefly, 100-500 ng of DNA was added to 100 µl competent cells and incubated on ice for 10 minutes, heat shocked at 42 °C for 45 seconds and incubated on ice for another 2 minutes. The reaction was added to 895 µL LB media and transformation reactions were recovered at 37 °C for 1 hr with shaking and plated on LB agar media containing the appropriate antibiotic for selection.

ii. P. aeruginosa Electroporation

All plasmids were delivered into *P. aeruginosa* using the rapid electroporation method as described previously by Choi *et al*., 2006. Briefly, 4x 3 mL *P. aeruginosa* overnight cultures of recipient cells were spun down at 16000 x *g* and washed twice with 1 mL of room temperature 300 mM sucrose and concentrated to a final volume of 200 µl. Cells (100 µL) were transferred to an electroporation cuvette (0.1 cm gap) (Fisher Scientific, Markham, ON, Canada), and 300 ng of plasmid DNA was added. Cells were shocked with a 2500 V electrical pulse for 5 milli-seconds using the Electroporation 2510 (Eppendorf, Westbury, NY, USA) electroporator, diluted immediately in 1 mL of room temperature LB and allowed to recover for 1 hour at 37 °C with shaking before plating on LB agar containing appropriate antibiotic for selection.

iii. Mini-Tn7 Based Single-Copy Gene Delivery System

Recipient *P. aeruginosa* overnight culture was concentrated from 12 to 2 ml and electroporated as described above with ~300 ng each of the mini-Tn*7* and the

transposase-encoding pTNS2 plasmids. Selection of cells containing successful integration was performed on medium containing 30 μg/mL gentamicin, and verified with Tn7R_Fwd and Pa_glmS_Rev primers that bind to the vector backbone and *P. aeruginosa glmS* gene (present immediately upstream of the insertion site) respectively, which generated a 292 bp PCR product. Once insertion was verified, the gentamicin resistance marker *aacC1* was removed by the *Flp*-FRT system as described previously (Hoang, *et al*. 1998). Briefly, overnight cultures of *P. aeruginosa* containing the marked insertions were electroporated with 50 ng-100 ng of *Flp*-recombinase-encoding pFLP2 and transformants were selected for on LB-agar supplemented with 200 μg/mL carbenicillin and screened for sensitivity to gentamicin by patching on LB agar supplemented with 30 μg/mL gentamicin and 200 μg/mL carbenicillin, respectively. pFLP2 plasmid was cured from patches displaying Gm^S, Cb^r phenotype. This was achieved by streaking cells on 10% sucrose plates and incubating overnight at 37 ^oC. Curing of the plasmid was confirmed by patching isolated colonies on LB agar supplemented with 200 μg/mL carbenicillin, 30 μg/mL gentamicin, and 10% sucrose, respectively. Insertion of the mini-Tn*7* plasmid in Gm^S, Cb^S, and $sucrose^r$ patches was confirmed once again by using PCR for mini-Tn*7* insertion as well as for the *aacC1* gene.

C. Quantitative-Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

i. RNA Extraction

Total RNA was extracted from cells using the RNeasy RNA isolation kit (Qiagen, Mississauga, ON, Canada). Briefly, cells were grown by subculturing an overnight culture 1:100 (v/v) into fresh LB broth. When required, cultures were induced at A_{600nm}~ 0.6 through the addition of 1.0 mM IPTG. At A_{600nm} ~0.8, 1.5 mL of cells were harvested by centrifugation at 17 000 x *g* at room temperature for 3 minutes. Cell pellets were frozen on dry ice and placed at -80 °C for 30 minutes to facilitate cell lysis. Cells were then thawed at room temperature and resuspended in a buffer (proprietary) containing 400 μg/mL lysozyme (Bioshop Canada Inc., Burlington, ON, Canada) and then transfered to a buffer containing guanidine-isothiocyanite (proprietary), which inactivates RNases. Total RNA was precipitated with 95-100% ethanol and the solution was applied to an RNeasy silica membrane. The membrane was washed with an ethanol containing wash

buffer (proprietary). RNA was eluted off of the column using RNase-free water. Quality and concentration of the sample were determined by spectrophotometry. RNA samples with concentrations no less than 400 ng/µl were used for further analysis.

ii. Complementary-DNA Synthesis

Contaminating genomic DNA was removed using the RNase-free DNase kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instruction. Briefly, 1 µg of total RNA was incubated with 1.0 unit of DNase for 40 minutes at 37 °C, DNase was heat inactivated at 70 °C for 5 minutes and 800 ng of RNA was reverse transcribed with the iScript Reverse Transcriptase (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. No reverse transcriptase (NRT) controls were included with every cDNA preparation to rule out genomic DNA contamination.

iii. Real-Time PCR

Real-Time PCR was performed in the CFX-96 Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) using Evagreen Sso-fast PCR supermix (BioRad Laboratories, Hercules, CA, USA). Cycling conditions were as follows: 95 °C 3 minutes; (95 °C 10 seconds; 60 °C 30 seconds) for 39 cycles. High-resolution melt conditions used were: 95 °C 10 seconds, 65 °C – 95 °C in 0.5 °C increments, 5 seconds/ °C. Primers used for detection of *oprM* (110 bp product), *mexK* (122 bp product), reference gene *rpsL* (242 bp product) and reference gene *proC* (106 bp product) were designed previously (Ganeshanantham, 2011). Efficiency of each primer set was determined by creating a standard curve using 10-fold dilutions of pooled cDNA samples. Primer sets with efficiencies below 95% or above 105% were omitted. No Reverse Transcriptase (NRT) controls for the housekeeping genes were included to rule out contamination by genomic DNA. A No Template Control (NTC) for each gene was also included. Expression of target genes under induced and uninduced conditions was assessed and normalized to a reference gene using the CFX Manager Software, Gene Expression Analysis tool (BioRad Laboratories, Hercules, CA, USA). The quality of the mRNA was assessed by ensuring the Ct-value remained constant for the housekeeping gene between runs. For gene expression analysis the ΔΔCt method was employed (Livak and Schmittgen, 2001).

D Protein Purification and Immunodetection

i. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS- PAGE was performed according to Laemmli (Laemmli, 1970), with some modifications. Briefly, 10 ng of protein (in a volume of 6 µL) was added to 2X SDS Sample Buffer (0.2% w/v bromophenol blue, 20% v/v glycerol, 0.6% v/v β-mercaptoethanol, 4% w/v SDS dissolved in a 62.5 mM Tris buffer pH 6.8) and incubated at 100 °C for 5 minutes, and centrifuged at 16 000 xg for 1 minute. The samples were electrophoresised using a 6% polyacrylamide stacking gel with a 12% polyacrylamide resolving gel. The stacking gel was composed of 2.18 mL mQH_2O, 0.38 mL 40% bis-acrylamide (6% final concentration), 0.38 mL 1.0 M Tris pH 6.8 (125 µM final concentration), 30 µl 10% SDS (0.1% final concentration), 30 µl 10% ammonium persulfate (0.1% final concentration) and 4 µl TEMED (0.1% final concentration). The resolving gel was prepared by combining 3.2 mL mQH_2O (milliQ water), 2.3 mL 40% bis-polyacrylamide (12% final concentration) (Bioshop, Canada Inc, Burlington, ON, Canada), 1.9 mL 1.5 M Tris pH 8.8 (0.38 M final concentration) (Bioshop, Canada Inc, Burlington, ON, Canada), 75 µl 10% SDS (0.1% final concentration) (Bioshop, Canada Inc, Burlington, ON, Canada), 75 µl 10% ammonium persulfate (0.1% final concentration) (Bioshop, Canada Inc, Burlington, ON, Canada) and 6 µl tetramethylethylenediamine (TEMED) (0.1% final concentration) (Bioshop, Canada Inc, Burlington, ON, Canada). 3 µl of pre-stained molecular weight standard (Bio-Rad, Mississauga, ON, Canada) was used. Gels were electrophoresised at 80 V for 30 mins followed by 120 V for 2.5 hrs in 25 mM Tris, 192 mM glycine, 0.1% SDS pH 8.3. Gels were then stained with either Coomassie Brilliant Blue (CBB) or silver stain as described below.

ii. Preparation of Whole Cell Lysate

Overnight cultures were subcultured 1:100 v/v, into a 4 mL volume of LB, and when required, induced by addition of 1.0 mM IPTG at an A_{600nm} of 0.4-0.5, and harvested at A_{600nm} 0.8-1.0. Cells were harvested by centrifugation at 17 000 xg for 3 min at room temperature and resuspended in 2X SDS Sample Buffer (SB) (Laemmli, 1970).

Cell number in each sample was standardized by adding 2X SB according to the formula: mL of 2x SB = 0.08x A_{600nm} of culture. Samples were boiled for 5 minutes, spun down, and 4 µl of each sample was resolved on a 10% SDS-PAGE.

iii. Extraction of Membrane Proteins

Membrane proteins were extracted as described by Cuenca and colleagues (Cuenca, *et al.*, 2003) with some adaptations. Briefly, cell pellets from 1 L *P. aeruginosa* cultures were harvested by centrifugation at 7440 x *g* for 10 mins at 4 °C and frozen at -20 ^{o}C overnight to facilitate lysis. Pellets were thawed for 30 minutes at room temperature and washed twice with 20% original culture volume (200 mL) phosphate buffer (800 mM K_2HPO_4, 20 mM KH_2PO_4, pH 7.2). Samples were spun at 4740 x *g* for 15 minutes at 4 °C. Pellets were resuspended in 20% v/v phosphate buffer and lysed using the French press (Thermo Scientific, Markham, ON, Canada) at 2000 psi on ice. Cell debris was removed by centrifugation, at 7440 x *g* for 10 min at 4 °C (repeated once). Membrane fractions were collected by centrifugation at 105 000 x *g* for 1 hr at 4 °C using the Sorvall® Discovery 100SE ultracentrifuge (Mandel Scientific, Guelph, ON, Canada). Pellets were solubilized in phosphate buffer containing 2% sodium lauryl sarcosinate and stored at -20 °C until use.

iv. Coomassie Brilliant Blue Staining

Gels were stained according to Meyer and Lambert (Meyer and Lambert, 1965). Briefly, the gels were placed in 0.05% CBB (MP Biomedicals LLC, Solon, OH, USA) dissolved in 50% methanol, 10% acetic acid, and incubated overnight, at room temperature, with shaking. Gels were destained for 1.5 hours in destaining solution (40% methanol, 50% water, 10% glacial acetic acid) prior to being photographed.

v. Immunodetection of Proteins

SDS-PAGE was performed as described above. Proteins were transfered (100 V for 2 hours) onto a nitrocellulose membrane (Thermo Scientific, Mississauga, ON, Canada) in pre-chilled transfer buffer (119 mM Tris, 4 M Glycine, 0.1% w/v SDS, 20% v/v methanol) containing an ice pack at room temperature. Membranes were recovered

and blocked overnight at 4 °C with shaking at 60 rpm, with either Phosphate Buffered Saline pH 7.2 containing 0.1% Tween 20 (PBST) and 10% skim milk (OprM) or PBST and 3% Bovine Serum Albumin (BSA) (MexK, OpmH) (Fisher Scientific, Markham, ON, Canada). Membranes were washed three times in 50 mL PBST for 5 mins. Membranes were then incubated in 1% w/v BSA, 20mL PBST and goat α-opmH (Chuanchuen, *et al.*, 2005) (1:10000 v/v), or rabbit α-OprM (1:10000 v/v) (a gift from Dr. Herbert Schweizer, Colorado State University, Fort Collins, CO, USA), or goat α-MexK (a gift from Dr. Herbert Schweizer, Colorado State University, Fort Collins, CO, USA) (1:20000 v/v) or rabbit α-His (Fisher Scientific, Markham, ON, Canada) (1:10000 v/v). Membranes were washed three times in 50 mL PBST for 5 mins. Membranes were incubated with secondary antibody for 1 hour with shaking at 60 rpm as follows: a 1:20000 v/v dilution of either 2 mg/mL horseradish peroxidase (HRP)-conjugated goat α-rabbit (Bioshop Canada Inc. Burlington, ON, Canada) or HRP- conjugated mouse α-goat (Thermo Scientific, Rockford, IL, USA) diluted in PBST + 3% w/v BSA. OpmH and MexK required α-rabbit while OprM required α-goat secondary antibodies. Membranes were washed three times in 50 mL PBST for 5 mins, and again for 5 min with PBS. Blots were developed by addition of 1:10 v/v diluted Pierce ECL detection reagent (Pierce Biotechnology, Rockford, IL, USA). X-ray film (CL-Xposure Film, Pierce Biotechnology, Rockford, IL, USA) was exposed to the blot for 30 secs up to 5 mins.

vi. Antibody Partial Purification using Ammonium Sulfate Precipitation

Ammonium sulfate precipitation was performed to remove debris from goat α-MexK IgG according to Harlow and Lane (Harlow and Lane, 1988). Briefly, 5 mL of antibody was centrifuged at 3000 xg for 30 min at 4 °C; supernatant was collected, stirred, and 0.5 X total volume of saturated ammonium sulfate was added drop-by-drop until a precipitate started to form. The solution was stored at 4 °C overnight to ensure a homogenous precipitation. The sample was centrifuged at 3000 xg for 30 min at 4 °C and the supernatant was collected in a clean beaker and stirred at room temperature. 0.5 X saturated ammonium sulfate was added drop-by-drop until a precipitate formed, and the solution was stored at 4 °C for approximately 6 hours. The precipitate was collected by centrifugation at 3000 xg for 30 min at 4 °C and the supernatant discarded. The pellet

was resuspended in 1X total of the original volume PBS and dialyzed using 12-14 kDa molecular weight cut off dialysis tubing at 4 °C overnight. Sample was removed from dialysis tubing and centrifuged at 3000 xg for 30 min at 4 °C. The supernatant was divided in 1 mL aliquots and kept at -20 °C until use.

E Domain Swapping Experiment

Previous studies using MexAB-OprM as a model had identified amino acids within OprM that when modified, changed the resistance profile and/or modified OprM production (Nehme and Poole, 2007). When an amino acid substitution was made in the form of T209A, both OprM production and resistance were changed (Nehme and Poole, 2007). However, when an amino acid substitution was made in the form of G216A or G424A only the resistance pattern was changed (Nehme and Poole, 2007). For these reasons, a range of amino acids from 192-204, 418-433 and 192-433 in OprM were replaced with corresponding domains from OpmH (192-206, 425-440, 192-440). A PCR-based mutagenesis protocol adapted from Elkins and Nikaido was used to construct the chimeras harbouring the swapped domain of OprM protein with those of OpmH (Figure 4) (Elkins and Nikaido, 2002). Briefly, primers were designed to amplify the nucleotide sequences encoding the target domain of OpmH. These primers contained 5'-overhangs with homology to the nucleotide sequence in *oprM* flanking the domain to be replaced. Gene construction software was used to ensure that the swapping of domains did not affect the reading frame of the *oprM* gene. Nucleotides encoding a domain in *opmH* were amplified using long primers that also contained sequences for the template *oprM.* The 3' ends of the primers were designed to bind to specific domains of *oprM,* whereas the 5' ends of the primers were designed to bind to the corresponding domains of *opmH*. The PCR product from first step was used as the primer in a second PCR reaction with the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Mississauga, ON, Canada). A plasmid containing the wild-type allele of *oprM* was used as a template ensuring that the entire plasmid was amplified with a precise replacement of the desired domain from OpmH (Figure 4). Cycle conditions were described by the manufacturer (denaturation at 95 °C for 2 mins, then 18 cycles of 95 °C for 20 sec, annealing at 60 °C for 10 secs, elongation at 68 °C for 30 sec/kb of plasmid length,

followed by a final extension at 68 °C for 5 mins). Following PCR reaction, the entire reaction mixture was treated with *DpnI* in order to remove the template plasmid, and 10 µl was transformed into *E. coli* DH5α competent cells to be screened by PCR for the replacement of domains.

F Antibiotic Susceptibility Testing

Antibiotic susceptibility assays were performed by three different methods as described below:

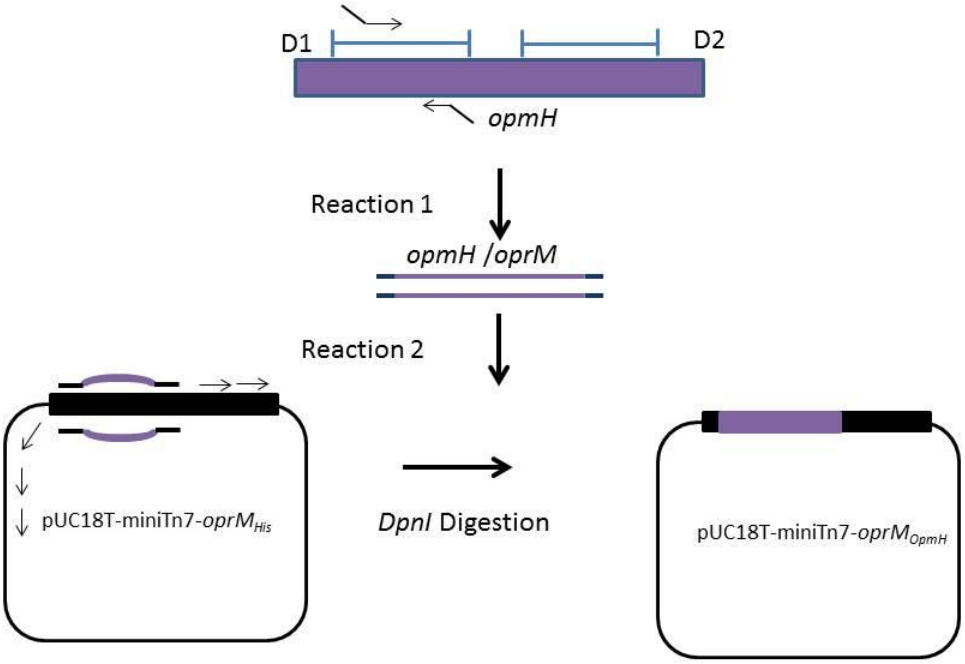
i. Agar Dilution Method

Overnight cultures were subcultured 1:100 v/v in a 3 mL volume of LB, grown to an A_{600nm}~0.5-0.6, standardized using a 0.5 McFarland standard in a 0.85% sodium chloride solution according to Clinical Laboratory Standards Institute (CLSI) guidelines (Clinical and Laboratory Standards Institute, 2011). Cells were then diluted 1:50 v/v in Mueller-Hinton Broth (MHB) and a 2 µl volume was spotted onto Mueller-Hinton Agar (MHA) plates in triplicate. Agar plates were made by adding antibiotic (erythromycin) (Biobasic Inc., Markham, ON, Canada), or antimicrobial (triclosan) (Biobasic Inc., Markham, ON, Canada) to a final concentration ranging from 1 µg/mL - 256 µg/mL in 2-fold serial increments. Stock solutions of erythromycin were prepared in ethanol while those of triclosan were prepared in methanol. MHA medium was supplemented with 1.0 mM IPTG to induce the expression of desired proteins. Plates were incubated at 37 °C for 18 hours. The MIC was determined to be the lowest concentration of antimicrobial that inhibited the growth of cells.

ii. Broth Dilution Method

Overnight cultures were standardized according to the *Agar Dilution Method* described above. Cells were added to the wells, induction of *oprM* expression was achieved by supplementing MHB with 1.0 mM IPTG. Antibiotic final concentration.

Figure 4. Schematic of protocol for swapping of OpmH domain into OprM. In reaction 1, amplification of *opmH* was accomplished using a pair of hybrid primers which produce *opmH* with small primer overhangs which are complimentary to *oprM*. This occurs because the primers contain complementary extensions (approximately 25 nt) to the intended start or end sites of *oprM*. In reaction 2, this product from reaction 1 was used to prime the second PCR reaction, thereby amplifying the entire pUC18T-mini-Tn7-$oprM_{His}$ plasmid containing the *opmH*/*oprM* chimera. The product from the second reaction was treated with *DpnI* to digest the methylated parental DNA. After digestion, the constructed plasmid was transformed into competent cells.


D1
D2
opmH
Reaction 1
opmH /oprM
Reaction 2
pUC18T-miniTn7-oprM$_{His}$
DpnI Digestion
pUC18T-miniTn7-oprM$_{OpmH}$

ranging from 1 μg/mL - 1024 μg/mL in 2-fold serial increments were prepared in 96-well plates (Sarstedt Canada, Montreal, QC, Canada) in triplicate for each condition tested ranging from 1 μg/mL - 1024 μg/mL in 2-fold serial increments were prepared in 96-well plates (Sarstedt Canada, Montreal, QC, Canada) in triplicate for each condition tested. Plates were incubated at 37 °C for 18 hours and MIC was determined to be the lowest concentration of antibiotic which was found to inhibit bacterial growth.

iii. Disc Diffusion Method

Overnight cultures were standardized in the same manner as for *Agar Dilution Method* described above. A sterile cotton swab was used to spread cells on an MHA plate for each condition tested (i.e. +/- 1.0 mM IPTG). Two discs of 15 μg/mL erythromycin were added to the plate at opposite ends. Plates were incubated at 37 °C for 18 hours and the zone of inhibition was measured around each of the discs and recorded.

G Virulence Assays

i. Biofilm Assay

Biofilm assays were performed according to O'Toole and Kolter (O'Toole and Kolter, 1998) with some modifications. Briefly, overnight cultures were subcultured 1:25 v/v in a 3 mL volume of MHB, grown to an A_{600nm}~0.5-0.6, and standardized in the same manner as for MICs described above. Cells were then diluted 1:100 v/v in MHB and a 100 μl volume was added in triplicate to a flat bottom 96-well plate. Plates were incubated at 37 °C for 24 hours without shaking. Medium was removed by inverting the plate and shaking gently, the plate was washed three times with distilled water. Crystal violet, 125 μl (0.5% [w/v]), was added to the wells and incubated at room temperature for 20 minutes. Crystal violet was washed out with distilled water three times and replaced with 150 μl of 95% ethanol. The ethanol incubated at room temperature for 20 minutes and was then transferred to a new plate. Absorbance was measured at A_{550nm}.

ii. N-Acyl Homoserine Lactone (AHL) Assay

AHL bioassays were performed using the method described by Anderson *et al.* 2001 with some modifications. Briefly, overnight cultures of *E. coli* MT102 harbouring pJBA132 and *P. aeruginosa* strain to be tested were standardized using a 0.5 McFarland standard in a 0.85% w/v sodium chloride solution according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2011). Five microliters of the standardized solution was streaked close to each other in the shape of a `T` on an LB plate ensuring that the cultures did not touch each other. Plates were incubated at 30 °C, and examined for green fluorescence after 48 hours on the Dark Reader Transilluminator (Clare Chemical, Dolores, CO, USA).

iii. Swimming and Swarming Assays

Swimming and swarming assays were carried out according to Rashid and Kornberg (Rashid and Kornberg, 2000). Briefly, overnight cultures were subcultured 1:100 v/v in a 3 mL volume of LB broth, grown to an A_{600nm} of 0.4-0.6 and standardized as above using a 0.5 McFarland standard. Standardized cells were spotted on LB agar plates containing 0.3% w/v agar (for swimming assay) and 0.5% w/v agar (for swarming assay). Plates were incubated for 48 hours and checked and photographed using a Canon Powershot SD750 every 24 hours.

H DNA Sequencing and Analysis

DNA sequencing was carried out at the Genome Quebec facility at McGill University, Montreal, QC. The sequence analysis was performed using Basic Local Alignment Search Tool (BLAST) and Gene Construction Kit Software (Textco Biosoftware).

I Modelling of Hypothetical Homology Models

Protein structure predictions were performed with Protein Homology/analog Y Recognition Engine v2.0 (Phyre2) (Kelley and Sternberg, 2009) available on the web (http://www.sbg.bio.ic.ac.uk/Phyre2/). Full amino acid sequences of (OpmH, 483 amino acid residues), ($OprM_{opmHH1}$, 461 amino acid residues), ($OprM_{opmHH3}$, 466 amino acid residues), ($OprM_{opmHH1:3}$, 464 amino acid residues), and ($OprM_{opmHH1-3}$, 474 amino acid

residues) were applied to PHYRE 2. All models were generated using the template of the crystal structure of OprM (at a 2.56 Å) (template 1wp1) with 100% confidence.

J Statistical Analyses

Data represents the mean ± standard deviation of at least two independent experiments unless otherwise stated. Statistical significance was determined using the student's t test or ANOVA as appropriate. $p < 0.05$ was considered statistically significant. All statistical analysis was performed using Microsoft Excel 2010 software.

III.

RESULTS

A. *Single-copy expression of OprM*

Expression of OprM in single-copy was achieved by the use of a mini-Tn*7*-based system. Insertion of the vector backbone and removal of the Gm-resistance cassette gene was confirmed by PCR (Figure 5a and 5b). Expression of OprM was confirmed by qRT-PCR (Figure 6b), SDS-PAGE and immunoblotting (Fig 7a and 7b) as described in later sections.

i. Quantitative Real-Time (qRT-PCR) Analysis of mRNA Expression

Expression of MexK was approximately 15-fold higher compared to wild-type in single-copy constructs, PA051 and PA058 (Figure 6a). PA051 and PA058 overexpress MexK, whereas PA01 does not express the MexJK pump, therefore a 15-fold increase in expression was expected.

qRT-PCR analysis of OprM demonstrated an approximately 30-fold overexpression of *oprM-H6* mRNA in PA058 strains that were induced compared to uninduced cells (Figure 6b) which correlated to an increase in protein concentration (Figure 7b). This demonstrates that the OprM expression mutant was functional, and capable of increasing mRNA levels under inducing conditions.

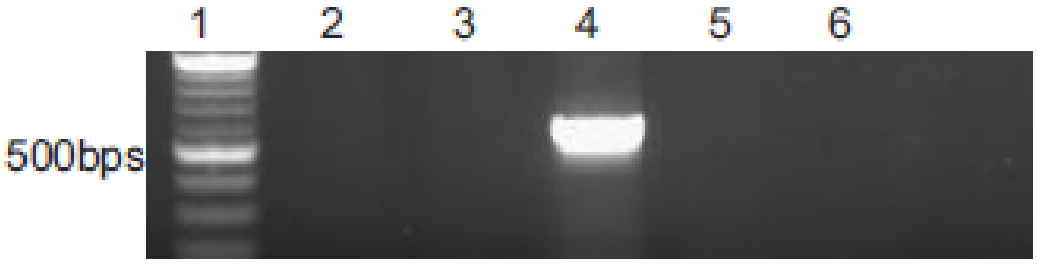
These results taken together, demonstrate an ideal system to examine OprM within the MexJK-OprM complex because PA058 constitutively overexpresses the MexJK proteins, and OprM expression can be controlled through induction.

ii. Protein Expression Analysis

In order to verify the qRT-PCR data, and to demonstrate that increases in transcription led to increases in translated protein, SDS-PAGE and immunoblotting were performed after membrane protein preparation on the two outer membrane proteins OprM, and OpmH along with the RND transporter, MexK. Upon induction of OprM from *P. aeruginosa* single-copy construct PA058 and a membrane protein preparation, a faint band of approximately 50 kDa, which is the expected molecular weight of OprM could be visualized by SDS-PAGE with CBB (Figure 7a). In order to confirm these

Figure 5. Verification of *P. aeruginosa* PA058 by PCR. (a) Removal of Gm resistance cassette. 1. 100 bp ladder; 2. PA058 (Isolate 1); 3. PA058 (Isolate 2); 4. PA052; 5. PA054 6. No Template Control (NTC) **(b) Confirmation of the presence of mini-Tn7 vector after removal of Gm cassette.** 1. 100 bp ladder; 2. PA058 (Isolate 1); 3. PA058 (Isolate 2); 4. PA054; 5. PA015; 6. NTC. PCR was carried out using primers GmFRT-UP and GmFRT-Dn in addition to Tn7R_Fwd and Pa_glmS_Rev (listed in Table 3) for confirmation of removal of Gm cassette, yielding a 548 bp product and for confirmation of mini-Tn*7* insertion, yielding a 292 bp product.

(a)



(b)

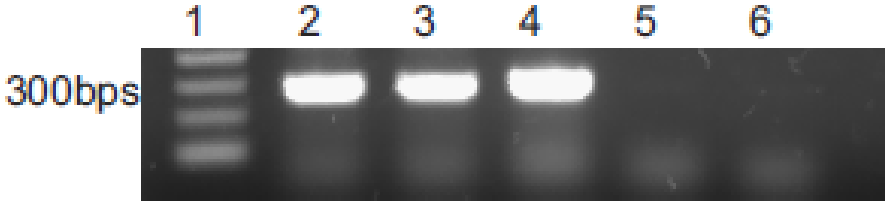
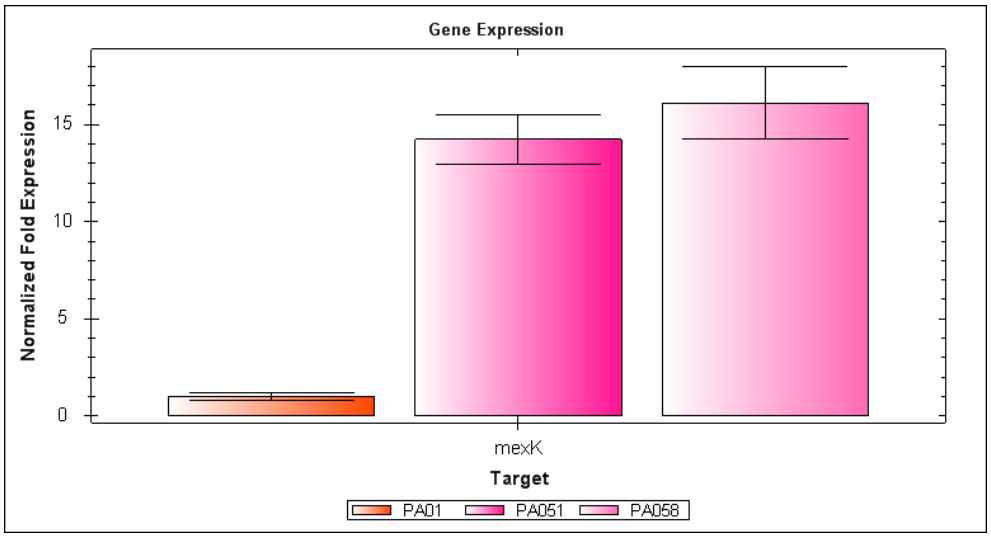
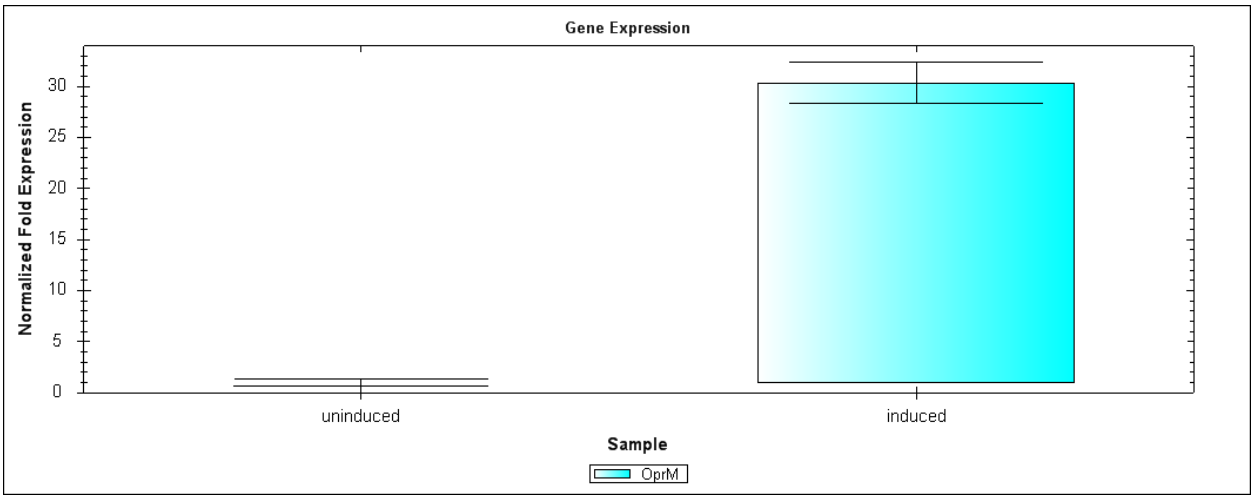


Figure 6. Quantitative-Real Time Polymerase Chain Reaction Analysis of mRNA Expression. Total RNA was extracted from induced and uninduced mid-log phase *P. aeruginosa* strains. (a) **MexK.** Normalized expression (using *proC* as housekeeping gene) analysis shows ~15-fold overexpression of MexK in PAO51 ($OpmH^+$) and PAO58 ($OprM^+$) when compared to the wild-type PAO1. (b) **OprM** (in PA058). Normalized expression (using *rpsL* as housekeeping gene) analysis shows a ~30-fold overexpression under inducing conditions (1.0 mM IPTG) compared to uninduced cells. Data shown is representative of at least 2 biological replicates.

(a)


Gene Expression
Normalized Fold Expression
15
10
5
0
mexK
Target
PA01
PA051
PA058

(b)


Gene Expression
Normalized Fold Expression
30
25
20
15
10
5
0
uninduced
induced
Sample
OprM

findings, further probing with rabbit α-OprM IgG antibody confirmed the presence of OprM (Figure 7b).

Upon induction of OpmH from *P. aeruginosa* single-copy construct PA051 and membrane protein preparation, a faint band of the expected molecular weight (also 50 kDa) could be visualized for OpmH. A more prominent band was observed after induction from a multi-copy system when subjected to SDS-PAGE and CBB (Figure 8a). In order to confirm the presence of OpmH, immunoblotting with Goat α-OpmH IgG antibody was performed. Although some non-specific protein binding was observed, a faint band of the expected 50 kDa molecular weight was produced by PA051, and a more prominent band of the same molecular weight was produced from the multi-copy system (Figure 8b). Taken together this suggests the 50 kDa protein band was indeed OpmH.

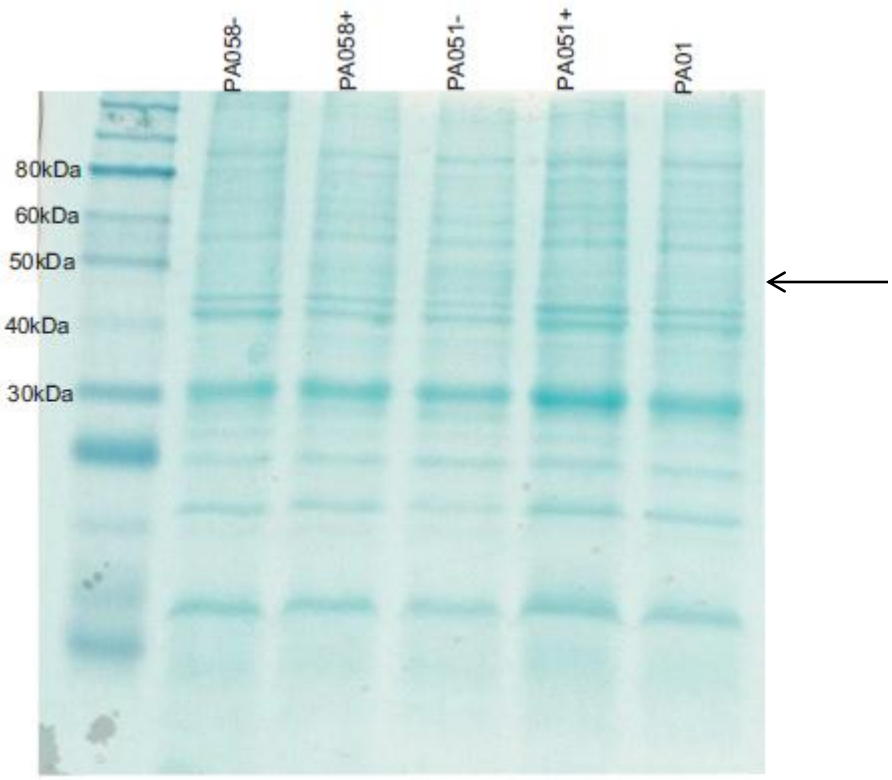
Both *P. aeruginosa* constructs PA051 and PA058 constitutively overexpress MexK from a single-copy plasmid under the control of the *mexL* repressor. MexK expression and membrane protein preparation resulted in a faint band at the expected molecular weight of 117 kDa after SDS-PAGE and CBB (Figure 9a). Although some difficulties were encountered, including non-specific binding, and apparent low affinity for MexK when probed with Goat α-MexK IgG antibody a very faint band could be observed at the approximate molecular weight of 120 kDa (Figure 9b) after antibody clean up protocols were performed.

iii. Antibiotic Susceptibility Profile

The phenotypic verification of the constructs was analyzed through antibiotic susceptibility assays where the MIC for each strain was determined by either broth dilution (erythromycin) or agar dilution (triclosan) according to the CLSI guidelines. The MexJK-OprM complex has been shown to efflux erythromycin (Chuanchuen, *et al.,* 2001); therefore resistance to this antibiotic was used as an indicator of a functional complex being formed. In contrast, the MexJK-OpmH complex specifically effluxes triclosan (Chuanchuen, *et al.,* 2001), therefore, resistance to triclosan was used as an indicator of a functional MexJK-OpmH complex being formed.

Figure 7. Protein Expression Analysis from Membrane Protein Preparation Demonstrating Protein Expression of OprM. Membrane protein analysis from *P. aeruginosa* PA0702 cells containing mini-Tn7-*oprM* (PA058) or mini-Tn7-*opmH* (PA051) were grown to A_{600nm} of 0.6, induced (1.0 mM IPTG) and harvested at A_{600nm} of 1.0 as described in the Materials and Methods.**(a) SDS-PAGE.** Membrane proteins were isolated as described in the Materials and Methods and subjected to denaturing 12% SDS-PAGE and stained with CBB. Membrane protein from PA051 -/+ 1.0 mM IPTG was included as a negative control and membrane protein from PA01 (Wild type expression of OprM) was included as a positive control. Arrow indicates the expected migration position for OprM. **(b) Immunoblot.** Immunoblot was probed with a 1:10000 dilution of polyclonal rabbit α-OprM antibody, and subsequently probed with a 1:20000 dilution of (HRP)-conjugated goat α-rabbit antibody.

(a)


PA058-
PA058+
PA051-
PA051+
PA01
80kDa
60kDa
50kDa
40kDa
30kDa

(b)

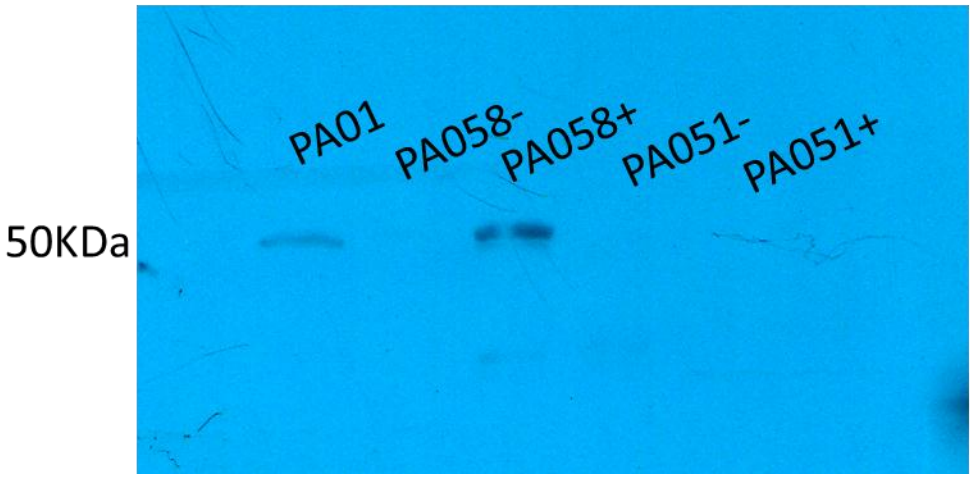
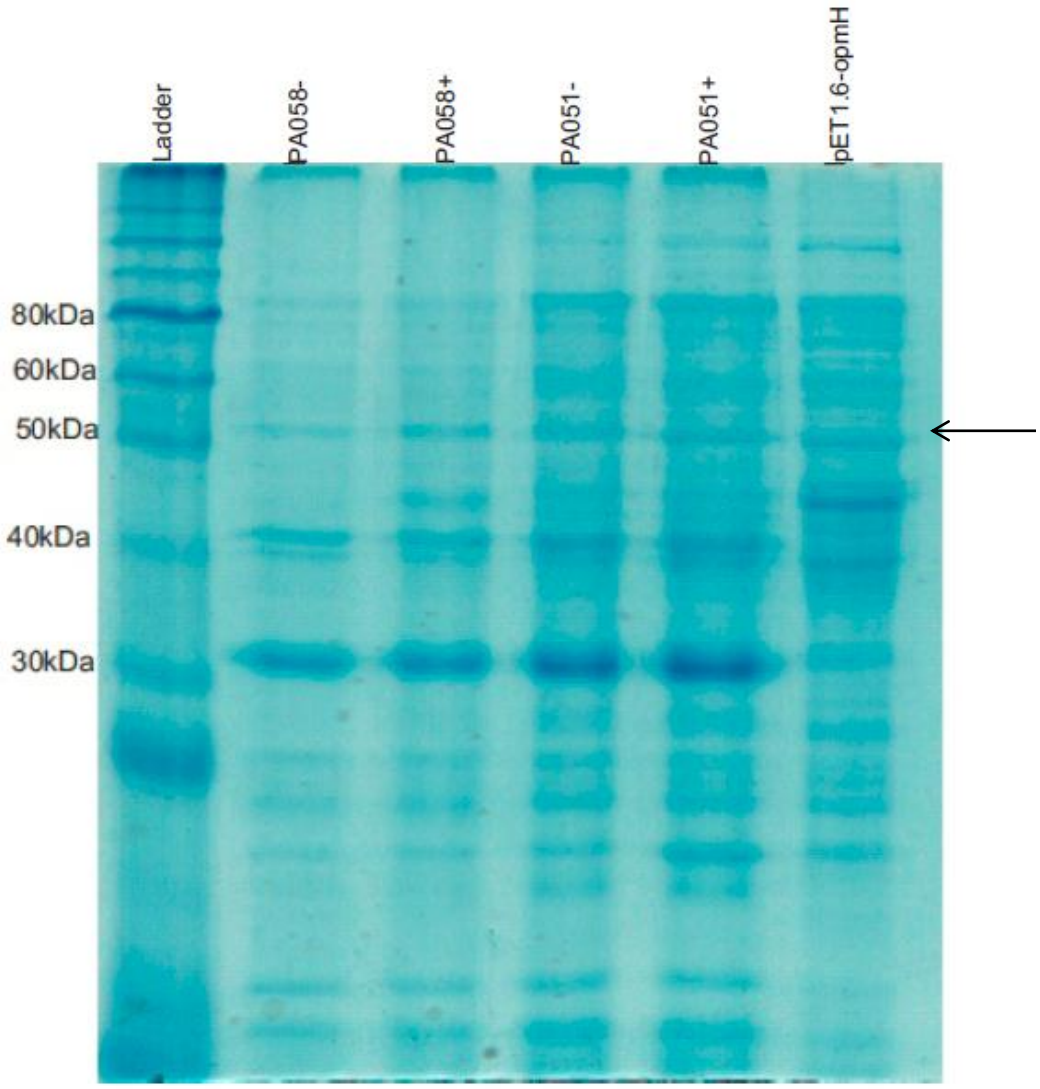
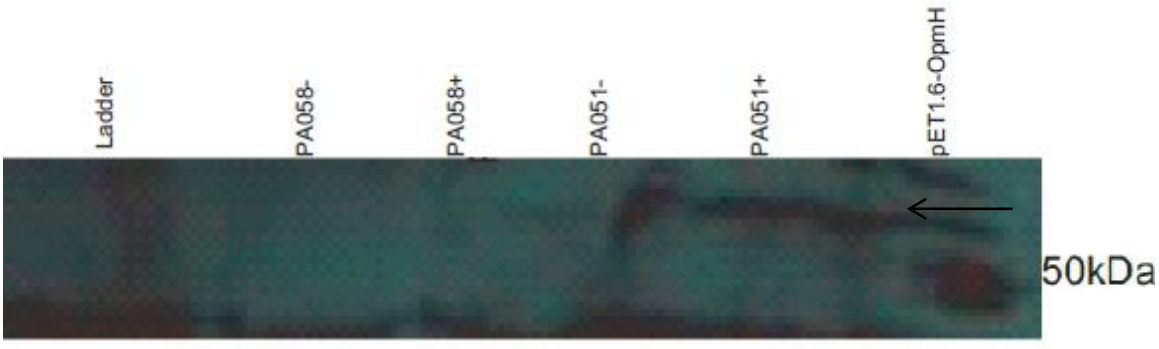

50KDa
PA01
PA058-
PA058+
PA051-
PA051+

Figure 8. Protein Expression Analysis from Membrane Protein Preparation Demonstrating Protein Expression of OpmH. Membrane protein analysis from *P. aeruginosa* PA0702 cells containing mini-Tn7-*oprM* (PA058) or mini-Tn7-*opmH* (PA051) were grown to A_{600nm} of 0.6, induced (1.0 mM IPTG) and harvested at A_{600nm} of 1.0 as described in the Materials and Methods.**(a) SDS-PAGE.** Membrane proteins were isolated as described in the Materials and Methods and subjected to denaturing 12% SDS-PAGE and stained with CBB. Membrane proteins from PA058 -/+ 1.0 mM IPTG were included as a negative control and whole cell lysate from pET1.6-*opmH* (multi-copy overexpression of OpmH) was included as a positive control. Arrow indicates the expected migration position for OpmH. **(b) Immunoblot.** Immunoblot were probed with a 1:10000 dilution of polyclonal Goat α-OpmH antibody, and subsequently probed with a 1:20000 dilution of (HRP)-conjugated mouse α-goat antibody.

(a)


Ladder
PA058-
PA058+
PA051-
PA051+
pET1.6-opmH
80kDa
60kDa
50kDa
40kDa
30kDa

(b)


Ladder
PA058-
PA058+
PA051-
PA051+
pET1.6-OpmH
50kDa

P. aeruginosa PA058 (expressing OprM) displayed resistance upon induction of OprM to erythromycin (Table 5) with a 4-fold change in MIC when compared to uninduced cells, which behaved similar to the control strain, PA050, which lacks both OprM and OpmH but expresses the MexJK proteins. This resistance was not observed in *P. aeruginosa* strain PA051 (expressing OpmH), indicating that the increase in resistance to erythromycin is due to the expression of the *oprM* gene specifically.

Conversely, *P. aeruginosa* PA051 cells displayed increased resistance to triclosan upon induction (Table 5) with an 8-fold change in MIC when compared to uniduced cells which behaved similiar to the control strain PA050. This enhanced resistance to triclosan was not observed in *P. aeruginosa* strain PA058 (expressing OprM), indicating that the increase in resistance to triclosan is due to the expression of the *opmH* gene specifically.

Taken together, this data shows that the MexJK pump forms functional complexes with OprM and OpmH proteins which is critical to the analysis of pump function *in vivo*.

B. Virulence Assays

RND efflux systems are notorious for having broad substrate specificities, and although antimicrobial efflux is the most prominent reason for studying RND efflux systems, some evidence has begun to accumulate which suggests that efflux pumps have important roles in a virulence capacity during infection, perhaps by effluxing various virulence factors. Indeed, other RND efflux systems such as the MexGHI-OpmD complex from *P. aeruginosa* have been shown to be critical for growth, antibiotic susceptibility and virulence by promoting cell-to-cell communications (Aendekerk, *et al.*, 2005). In order to assess the role MexJK-OpmH/OprM plays in virulence, a variety of assays were performed using various RND efflux mutants.

i. Biofilm Formation

All strains tested appeared to be capable of producing biofilm. PA050, which is deficient in an outer membrane protein for MexJK was capable of biofilm production, demonstrating that an intact RND pump with its outer membrane protein is not absolutely required for biofilm production. The MexJK-OpmH strain, PA051, showed a significant

1.7-fold (p=0.009) increase in biofilm production compared to PA050 in the absence of IPTG or a significant 1.4-fold (p=0.0009) increase in the presence of IPTG compared to PA050 (Figure 10a), suggesting that the OpmH protein may play a role in biofilm production, we hypothesize through cell-to-cell signalling. The MexJK-OprM strain, PA058, produced an even more dramatic 2.6-fold (p=0.008) increase in biofilm production compared to PA050 in the absence of IPTG, and a significant 2.2-fold ($p<0.0001$) increase in the presence of IPTG (Figure 10a). Additionally, PA058 showed a significant 1.6-fold (p=0.05) increase in biofilm compared to PA051 in the absence of IPTG, and a significant 1.5-fold (p=0.0004) increase in the presence of IPTG (Figure 10a). This demonstrates that the MexJK-OprM strain PA058 produced the most biofilm regardless of induction, and that the MexJK only strain PA050 produced the least amount of biofilm. However, there were no significant differences between uninduced or induced growth conditions amongst the 3 strains, underlying reasons for which were not investigated in this work.

Given that it is suspected that the interaction of MexJK pump with OprM or OpmH is substrate-driven, we decided to further test each strain's ability to form biofilm in the presence of erythromycin. Erythromycin is a common antibiotic used to treat Gram-positive lung infection. Many Gram-positive lung infections tend to be polymicrobial, meaning that they often contain Gram-negative bacteria as well, we wanted to test the effect that treating these infections would have on *P. aeruginosa* biofilm formation as *P.* aeruginosa is not only commonly found in the lung, but also readily forms biofilm in the lung. Curiously, all strains tested showed the general trend of increasing biofilm production as the concentration of erythromycin increased (Figure 10b-d), although the concentrations of erythromycin used here were no less than 1/8 the MIC depending on the strain, and were increased up to the MIC concentration in some strains.

The MexJK strain which lacks an outer membrane protein, PA050, generated significantly more biofilm in the presence of erythromycin at all concentrations independent of induction (Figure 10b). Unexpectedly, there was a significant difference in biofilm production between induced and uninduced cultures at 2 µg/ml erythromycin.

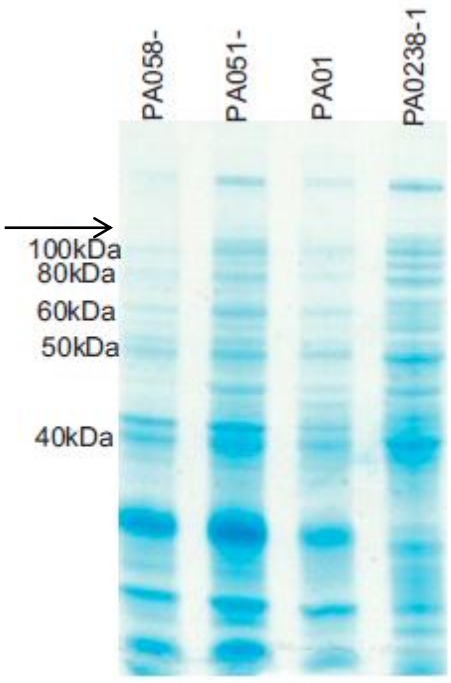
Further replicates are needed to confirm this result, as no significant differences in biofilm production between induced and uninduced cultures was observed at 0, 1, 4 and 6 µg/ml, which is expected because no proteins are under induction control, suggesting the change at 2 µg/ml may be artifactual.

PA051(OpmH expressing) also demonstrated an increase in biofilm production in a dose dependent fashion as concentrations of erythromycin increased, with the exception of 6 µg/ml in uninduced cells, which actually produced significantly less biofilm (Figure 10c). Unlike PA050, PA051 increases expression of OpmH upon induction, and curiously induction of OpmH led to a significant 1.7-fold ($p=0.002$), and 1.8-fold ($p=0.01$) decrease in biofilm production at 2 and 4 µg/ml erythromycin respectively. This data suggests that OpmH expression decreases biofilm production. The decrease in biofilm production could be caused by MexJK-OpmH efflux of quorum sensing molecules which have been shown to accumulate during biofilm production (de Kievit, 2009), as the antibiotic itself is not compatible with OpmH mediated efflux. At 6 µg/ml erythromycin, this trend reversed and the induced cultures produced a significant 8.1-fold ($p=0.0005$) increase in biofilm, which was hypothesized to be a result of growth inhibition in the uninduced culture. Taken together, this data supports a role for OpmH in biofilm production when *P. aeruginosa* is grown in the presence of erythromycin. Although mechanistic studies were not performed, it is hypothesized that OpmH is contributing to cell-to-cell communication, as erythromycin is not a substrate for MexJK-OpmH efflux.

In the absence of erythromycin, PA058 (OprM expressing) demonstrated significantly higher levels of biofilm production compared to the other strains which were tested. In the presence of erythromycin, PA058 behaved similarly to both PA050 and PA051, showing dose dependant increases in biofilm production as erythromycin concentration increased, however, unlike PA051, no significant differences were detected upon induction (Figure 10d). PA058 was capable of producing more biofilm in the absence of erythromycin than both PA050 and PA051, this trend was also maintained in the presence of erythromycin.

Figure 9. Protein Expression Analysis from Membrane Protein Preparation Demonstrating Protein Expression of MexK. Membrane protein analysis from *P. aeruginosa* PA0702 cells containing MexJK were grown to A_{600nm} of 1.0 as described in the Materials and Methods. **(a) SDS-PAGE.** Membrane proteins were isolated as described in the Materials and Methods and subjected to denaturing 12% SDS-PAGE and stained with CBB. Membrane proteins from PA01 (which does not normally express MexJK) was included as a negative control and whole cell lysate from PA0238-1 (Wild type expression of MexJK) was included as a positive control. Arrow indicates the expected migration position for MexK. **(b)Immunoblot.** Immunoblot was probed with a 1:20000 dilution of polyclonal rabbit α-MexK antibody, and subsequently probed with a 1:20000 dilution of (HRP)-conjugated mouse α-goat antibody.

(a)


PA058-
PA051-
PA01
PA0238-1
100kDa
80kDa
60kDa
50kDa
40kDa

(b)

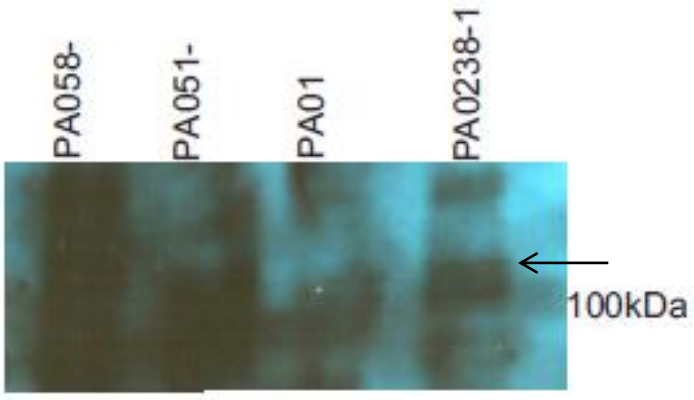
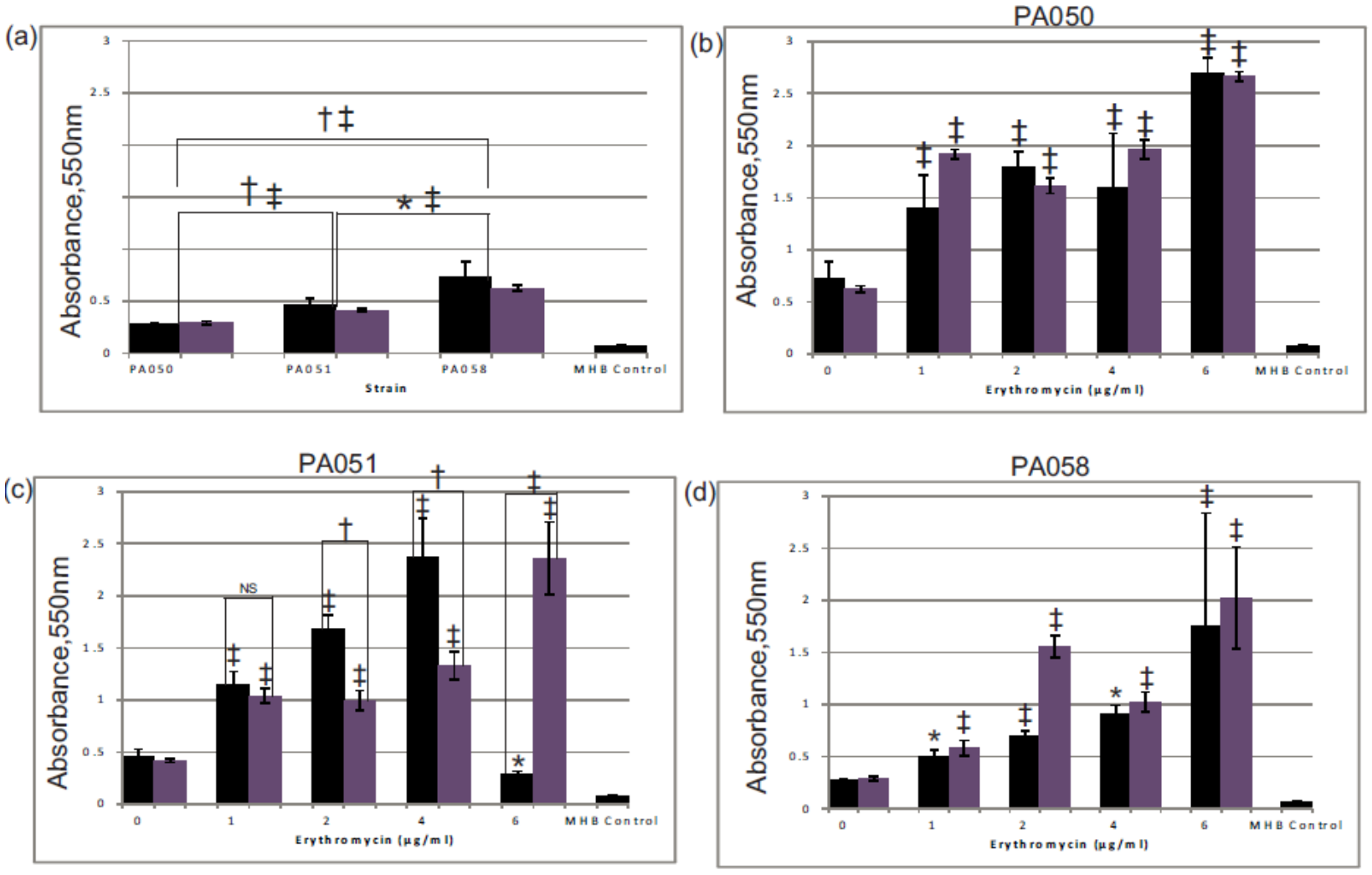

PA058-
PA051-
PA01
PA0238-1
100kDa

Table 5. Antibiotic Susceptibility Profile. Minimum inhibitory concentrations for Triclosan and Erythromycin, substrates for MexJK-OpmH (PA051) and MexJK-OprM (PA058) respectively were determined according to the CLSI standards as described in the Materials and Methods. MIC values represent the lowest concentration of antibiotic or compound required to inhibit growth of bacteria. The agar dilution method was used to determine triclosan MIC**.** The broth dilution method was used to determine the erythromycin MIC. Data shown is representative of a minimum of two biological replicates.

Strain	**Efflux Components Expressed**	**Triclosan (µg/mL)**			**Erythromycin (µg/mL)**		
		-IPTG	+IPTG	Fold Change	-IPTG	+IPTG	Fold Change
PA050	MexJ, MexK	2	2	No Change	4	4	No Change
PA051	MexJ, MexK, OpmH	4	32	8-fold	4	4	No Change
PA058	MexJ, MexK, OprM	4	4	No Change	2	8	4-fold

Figure 10. The Effect of MexJK-OpmH/OprM on Biofilm Formation in *P. aeruginosa* Constructs Containing Different Outer Membrane Components (a) Biofilm formation of *P. aeruginosa* isolates PA050 (MexJK^{+} OprM^{-} / OpmH^{-}), PA051 (MexJK-OpmH), and PA058 (MexJK-OprM) were determined +/- induction with 1.0 mM IPTG as discussed in the Materials and Methods. In the presence of IPTG, data is represented as mean±standard deviation, p=0.05 for IPTG- and p=0.0004 for IPTG+. Biofilm was assessed in the same fashion as (a) however, various concentrations of erythromycin during biofilm formation were also assessed. Erythromycin concentrations of 1.0, 2.0, 4.0, and 6.0 μg/ml were used for strains PA050 (b), PA051 (c), and PA058 (d), which have no outer membrane RND protein, inducible OpmH, and inducible OprM respectively. Data shown is representative of a minimum of two biological replicates. Bars represent the mean ± standard deviation, and statistical analysis was performed using the student T-test, with $p \leq 0.05$ being the cut off for significance.


(a)
Absorbance,550nm
PA050
PA051
PA058
MHB Control
Strain
(b)
PA050
Absorbance,550nm
Erythromycin (µg/ml)
(c)
PA051
NS
Absorbance,550nm
Erythromycin (µg/ml)
(d)
PA058
Absorbance,550nm
Erythromycin (µg/ml)
IPTG -
IPTG +
* p≤ 0.05
† p≤ 0.01
‡ p ≤ 0.001
NS not significant

While the introduction of *oprM* in PA050 resulted in an increase in biofilm, no significant difference was observed upon induction of the *oprM* gene compared to when it was not induced. It is not clear why we did not observe a further increase in biofilm formation upon the induction of *oprM* when the mere presence of this gene causes an increased biofilm formation and further investigation is necessary to study the reasons thereof.

P. aeruginosa is notorious for biofilm production, which is important during infection, and in some environmental systems. The data reported here demonstrates that the outer membrane RND protein in complex with MexJK is important for biofilm production. The strain containing OprM was capable of generating more biofilm than the OpmH containing strain, which produced more biofilm than the strain lacking both. Curiously though, OprM induction did not significantly affect biofilm production in the presence of erythromycin, whereas, OpmH induction led to a decrease in biofilm production in the presence of erythromycin, suggesting a possible role in cell-to-cell communications. Further investigation is required to completely assess the role of the MexJK outer membrane protein in biofilm production.

The outer membrane component of MexJK-OprM/OpmH had a strong effect on *P. aeruginosa* biofilm production, which was amplified in the presence of erythromycin. These observations led us to examine the effects of erythromycin on the production of biofilm with a variety of strains containing deletions of various RND efflux components (Figure 11). Wild-type *P. aeruginosa* PA01 demonstrated a significantly decreased capacity to produce biofilm in the presence of 6 µg/ml erythromycin when compared to biofilm production in the absence of erythromycin (Figure 11) which was expected based on the observations of others (Kondoh and Hashiba, 1998). All of the deletion strains tested showed significantly enhanced biofilm formation in the presence of erythromycin. In the absence of erythromycin, none of these strains generated significantly different biofilm when compared to each other.

Taken together, this data demonstrates that the RND efflux pumps have important roles when *P. aeruginosa* encounters antibiotics, particularly erythromycin. RND efflux pumps are not only capable of enhancing survival through efflux of antibiotic molecules, but may also play a role in mediating biofilm formation during erythromycin treatment.

Wild-type strains reduce the amount of biofilm production in the presence of erythromycin. However, strains deficient in one or more component of the RND efflux complexes typically generated more biofilm in the presence of erythromycin, possibly implicating a complex compensation mechanism, as biofilm has been shown to enhance antibiotic resistance (Hoiby, *et al.*, 2011). Biofilm production has been linked to quorum sensing, and it is possible that when the efflux pumps are absent, erythromycin accumulates within the cell, enhancing signaling molecule production. These signaling molecules cannot be secreted due to various deletions in the efflux components, and therefore they positively regulate biofilm production within the cell.

ii. Bioassay for AHL Secretion

RND efflux pumps have been associated with cell-to-cell communications, through their ability to efflux quorum sensing molecules (Alvarex-Ortega, *et al.*, 2013). Here, we demonstrate that multiple strains with differing efflux pump compositions were capable of effluxing AHLs. Various *P. aeruginosa* strains were grown next to *E. coli* MT102 which harbours the plasmid pJBA132, an AHL reporter plasmid which expresses GFP when exposed to AHL. The presence of increased fluorescence indicates increased secretion of AHL by the *P. aeruginosa* strain. Strains PA01, PA050, PA051, PA058, PA0702 and PA01172 all demonstrated secretion of the AHL, which was apparent by the green fluorescence at the tip of the reporter *E. coli* streak (Figures 12a-d, g, and h). PA0200 and PA0238 were not capable of inducing GFP expression in the *E. coli* reporter (Figure 12e and f) indicating they did not secrete AHL into the medium. This result remains somewhat convoluted, as PA0200 and PA0238 have deletions in MexAB-OprM (PA0200), or MexAB-OprM and MexCD-OprJ (PA0238). The common factor between these strains is the deletion in MexAB-OprM, however, MexAB-OprM is deleted in all other strains tested except PA01, suggesting some other causative agent is responsible.

Figure 11. Erythromycin Affects Biofilm Formation in *P. aeruginosa* Isolates. The effect of erythromycin on biofilm formation using 6.0 μg/mL was measured on *P. aeruginosa* strains harbouring RND pump knockouts. Data shown is representative of a minimum of two biological replicates. Bars represent the mean ± standard deviation, and statistical analysis was performed using the student T-test, with p=0.05 being the cut off for significance.

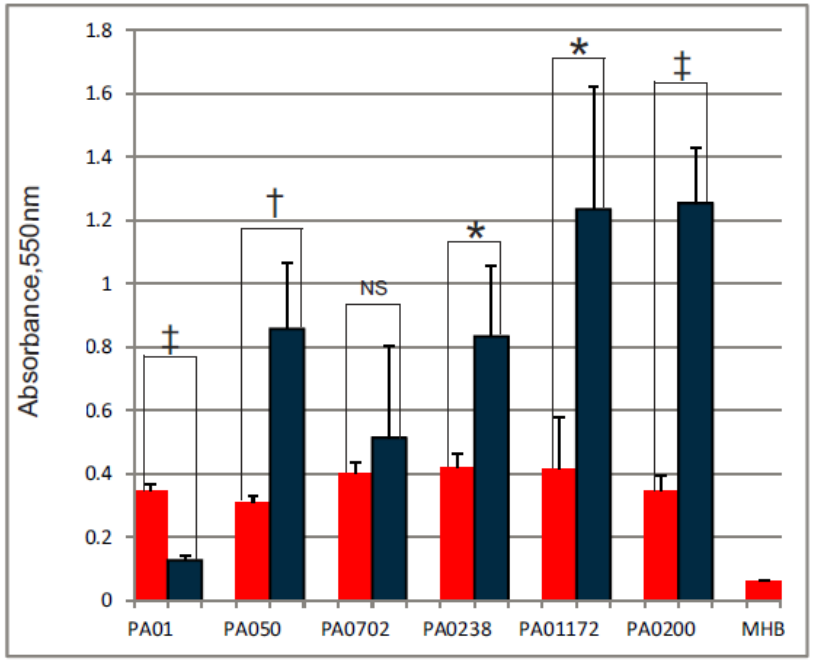
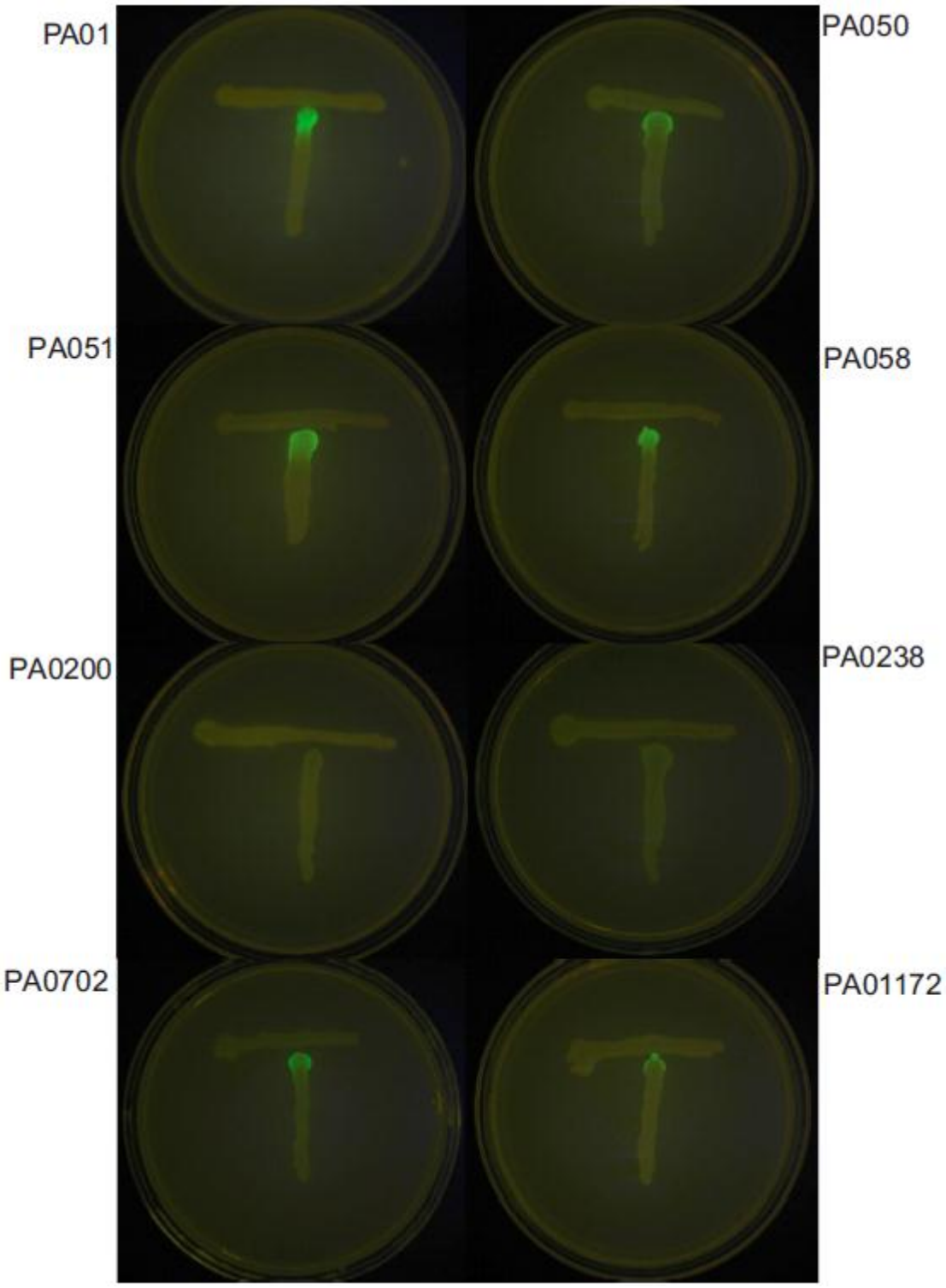

Absorbance,550nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PA01
PA050
PA0702
PA0238
PA01172
PA0200
MHB
‡
†
NS
*
*
‡
Erythromycin -
Erythromycin+
* p≤ 0.05
† p≤ 0.01
‡ p ≤ 0.001
NS not significant

Figure 12. Bioassay for AHL Secretion. *E. coli* strain harbouring the reporter plasmid pJBA132 which produces GFP if exposed to AHL were streaked vertically. *P. aeruginosa* strains PA01, PA050, PA051, PA058, PA0200, PA0238, PA0702, and PA01172 were streaked horizontally and green fluorescence at the junction between the sample and the reporter was monitored with a blue light transilluminator**.** Data shown is representative of a minimum of two biological replicates.


PA01
PA050
PA051
PA058
PA0200
PA0238
PA0702
PA01172

iii. Swimming and Swarming Motility

Swimming motility was analyzed with and without the addition of 6 µg/ml erythromycin. In *P. aeruginosa* a single, polar flagellum is thought to drive swimming motility (Murray and Kazmierczak, 2006) independently of cell-to-cell communications (O'May and Tufenkji, 2011). All strains tested demonstrated swimming motility in the presence or absence of 6 µg/ml erythromycin (Figure 13). In the absence of erythromycin wild-type PA01 demonstrated limited controlled swimming in a characteristic circular pattern. However, when swimming was assessed in the presence of 6 µg/ml erythromycin PA01 demonstrated enhanced motility, and sporadic growth reaching the perimeter of the plate. PA050 (lacking OpmH, MexAB-OprM, MexXY, MexCD-OprJ, with mini-Tn7-lac), PA0702 (lacking OpmH, MexAB-OprM, MexXY, MexCD-OprJ), and PA01172 (lacking MexAB-OprM, MexCD-OprJ, MexJK, MexEF-OprN, MexXY, OpmH, and TriABC) swimming motility was relatively unaffected when compared to PA01. Noteably, PA050, showed unorganized outgrowth in the absence of erythromycin when compared to samples grown in the presence of erythromycin. Also a green pigment, which is assumed to be pyocyanin, a toxin and quorum sensing molecule (Gunaratnam, *et al.*, 2011), appeared to be enriched in the presence of erythromycin, particularly in PA01172, which may be a stress response to erythromycin. Quantification of pyocyanin production would be required to support this hypothesis.

Swarming motility was also analyzed with and without the addition of 6 µg/ml erythromycin. In contrast to swimming motility, swarming motility in *P. aeruginosa* is a community behavior, requiring quorum sensing, which allows the bacterium to effectively work together to promote organized flagellum-dependent motility on surfaces (Murray and Kazmierczak, 2006). Unlike swimming, which uses a single polar flagellum for motility, swarming is associated with multiple, lateral flagella (Bardy, *et al.*, 2000) in the majority of bacterium. In *P. aeruginosa* swarming is accomplished through twitching motility, usually by two polar flagella (Kohler, *et al.*, 2000), however, type IV pili have also been shown to contribute (Mattick, 2002). In the absence of erythromycin all strains demonstrated out-growth, however, this growth did not appear organized, and resembled swimming (Figure 14). Curiously, wild-type PA01 did not appear to swarm, however, PA050 (lacking OpmH, MexAB-OprM, MexXY, MexCD-OprJ, with mini-Tn7 lac),

PA0702 (lacking OpmH, MexAB-OprM, MexXY, MexCD-OprJ), and PA01172 (lacking MexAB-OprM, MexCD-OprJ, MexJK, MexEF-OprN, MexXY, OpmH, and TriABC) demonstrated characteristic dendritic fractal-like growth indicating effective, organized, quorum sensing was taking place. This was expected because the RND efflux pumps are associated with efflux of quorum sensing molecules which limits their effectiveness. Although further experimentation is required, it appears that MexJK-OprM/OpmH may have an important role in quorum sensing molecule efflux, as all strains which lack a functioning MexJK-OprM/OpmH complex showed increased swarming motility which suggests an increase in quorum sensing, under antibiotic stress with 6 µg/ml erythromycin.

C. Domain Swapping Experiment

Triclosan, a commonly used biocide in household products, is quickly becoming ineffective against several strains of *P. aeruginosa* in part because of this bacterium's ability to actively efflux the molecule. Triclosan inhibits a highly conserved enzyme enoyl-ACP reductase (FabI) which is involved in bacterial fatty-acid biosynthesis (Heath and Rock, 2000). Triclosan is becoming ubiquitous in the environment, and is of particular concern in wastewater as the rate of species resistant to this biocide continues to grow. MexJK-OpmH is capable of triclosan efflux and we sought to identify the domains within OpmH that are responsible for triclosan efflux, and therefore resistance.

i. Creation of the chimeras:

Creation of the chimeras was carried out by performing a PCR-based domain swapping protocol (Elkins and Nikaido, 2002) followed by inserting the His-tagged genes into the chromosome of *P. aeruginosa* PAO702 cells, as described in the Materials and Methods. PCR was performed in order to confirm the successful swap of the domains. For reference, primer binding sites are highlighted in Figure 15a. PCR products for the primers were run out on a 0.8% agarose gel in Figure 15b confirming that domain 1 and domain 2, which correlates to helix 1 and 3, respectively, were inserted (also confirmed by sequencing; see Figure 16). A unique set of primers was used to confirm insertion of the

Figure 13. The Effect of Erythromycin on Swimming Motility of Various *P. aeruginosa* Strains with Deletions in their RND Efflux Components. Swimming zones were measured after 48 hours on 0.3% LB agar plates with or without 6 µg/ml erythromycin as discussed in the Materials and Methods. **PA01**, **PA050**, **PA0702**, **PA01172**, **PA01 with erythromycin**, **PA050 with erythromycin**, **PA0702 with erythromycin**, and **PA01172 with erythromycin** were analyzed. Data shown is representative of a minimum of two biological replicates.

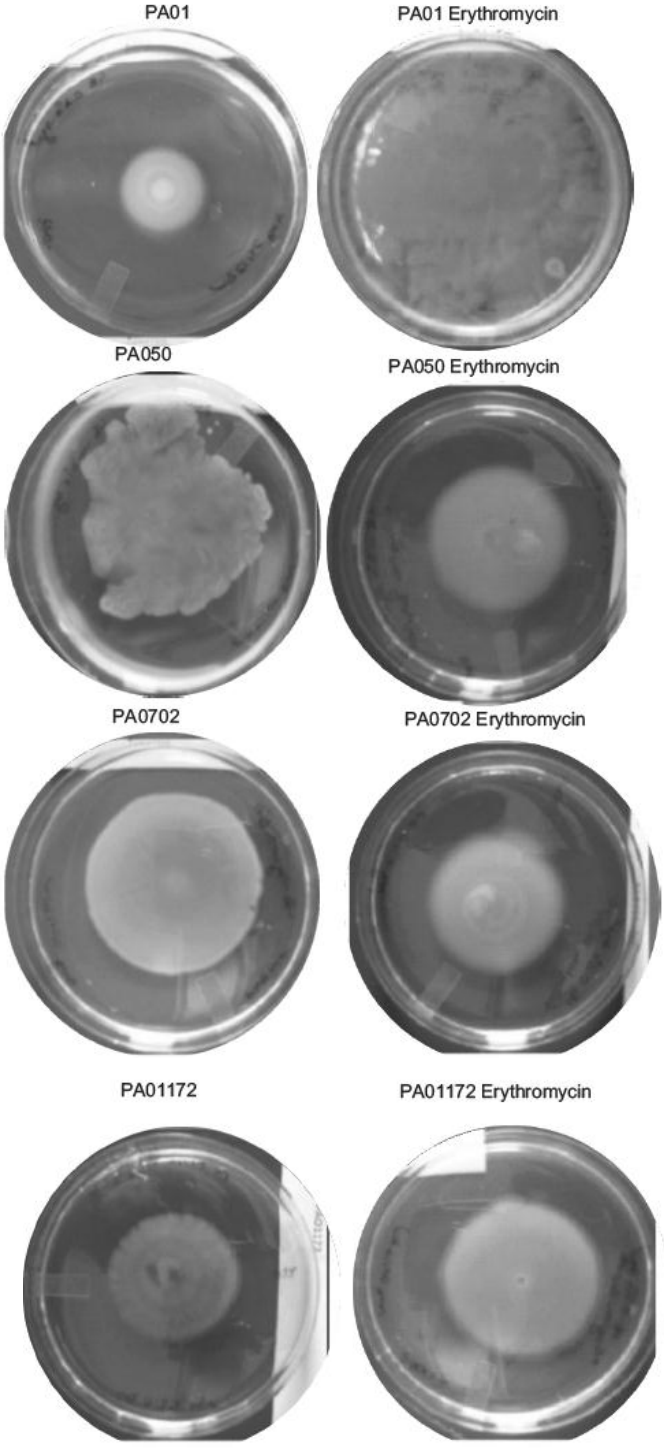

PA01
PA01 Erythromycin
PA050
PA050 Erythromycin
PA0702
PA0702 Erythromycin
PA01172
PA01172 Erythromycin

Figure 14. The Effect of Erythromycin on Swarming Motility of Various *P. aeruginosa* Strains with Deletions in their RND Efflux Components. Swarming zones were measured after 48h on 0.5% LB agar plates with or without 6 µg/ml erythromycin as discussed in the Materials and Methods. **PA01**, **PA050**, **PA0702**, **PA01172**, **PA01 with erythromycin**, **PA050 with erythromycin**, **PA0702 with erythromycin**, and **PA01172 with erythromycin** were analyzed. Data shown is representative of a minimum of two biological replicates.

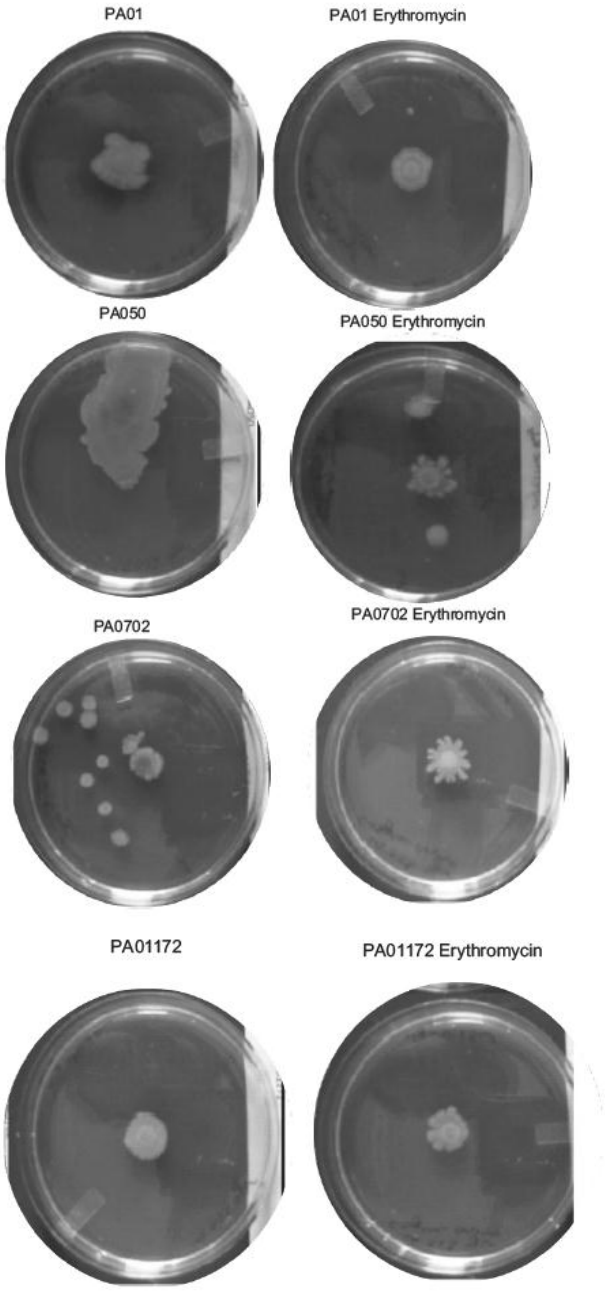
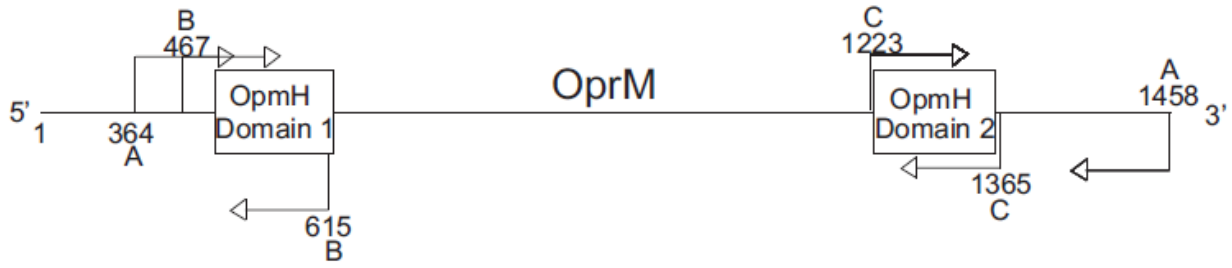
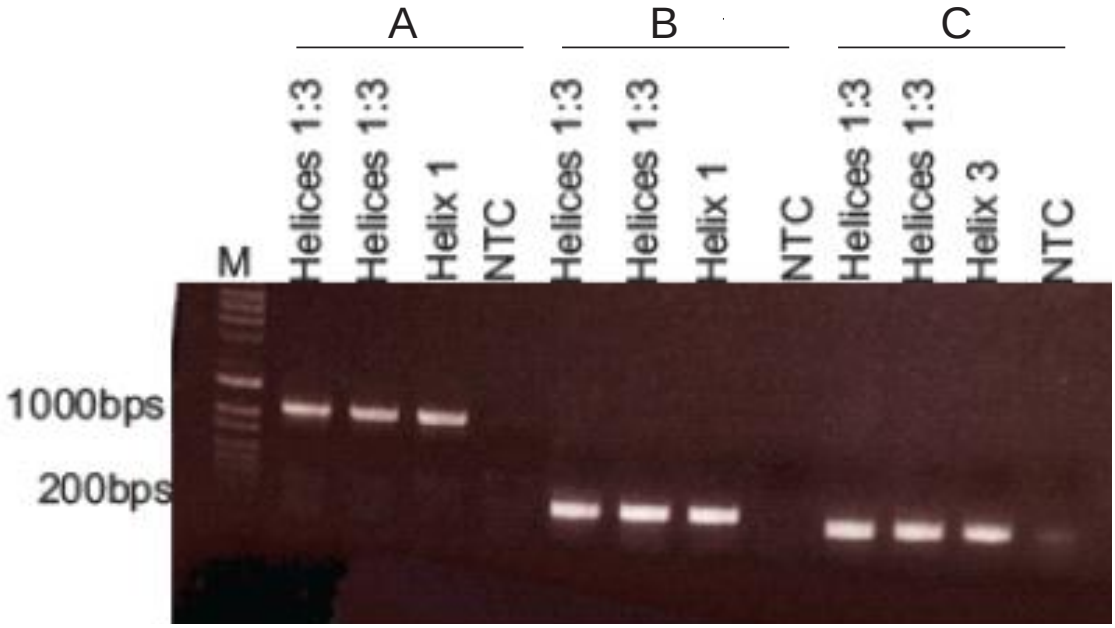

PA01
PA01 Erythromycin
PA050
PA050 Erythromycin
PA0702
PA0702 Erythromycin
PA01172
PA01172 Erythromycin

Figure 15. Verification of the Various Domains of the OprM/OpmH C*himeras* by 0.8% Agarose Gel Electrophoresis of PCR products. (a) Panel B primer binding sites. To simplify, primer binding sites are denoted in Panel A. **(b) Verification of domain 1, domain 2 and domain 1 and 2 insertion.** Sample 'Helix 1' contains an OpmH insert at the Helix 1 position of OprM (5' end; domain 1), sample 'Helix 3' contains an OpmH insert at the Helix 3 position of OprM (3' end; domain 2), and sample 'Helix1:3' is a hybrid containing an OpmH insert at both Helix 1 and Helix 3 (domains 1 and 2). PCR products using the various primer sets are denoted in Panel B (oprM_T117F and OprM_E481R 1100 bp amplicon (Panel A: primer set A(also sequencing primers)); OprM_A200R and opmH_L164F 148 bp amplicon (Panel A: Primer set B); oprM_R426F and opmH_N409R 142bp amplicon (Panel A: Primer set C). **(c) Verification of the chimera spanning from the beginning of domain 1 to the end of the domain 2 insert**. Samples '1-4' and '5-8' are replicates, sample '9' is a no template control. Sample '1-4' are a 292 bp amplicon of the mini-Tn7 vector, confirming insertion into *P. aeruginosa* chromosome (primers: Tn7R_Fwd and Pa_glmS_Rev), and samples '5-8' are a 1800 bp amplicon spanning from the 5' OprM region to the 3' OpmH region (primers: OprM_T117F and OpmH_A202R). (All primers listed in Table 3)

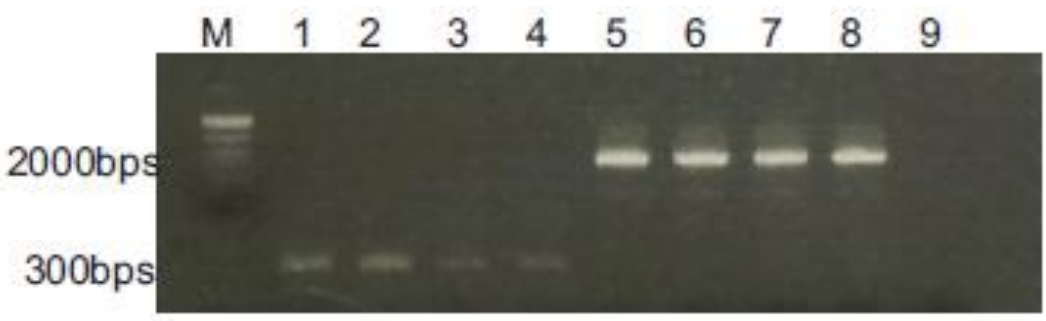
(a)


B
467
C
1223
A
1458
5'
1
364
A
OpmH
Domain 1
OprM
OpmH
Domain 2
3'
615
B
1365
C

(b)


A
B
C
M
Helices 1:3
Helices 1:3
Helix 1
NTC
Helices 1:3
Helices 1:3
Helix 1
NTC
Helices 1:3
Helices 1:3
Helix 3
NTC
1000bps
200bps

(c)


M
1
2
3
4
5
6
7
8
9
2000bps
300bps

hybrid spanning domain 1 to 2, or helix 1 to 3 respectively (Figure 15c; also confirmed by sequencing; see Figure 16).

ii. Sequencing Analysis

The swapped domains were amplified using PCR from all four strains harbouring chimeras and the products were sequenced at Genome Quebec Facility, McGill University (Montreal, QC) (Figure 16a-d) to confirm the constructs.

iii. Hypothetical Homology Models

Hypothetical homology models of the monomeric component of the homo-trimeric structure were generated using Protein homology/analog Y recognition engine V 2.0 (Phyre2), using the PHYRE2 Protein Fold Recognition Server (www.sbg.bio.ic.ac.uk/phyre2/), displaying the location of the swapped domains (Figure 17a), and the domain's origin (Figure 17b). Site-directed mutagenesis studies were previously performed by Poole (Nehme and Poole, 2007) and were included for reference (Figure 17c). Models were also generated showing the location of the helix 1 swap (domain 1), the helix 3 swap (domain 2), swapping both helix 1 and 3 (domain 1 and 2) together and swapping the residues from helix 1 to helix 3 (domain 1 star to domain 2 end) (Figure 17d-g).

Figure 16. Sequencing Results of Chimeras Harbouring Swapped Domains. Highlighted regions represent region swapped. (a) **Single domain (Domain 1) swapped in helix 1.** (b) **Single domain (Domain 2) swapped in helix 3.** (c) **Both domains (Domain 1 and 2) swapped in helices 1 and 3.** (d) **Domains in helices 1 and 3 were swapped including the region between them (Domain 1 to Domain 2).**

(a)

gactcgatctcttcggccgcctgcgcagcctgcgcgaccaggccctggagcagtacctggcgaccgaacaggcgcagcgca
gcgcgcagaccaccctggtggccagcgtggcgaccgcctacctgacgctgaaggccgaccaggcgcagctggccaccagc
aaggccgaggaagccgccttcaagcgccagctggaccaggccaacgagcgcttcgacgtgggcctttccgacaagaccgac
gtgctcgaggcccagaccgccgtggaaggcgcccgcgcgaccctggcgcagtacacccgcctggtagcccaggaccagaa
tgcgctggtcctgctgctgggctccgggatcccggcgaacctgccgcaaggcctgggcctggaccagaccctgctgaccgaa
gtgccggcgggtctgccgtcggacctgctgcaacggcgcccggacatcctcgaggccgagcaccagctcatggctgccaac
gccagcatcggcgccgcgcgcgcggcgttcttcccgagcatcagcctgaccgccaacgccggcaccatgagccgccaactg
tccggcctgttcgacgccggttcgggttcctggttgttccagccgtcgatcaacctgccgatcttcaccgccggcagcctgcgtg
ccagcctggactacgcgaagatccagaaggacatcaacgtcgcgcagtacgagaaggcgatccagacggcgttccaggaag
tcgccgacggcctggccgcgcgcggtaccttcaccgagcagttgcaggcgcagcgcgatctggtcaaggccagcgacgagt
actaccagctcgccgacaagcgctatcgcacgggggtggacaactacctgaccctgctcgacgcgcaacgctcgctgttcacc
gcgcagcagcaactgatcaccgaccgcctcaatcagctgaccagcgaggtcaacctgtacaaggc

(b)

gactcgatctcttcggccgcctgcgcagcctgcgcgaccaggccctggagcagtacctggcgaccgaacatgcgcagcgca
gcgcgcagaccaccctggtggccagcgtggcgaccgcctacctgacgctgaaggccgaccaggcgcagttgcagctgacca
aggacaccctgggcacctaccagaagagtttcgacctgacccagcgcagctacgacgtcggcgtcgcctccgcgctcgNN
NNgcgccaggcgcagaccgccgtggaaggcgcccgcgcgaccctggcgcagtacacccgcctggtagcccaggaccag
aatgcgctggtcctgctgctgggctccgggatcccggcgaacctgccgcaaggcctgggcctggaccagaccctgctgaccg
aagtgccggcgggtctgccgtcggacctgctgcaacggcgcccggacatcctcgaggccgagcaccagctcatggctgcca
acgccagcatcggcgccgcgcgcgcggcgttcttcccgagcatcagcctgaccgccaacgccggcaccatgagccgccaac
tgtccggcctgttcgacgccggttcgggttcctggttgttccagccgtcgatcaacctgccgatcttcaccgccggcagcctgcgt
gccagcctggactacgcgaagatccagaaggacatcaacgtcgcgcagtacgagaaggcgatccagacggcgttccaggaa
gtcgccgacggcctggccgcgcgcggtaccttcaccgagcagttgcaggcgcagcgcgatctggtcaaggccagcgacgag
tactaccagctcgccaccgagatcggctaccaggtcggcacccgcaacatcgtcgacgtgctcaacgcccagcgccagctgta
cgccgccgtgcgcgactacaacaccgaccgcctcaatcagctgaccagcgaggtcaacctgtacaaggccctcggcggcgg

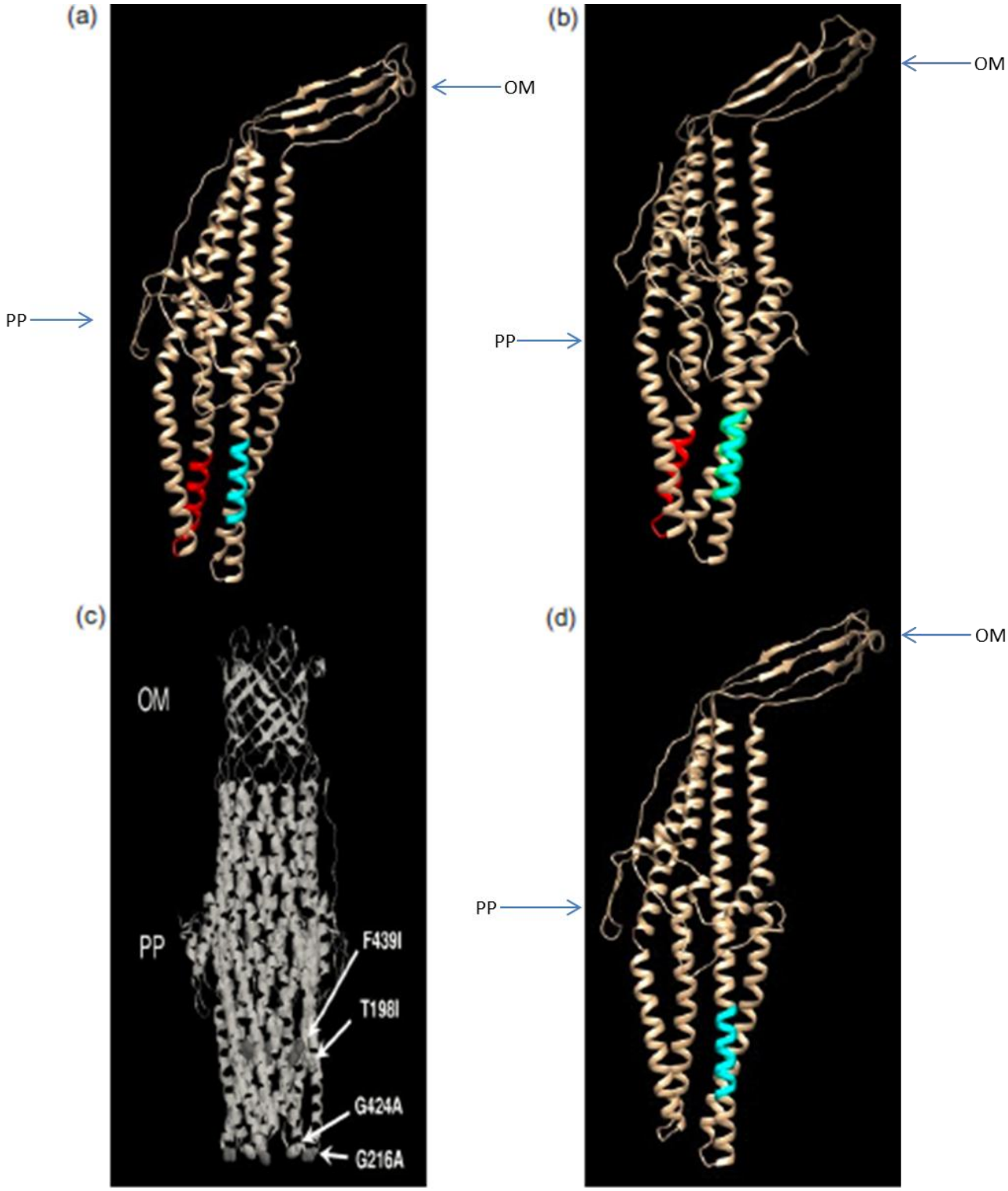
(c)

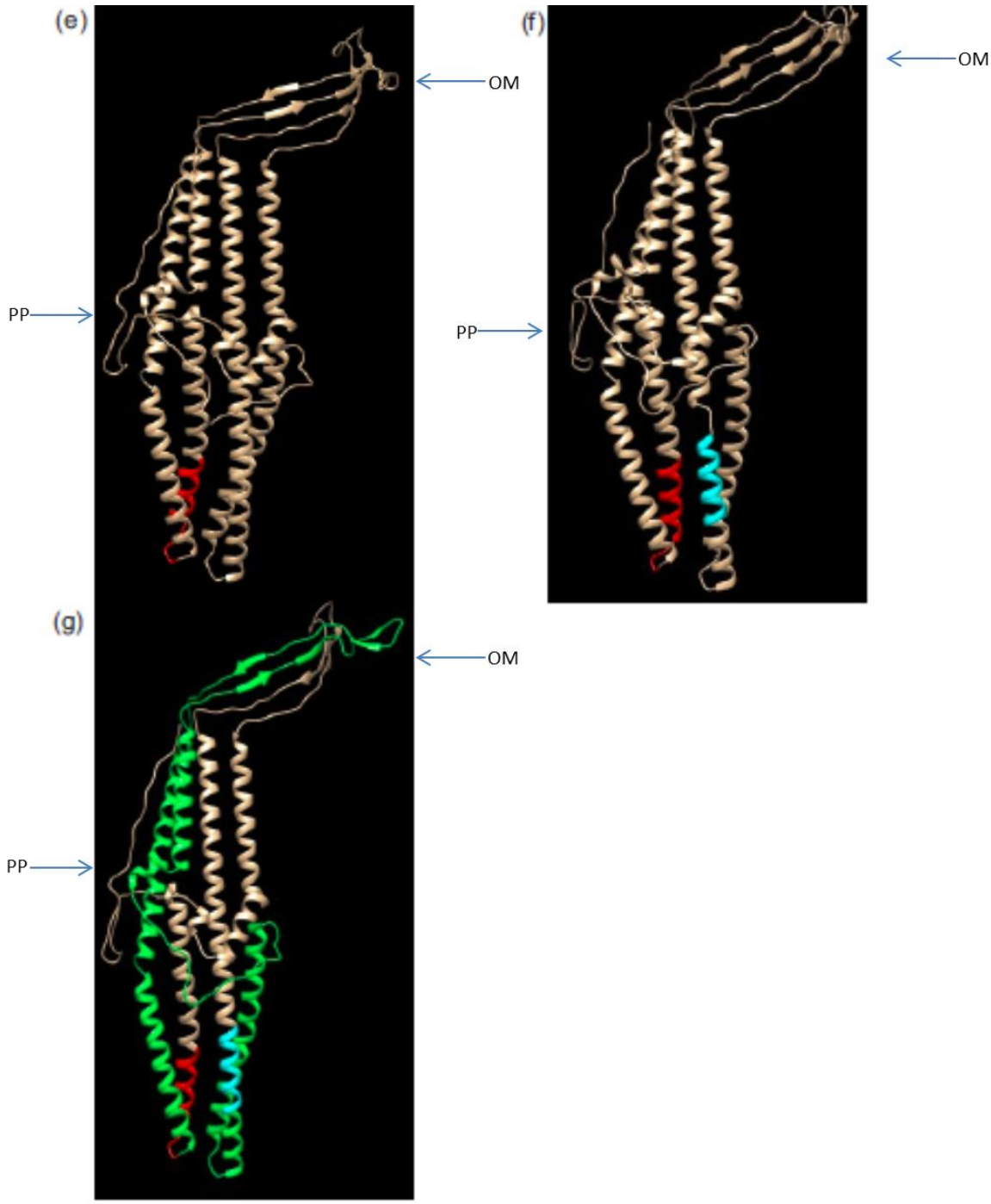
ggactcgatctcttcggccgcctgcgcagcctgcgcgaccaggccctggagcagtacctggcgaccgaacatgcgcagcgc
agcgcgcagaccaccctggtggccagcgtggcgaccgcctacctgacgctgaaggccgacccgctggccaccagcaaggc
cgaggaagccgccttcaagcgccagctggaccaggccaacgagcgcttcgacgtgggcctttccgacaagaccgacgtgctc
gaggcccagaccgccgtggaaggcgcccgcgcgaccctggcgcagtacacccgcctggtagcccaggaccagaatgcgct
ggtcctgctgctgggctccgggatcccggcgaacctgccgcaaggcctgggcctggaccagaccctgctgaccgaagtgcc
ggcgggtctgccgtcggacctgctgcaacggcgcccggacatcctcgaggccgagcaccagctcatggctgccaacgccag
catcggcgccgcgcgcgcggcgttcttcccgagcatcagcctgaccgccaacgccggcaccatgagccgccaactgtccgg
cctgttcgacgccggttcgggttcctggttgttccagccgtcgatcaacctgccgatcttcaccgccggcagcctgcgtgccagc
ctggactacgcgaagatccagaaggacatcaacgtcgcgcagtacgagaaggcgatccagacggcgttccaggaagtcgcc
gacggcctggccgcgcgcggtaccttcaccgagcagttgcaggcgcagcgcgatctggtcaaggccagcgacgagtactac
cagctcgccaccgagatcggctaccaggtcggcacccgcaacatcgtcgacgtgctcaacgcccagcgccagctgtacgccg
ccgtgcgcgactacaacaccgaccgcctcaatcagctgaccagcgaggtcaacctgtacaaggcNNNcggcggcgg

(d)

actcgatctcttcggccgcctgcgcagcctgcgcgaccaggccctggagcagtacctggcgaccgaacaggcgcagcgcag
cgcgcagaccaccctggtggccagcgtggcgaccgcctacctgacgctgaaggccgaccaggcgcagctggccaccagca
aggccgaggaagccgccttcaagcgccagctggaccaggccaacgagcgcttcgacgtgggcctttccgacaagaccgacg
tgctcgaggcccaggccagctacgacaccgcccgcgccaaccggttgatcgccgaacagcgcgtggacgatgccttccagg
ccctggtgaccctgaccaaccgcgactacagcgccatcgagggcatgcgccacaccctgccggtggtgccgccggcgccga
acgacgccaaggcctgggtcgacaccgcggtgcagcagaacctgcgcctgctggccagcaactacgcggttaacgccgccg
aggaaaccctccgccagcgcaaggccgggcacctgccgaccctcgatgccgtggcccagtaccagaagggcgacaacgac
gccctcggcttcgccaacagcgccgccaatccgctggtgcactatggcaagtatgtcgacgagcgcagcattggcctggaact
gaacatcccgatctacagcggcggcctgacctcctcccaggcccgcgagtcctaccagcgcctcaaccagagcgagcaatcc
cgcgaaggccagcgccgccaggtggtgcaggatacccgcaacctgcaccgcgcggtgaataccgacgtcgagcaggtcca
ggcgcggcgccaggcgatcatctccaaccagagttcgctggaagccaccgagatcggctaccaggtcggcacccgcaacat
cgtcgacgtgctcaacgcccagcgccagctgtacgccgccgtgcgcgactacaacaccgaccgcctcaatcagctgaccagc
gaggtcaacctgtacaaggccctcggcggcg

Figure 17. Homology Models of Chimeras Harbouring Swapped Domains. The chimeras were modelled using *P. aeruginosa's* OprM as a template as described in the Materials and Methods. Helix 1 swap is represented by blue, helix three swap is represented by red and the region located between the two helices is represented by green. (a) Native OprM. (b) Native OpmH (c) Reference: mutated residues within OprM (Nehme and Poole, 2007) (d) Structure showing Helix 1 swap (Domain 1). (e) Structure showing helix 3 swapped (Domain 2). (f) Structure showing helices 1 and 3 swapped together (Domain 1 and 2). (g) Structure showing helices 1 and 3 including the area between them swapped (Domain 1 to Domain 2). OM stands for outer membrane and PP stands for periplasm.


(a)
OM
PP
(b)
OM
PP
(c)
OM
PP
F439I
T198I
G424A
G216A
(d)
OM
PP


(e)
OM
PP
(f)
OM
PP
(g)
OM
PP

iv. Insertion of Chimeras into P. aeruginosa Single-copy Vectors

Insertion of the vector backbone and removal of the Gm resistance gene cassette was confirmed by PCR (Figure 18). Expression of this system was confirmed by SDS-PAGE and immunoblotting as described in Figure 19.

D. Phenotypic Analysis of Strains Harbouring Chimeras

i. Antibiotic Susceptibility Profile

The phenotypic verification of the constructs was analyzed through antibiotic susceptibility assays where the MIC for each strain was determined by either broth dilution (erythromycin), disc-diffusion (erythromycin) or agar dilution (triclosan) methods according to the CLSI guidelines. The MexJK-OprM complex has been shown to efflux erythromycin but not triclosan; therefore resistance to erythromycin was used as an indicator of a functional MexJK-OprM complex being formed. Alternatively, the MexJK-OpmH complex has been shown to efflux triclosan but not erythromycin; therefore resistance to triclosan was used as an indicator of a functional MexJK-OpmH complex being formed.

P. aeruginosa strain PA058 harboring MexJK-OprM, displayed resistance to erythromycin (Table 6) with a 4-fold change in MIC upon induction (4 µg/ml to 16 µg/ml) which was similar to the change reported in Table 5 (2 µg/ml to 8 µg/ml). As expected, the MexJK-OpmH harboring strain, PA051, did not efflux erythromycin in an induction dependant manner. Erythromycin resistance was lost in the strains harbouring a Domain 1 swapped chimeric OprM protein (PA071, PA073 and PA074), as indicated by the MIC remaining at 4 µg/ml. This indicates that the amino acids contained within Domains 1 (192-204) and 2 (418-433) are critical for MexJK-OprM dependent efflux. PA072 contained a modified domain 2, which requires further investigation, as MIC changed to that of the induced control (16 µg/ml), in both induced and uninduced samples. This change could be due to active MexJK-chimericOprM efflux, or the lack of induction could indicate that amino acids with Domain 2 (425-440) are also critical for function, given that induction was confirmed to have an effect on triclosan efflux. Further

Figure 18. Verification of Chimera Insertion into *P. aeruginosa.* Insertion of mini-Tn7 vector was assessed by PCR using primers Tn7R_Fwd and Pa_glmS_Rev (listed in Table 3), yielding a 292bp product. 1. **100-1.5kb ladder**. 2. **PA071** (Domain 1). 3**. PA072** (Domain 2). 4. **PA073** (Domain 1 to Domain 2). 5. **PA074** (Domain 1 and 2). 6. **No Template Control.**

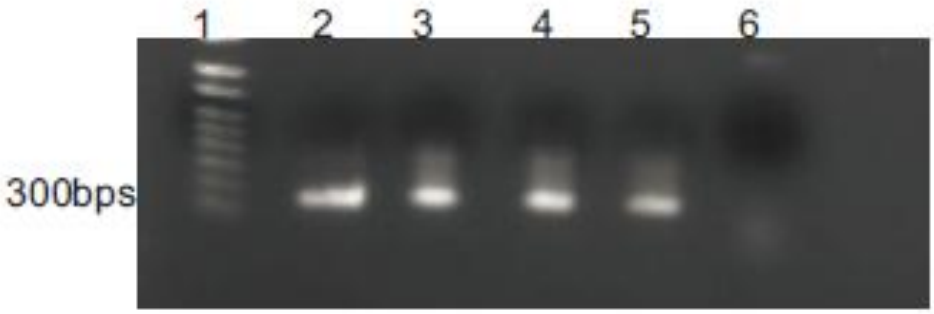

1
2
3
4
5
6
300bps

Table 6. Antibiotic Susceptibility Profile for Chimeras. Minimum inhibitory concentrations for triclosan and erythromycin, substrates for MexJK-OpmH and MexJK-OprM respectively were determined for the chimeras harbouring the swapped domains according to the CLSI standards as described in the Materials and Methods. MIC values represent the lowest concentration of antibiotic or compound required to inhibit growth of bacteria**.** The agar dilution method was used to determine triclosan MIC, both disc diffusion and broth dilution methods were used to determine erythromycin MIC.

Strain	**Efflux Components Expressed**	**Erythromycin (Broth Dilution)**			**Triclosan (Agar Dilution)**		
		-IPTG	+IPTG	Fold-change	-IPTG	+IPTG	Fold-change
PA071	MexJ,MexK, OprM, Helix 1	4	4	No Change	2	2	No Change
PA072	MexJ, MexK, OprM, Helix 3	16	16	No Change	2	8	4-fold
PA073	MexJ, MexK, OprM, Helix 1 to Helix 3	4	4	No Change	2	2	No Change
PA074	MexJ, MexK, OprM, Helix 1 and Helix 3	4	4	No Change	2	2	No Change
PA050	MexJ, MexK only	8	8	No Change	2	2	No Change
PA051	MexJ, MexK, OpmH	4	4	No Change	4	32	8-fold
PA058	MexJ, MexK, OprM	4	16	4-fold	2	2	No Change

experimentation is required to elucidate the mechanism responsible for the changes in MIC, and to specifically pinpoint which amino acids are critical to erythromycin efflux.

Conversely, *P. aeruginosa* strains PA051 harboring MexJK-OpmH, displayed resistance to triclosan with an 8-fold change in MIC upon induction (4 µg/ml to 32 µg/ml) which was identical to the 8- fold change reported in Table 5 (4 µg/ml to 32 µg/ml), however, it should be noted that some variability in triclosan MIC replicates was observed, and up to 32-fold changes in MIC were occasionally seen (2 µg/ml to 64 µg/ml) (Appendix F), perhaps because triclosan MIC must be performed on solid media, making standard serial dilutions impractical. All other strains (PA071, PA073, PA074, PA050, PA058) except PA072 were not capable of increased triclosan resistance, under inducing or noninducing conditions. The MexJK-chimeric OprM PA072 strain has amino acids 418-433 of the OprM protein (DKRYRTGVDNYLTLLD) replaced with OpmH amino acids 425-440 (GTRNIVDVLNAQRQLY), and showed a 4-fold increase in triclosan resistance upon induction (2 µg/ml to 8 µg/ml) in a reproducible fashion. MexJK dependant efflux of triclosan was previously thought to be OpmH dependent, however, for the first time a chimericOprM protein is reported to also be capable of triclosan efflux in connection with the MexJK complex. The wild type phenotype was not completely restored, suggesting that the chimeric OprM protein is not as effective as OpmH at triclosan efflux. Helix 3 of OpmH/OprM appears to play a critical role for substrate specificity and efficiency of the MexJK pump, possibly implicating it as a binding site between components, or as a critical component in channel orientation.

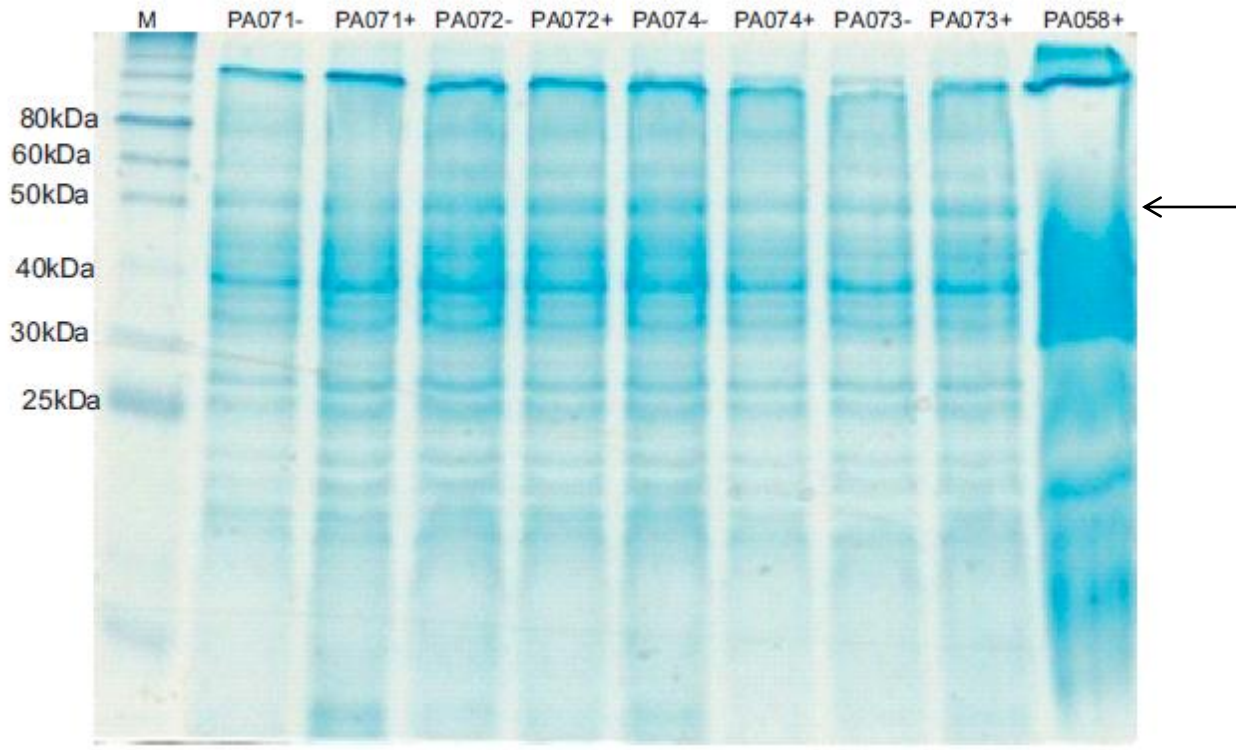
ii. Protein Expression Analysis

SDS-PAGE with CBB staining demonstrated a 50 kDa protein band corresponding to the expected molecular weight of OprM/OpmH for all strains (Figure 19a). Upon induction of OprM and probing with an α-OprM antibody a band could be seen at approximately 50 kDa from *P. aeruginosa* strains PA058, PA072 and PA074 (harbouring wild type or chimeric OprM) but not PA071 and PA073 (harbouring chimeric OprM with swapped domain 1 or swapped domain 1 to domain 2). This indicated that modification to domain 1 may destabilize the protein, or interfere with its transport to the membrane. It is also possible that domain 1 is required for epitope

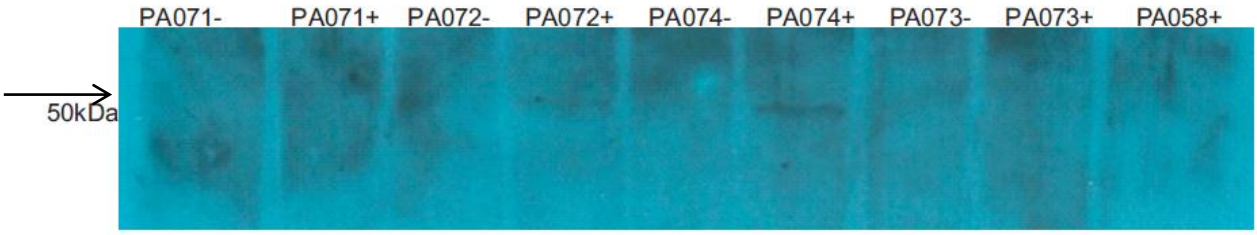
recognition by the antibody, however, the fact that PA074, a strain containing swapped domain 1 and 2 showed expression adds uncertainty.

Figure 19. Protein Expression Analysis from Whole Cell Lysate Demonstrating OprM. Whole cell lysate analysis from *P. aeruginosa* PA0702 cells harbouring swapped domains were grown to A_{600nm} of 0.6, induced (1.0 mM IPTG) and harvested at A_{600nm} of 1.0 as described in the Materials and Methods. **(a) SDS-PAGE.** Whole cell lysates were analysed on a denaturing 12% SDS-PAGE, membrane protein from PA058 +1.0 mM IPTG was included as a positive control. Arrow indicates the expected migration position for OprM. **(b) Immunoblot.** Immunoblot was carried out using a 1:10000 dilution of purified rabbit α-OprM primary antibody, and subsequently probed with a 1:20000 dilution of (HRP)-conjugated goat α-rabbit antibody.

(a)


M
PA071-
PA071+
PA072-
PA072+
PA074-
PA074+
PA073-
PA073+
PA058+
80kDa
60kDa
50kDa
40kDa
30kDa
25kDa

(b)


PA071-
PA071+
PA072-
PA072+
PA074-
PA074+
PA073-
PA073+
PA058+
50kDa

IV.

DISCUSSION

A. Antimicrobial Resistance and P. aeruginosa

The discovery of antibiotics was one of the most influential breakthroughs of modern medicine. So much so that it led the 1967 Surgeon General William H. Steward to proclaim that "the war against infectious diseases has been won" (Upshur, 2008). However, the gross misuse of antibiotics has given rise to an epidemic of antibiotic resistance, so much so, that the science ministers at the G8 world leader's summit have proclaimed that antimicrobial drug resistance will be a major health security challenge in the 21st century (Hunt, 2013).

The increasing prevalence of RND efflux pumps and their ability to pump out a wide range of antimicrobial substrates make them particularly important to study as antimicrobial resistance agents. Different RND efflux pumps from Gram-negative pathogens such as *P. aeruginosa, E. coli* and *A. baumannii* have been identified which in combination efflux all classes of clinically relevant natural, semi-synthetic, and completely synthetic antibiotics (Wright, 2005). All outer membrane proteins within the RND efflux protein family are trimeric with each protomer having a long β-barrel domain anchored to the outer membrane and a long α-helical domain containing 12 coiled coils projecting into the periplasm (Blair and Piddock, 2009). The majority of RND efflux complexes have a broad substrate range, making the mechanisms of efflux challenging to study. However, there are some pump complexes such as the MexJK-OprM and the MexJK-OpmH complex that have narrow substrate specificity. It is this characteristic that makes MexJK-OprM/OpmH ideal for studying how RND efflux pumps recognize their substrates.

P. aeruginosa is a Gram-negative opportunistic pathogen, prevalent in nosocomial infections particularly in patients suffering from cystic fibrosis (Davies, 2002). The success of *P. aeruginosa* as a nosocomial pathogen can be attributed to its intrinsic resistance to several antimicrobial agents including antibiotics, biocides and heavy metals. Multidrug efflux systems significantly contribute to this intrinsic multidrug resistance of *P. aeruginosa,* and in the clinic this has promoted enhanced acquired multidrug resistance. These pumps are able to export not only antibiotics, but dyes, detergents, disinfectants and homoserine lactones involved in quorum sensing

(Lamarche, *et al.*, 2011). An increased understanding of how the RND efflux pumps of *P. aeruginosa* function is critical given their clinical significance and roles in antibiotic resistance.

B. Single-Copy Expression System

Rather than using a multi-copy expression system, which may give rise to artificial or sometimes even lethal phenotypes; the construction of a single-copy expression system to incorporate and express the target gene was chosen because it more accurately represents protein levels that are biologically relevant to the cell. Choi *et al.* designed a single-copy system utilizing the Tn7 bacterial transposon which is capable of transposition into the bacterial chromosome at the neutral Tn7 attachment site (*attTn7*) (Choi, *et al.*, 2005). We have used this system to express the efflux components OprM and OpmH along with several chimeric proteins containing combinations of the two, in an attempt to better understand how these proteins operate within the MexJK-OprM/OpmH system.

To assess the expression of the outer membrane protein OprM, along with the RND-transport protein MexK, qRT-PCR was used. As expected, qRT-PCR indicated levels of OprM mRNA which was comparable with another single copy system, OpmH, reported from our laboratory (Ganeshanantham, 2011) and were approximately 5-fold lower than multi-copy OpmH expression systems (Ganeshanantham, 2011).

The parent strain PA0702 for which OprM and OpmH were inserted; constitutively overexpresses MexK. qRT-PCR of MexK demonstrated 15-fold higher transcription for PA051 (OpmH) and PA058 (OprM) compared to PA01 which expresses native levels of MexK (Figure 6a). Induction of OpmH resulted in a 20-fold increase in expression (Ganeshanantham, 2011) and a 30-fold increase in expression of OprM when compared to uninduced cells (Figure 6b). Taken together these results indicate that the single copy systems were functional, and although they still produce relatively higher levels of the desired transcript, they are capable of more closely imitating the natural biological system.

Protein analysis by SDS-PAGE and CBB staining demonstrated an approximately 50 kDa protein which correlates to the expected size of the outer membrane protein OprM (50 kDa), after membrane protein isolation (Figure 7a). After probing with rabbit α-OprM antibodies a band correlating to OprM was observed in PA058, a strain constructed to express OprM from a single copy vector (Figure 7b). A 50 kDa band correlating to OpmH was also observed by SDS-PAGE with CBB staining in PA051 (Figure 8a), a strain constructed to express OpmH from a single copy vector. Also, a band correlating to OpmH after probing with goat α-OpmH antibodies was observed (Figure 8b). The band correlating to OpmH was faint, because as antibody concentration increased, non-specific binding also increased, making procedure optimization challenging. SDS-PAGE and CBB staining revealed a protein of 100-120 kDa which correlates to MexK (Figure 9a). After partial purification of goat α-MexK antibodies from blood, a 100-120 kDa protein correlating to MexK was also observed with immunoblotting of membrane proteins from PA051 and PA058 which both constitutively express MexK (Figures 9a and 9b). In combination with the qRT-PCR data described above, this data demonstrates that the single-copy expression systems designed in this study, to analyze the outer membrane proteins OpmH and OprM in the MexJK-OpmH/OprM complex is completely functional. mRNA transcripts were elevated compared to wild type expression levels, however, not to the extent of multi-copy systems, and the transcribed message was being translated into protein which migrated to the membrane. This system provides several advantages over traditional multi-copy systems.

Traditional multi-copy systems were developed for high levels of gene expression from plasmids, which is often desirable particularly if one intends on purifying the protein being expressed. However, if protein purification is not the goal of a particular study, overproduction of protein may generate undesired phenotypes, such as decreased growth rate, induction of stress responses, and altered properties of the protein itself, resulting in observations which may not be physiologically relevant, and hence misleading (Boyd, *et al.*, 2000). This is particularly important when studying the RND efflux complex, as the pumps activity is measured *in vivo* using assays such as MIC. Overexpression of the RND efflux proteins may result in loss of cell viability, or artifacts

that may convolute the natural phenotype. In an attempt to avoid the negative complications associated with multi-copy gene expression systems, we developed a single copy system which is particularly accommodative for studying the outer membrane components of the MexJK-OprM/OpmH efflux pump, with marked reduction in expression levels compared to more traditional multi-copy systems. In these systems, the outer membrane protein (OprM or OpmH) may be induced allowing specifically for the study of its role in the RND complex. The membrane fusion protein MexJ and the resistance nodulation division transporter protein MexK are constitutively expressed, remaining constant amongst the strains.

As a final confirmation, antibiotic susceptibility data was compared, and indicated that these strains are able to form functional complexes capable of efflux; promoting a resistant phenotype. Upon induction of the outer membrane proteins of the MexJK-OprM complex there was a 4-fold increase in erythromycin MIC compared to uninduced cultures and an 8-fold increase in triclosan MIC after induction of the outer membrane protein of the MexJK-OpmH complex compared to uninduced cultures (Table 5). The enhancement of MIC after induction of the outer membrane protein is indicative of functional complex formation with MexJK. These functional complexes were capable of erythromycin efflux (OprM) or triclosan efflux (OpmH). Others have reported comparable findings after induction of a single copy vector containing OpmH (4-fold increase triclosan vs. 8-fold in this study), and OprM (3-fold increase in triclosan vs. 8-fold in this study) (Chuanchuen, *et al.*, 2005).

Taken together this data demonstrates successful construction of a single copy expression system for the analysis of OprM/OpmH in the MexJK-OprM/OpmH RND efflux complex. Transcripts from the various RND components were measured, and although elevated compared to wild-type expression levels, were more representative of the biological system than traditional multi-copy systems. Translation of the transcripts and migration of the protein to the membrane fraction of the cell was also confirmed. Finally, the activity of the MexJK-OprM/OpmH complexes were measured using MIC, and conclusively demonstrated functional complex formation, as the strains were able to efflux the expected antibiotic in an inducible fashion.

C. P. aeruginosa Virulence

i. Biofilm Formation

Biofilms are surface-associated microbial communities in which the cells are typically encased in an extracellular matrix composed of DNA, RNA, protein, and polysaccharides (Ma, *et al.,* 2009). Biofilms have broad reaching importance, not only in the clinic but also in the environment, as they may adhere to either biotic (i.e. patient) or an abiotic (i.e. water pipe) surfaces (Flemming and Wingender, 2010). *P. aeruginosa* biofilms are particularly important in the cystic fibrosis lung, allowing for permanent colonization, even through aggressive antibiotic treatment (Singh *et al.,* 2000). Biofilm production during infection is associated with an increase in antimicrobial resistance. Several mechanisms have been reportedly responsible for the enhanced antimicrobial resistance within the biofilm structure. (1) Biofilms decrease the permeability of most antibiotics through the polysaccharide matrix (Anderl, *et al.,* 2000). (2) Biofilms cause physiological changes such as decreased growth rate and starvation responses which are associated with enhanced resistance (Brown, *et al.,* 1988; Walters, *et al*, 2003). (3) Biofilms enhance the expression of efflux pumps within the matrix (Gilbert, *et al.,* 2002), and (4) enhance survival of persistor cells within the matrix (Lewis, *et al.,* 2007), something which is particularly important in *P. aeruginosa* infections in the cystic fibrosis lung, where complete erradication has proven almost impossible in some patients. Quorum sensing has been shown to be critical to effective biofilm production (Brooun, *et al.,* 2000), and quorum sensing inhibitors are currently being explored as an attractive therapeutic which may limit biofilm production during *P. aeruginosa* infection of the cystic fibrosis lung (Jakobsen, *et al.,* 2013), thereby making them more susceptible to antibiotic therapies.

The MDR RND efflux pumps of *P. aeruginosa* play an important role in the antibiotic resistance of non-biofilm associated planktonic cells, and are also important in *P. aeruginosa* biofilm associated cells, exhibiting resistance to all classes of antimicrobials. Aside from an important role in antimicrobial resistance, RND efflux pumps also have a role in influencing biofilm production in several organisms including *P. aeruginosa*. The RND efflux pumps MexAB-OprM (Evans, *et al.,* 1998) and MexEF-

OprN (Lamarche and Deziel, 2011) are able to efflux AI molecules, which are critical to quorum sensing and biofilm production (Miller and Basser, 2001). Curiously, strains which overexpress these pumps show a decrease in AI molecule accumulation within the cell, causing downstream effects, such as attenuated virulence and a reduced capacity to form biofilms (Minagawa, *et al.*, 2012). To date, the role of the MexJK-OprM/OpmH RND efflux pump on biofilm formation has not been evaluated. Therefore, we sought to determine the role of MexJK-OprM/OpmH on biofilm production using the single copy construct (PA050, PA051 and PA058). A variety of *P. aeruginosa* strains containing alternative RND efflux pump compositions were used to more generally assess the role of RND efflux pumps on biofilm production.

Upon induction of OpmH from PA051, biofilm formation was shown to be significantly increased when compared to the uninduced cells or the MexJK only strain (PA050) which does not contain an RND outer membrane protein (Figure 10a). Upon induction of OprM from PA058, biofilm formation was shown to be significantly increased when compared to the empty strain PA050 (Figure 10a). PA058 was also capable of producing significantly more biofilm than both PA050 and PA051 suggesting that MexJK-OprM contributes more effectively to biofilm production than MexJK-OpmH (Figure 10a).

Long-term erythromycin treatment (up to 80 days) has been proposed to treat diffuse panbronchiolitis, a chronic lower respiratory tract infection associated with persistent *P. aeruginosa* infection in the cystic fibrosis lung, because it reduces biofilm formation (Naqata *et al.*, 2004). Here we assessed the role of MexJK-OpmH/OprM in biofilm production grown in the presence of varying concentrations of erythromycin. Contrary to the findings of Nagata *et al*. in wild-type *P. aeruginosa*, we observed significant increases in biofilm production in all of our constructed strains, which increased in an apparent dose dependant fashion with increasing concentration of erythromycin (Figure 10b-d). Increasing concentrations of erythromycin led to increased biofilm production in PA050, a strain which lacks OpmH or OprM, however, this effect appeared to be more pronounced in PA051 and PA058, strains containing OpmH and OprM respectively. Curiously, PA058 did not show any significant differences after

induction of OprM, however, induction of OpmH in PA051 led to significant differences in biofilm production at 2, 4, and 6 µg/ml (Figure 10 c). At 2 and 4 µg/ml erythromycin, PA051 biofilm production was significantly decreased. Efflux of quorum sensing molecules by RND efflux pumps has been shown to limit the accumulation of quorum sensing molecules within the cell which results in decreased biofilm production (Lamarche and Deziel, 2011). Therefore, our results suggest that the MexJK-OpmH pump may be involved in the efflux of quorum sensing molecules because after induction of OpmH there was a decrease in biofilm production. At 6 µg/ml erythromycin, biofilm production was significantly enhanced upon induction, which is likely a result of enhanced cell viability, because the uninduced strain would be unlikely to survive at this concentration given our previously reported MIC of 4 µg/ml (Table 5). The role of the MexJK-OprM/OpmH efflux pump on biofilm production remain somewhat convoluted by the fact that there were no significant differences upon induction of the respective pumps except with erythromycin treatment of PA051, which implicates other important contributing factors. This in combination with the relatively non-specific nature of the assay leaves several questions as to the specific role of OpmH and OprM, which must be addressed in additional studies.

Antimicrobials are consistently found at low, sub-inhibitory concentrations within the soil. However, these concentrations are typically not at critical concentrations required for complete inhibition of microbial growth. This observation has led to the hypothesis that antibiotics have alternative roles which are dependent on their concentration. At lower concentrations antibiotics may be acting as signalling molecules and are able to modulate gene expression, whereas at clinically relevant higher concentrations they may inhibit a variety of processes (Davies *et al.*, 2006; Fajardo and Martinez, 2008). Erythromycin is an antibiotic used to treat non-cystic fibrosis infections in the lung such as bronchiectasis (a condition characterized by damage and scarring to the airway) (Serisier, *et al.*, 2013) and has also been explored as a biofilm reducing agent for *P. aeruginosa* infections (Naqata, *et al.*, 2004). Infections of the lung are known to be polymicrobial, and may contain both Gram-positive and Gram-negative organisms. Erythromycin is an antibiotic commonly used to treat Gram-positive infections of the lung. Although erythromycin is not typically used to treat *P. aeruginosa* infections, *P.*

aeruginosa may be inadvertently exposed to erythromycin during treatment. We wanted to investigate whether erythromycin would enhance biofilm production in *P. aeruginosa* when treating these infections.

The wild-type strain PA01 displayed decreased biofilm production in the presence of 6 µg/ml erythromycin, compared to cells grown in the absence of erythromycin (Figure 11), an effect which has been observed by others (Nagata, *et al.*, 2004). The parent strain PA0702 (lacking OprM/OpmH) showed a non-significant increase in biofilm formation upon addition of 6 µg/ml erythromycin compared to no erythromycin, however, strains PA0238 (lacking MexAB-OprM and MexCD-OprJ), PA01172 (lacking MexAB-OprM, MexCD-OprJ, MexJK, MexEF-OprN, MexXY, OpmH, and TriABC), and PA0200 (lacking MexAB-OprM) all showed a significant increase in biofilm formation with the addition of erythromycin (Figure 11). In the absence of erythromycin, all strains showed relatively similar biofilm production. These results are interesting and somewhat unexpected. Although further experimentation is required, it is tempting to speculate that erythromycin may upregulate RND efflux components in wild-type cells such as PA01 to ensure survival. The upregulation of efflux components may lead to an increase in the efflux of quorum sensing molecules such as AI's, and a decreased accumulation of biofilm generating signals within the cell. In the deletion strains, the opposite effect occurs, suggesting that erythromycin may actually trigger biofilm producing signaling molecules, and that the cell can no longer efflux the biofilm producing molecule, allowing for accumulation within the cell, and increased biofilm production. This could be a result of the MexAB-OprM pump, in those strains containing MexAB-OprM (PA01) biofilm production decreases in the presence of erythromycin, in those mutant strains (PA0702, PA0200, PA0238, PA01172) lacking MexAB-OprM, the cells start behaving differently in the presence of erythromycin, increasing the production of biofilm.

ii. Quorum Sensing and Virulence

Quorum sensing is the mechanism by which single celled organisms communicate with one another in a fitness enhancing matter to adapt to environmental stressors (Miller and Bassler, 2001). Amongst *P. aeruginosa* isolates, evidence is accumulating which

implicates the RND-efflux systems as being an important component of quorum sensing, through efflux of quorum sensing molecules (Lamarche, *et al.*, 2011). Quorum sensing has been shown to play a critical role in a variety of mechanisms which are important during infection, most notably in biofilm production and virulence factor regulation (Smith and Iglewski, 2003). This broadens the role of RND efflux pumps during pathogenesis, because not only are they critical to antibiotic efflux, but also important virulence factor modulators. In an attempt to better understand the role of MexJK-OprM/OpmH during infection, we evaluated a potential link between expression of MexJK-OprM/OpmH efflux pumps and virulence of the pathogen *in vitro*.

The importance of *P. aeruginosa* infections have led to its use as a model organism for the study of biofilms (De Kievit, 2009). *P. aeruginosa* quorum sensing has been shown to play a critical role in effective biofilm formation, which was explored in the previous section. Three quorum sensing systems have been described for *P. aeruginosa* to date, the Las, Rhl and PQS systems. These systems rely on signalling molecules which are self-generated and permeable through the membrane, and when in complex with their receptor, coordinate gene expression in response to changes in population density (Mangwani, *et al.*, 2012).

AHLs are a group of signalling molecules which participate in quorum sensing. It has been demonstrated that certain efflux pumps such as MexAB-OprM are able to recognize AHLs as a substrates (Pearson, *et al.*, 1999), whereas others such as MexEF-OprN, efflux precursor AHL molecules limiting the production of AHL in MexEF-OprN containing cells (Kohler, *et al.*, 2001). To date the role of MexJK-OprM/OpmH in AHL efflux has not been explored, we, therefore, sought to evaluate the role of MexJK-OprM/OpmH in AHL efflux, using a reporter strain which expresses GFP in the presence of the signaling molecules. Using the single copy constructs (PA050, PA051 and PA058) we tested the ability of MexJK-OprM/OpmH to secrete AHLs. Deletion strains were also compared.

P. aeruginosa strains were grown next to *E. coli* MT102 which harbours the plasmid pJBA132. In this system, LuxR is produced, and when in the presence of AHLs, binds to the LuxI promoter in a transcription activating fashion, which has been

engineered to control GFP production. In the absence of AHL, LuxR cannot bind to LuxI, and no GFP is produced. Therefore, GFP production in *E. coli* MT102 indicates AHL secretion by the test strain. Wild-type PA01, and the constructs PA050, PA051, PA058, harboring MexJK alone, MexJK-OpmH, and MexJK-OprM respectively all demonstrated AHL secretion, causing the reporter to generate GFP (Figure 12). This does not completely eliminate a possible role for MexJK-OprM/OpmH in AHL efflux, however, it does rule out efflux of a precursor AHL molecule. If an AHL precursor molecule was being secreted then AHL synthesis would be inhibited and no florescence would be expected, an effect which is observed in MexEF-OprN containing strains. Additionally, similar to biofilm formation, when MexAB-OprM is knocked out, the cells begin behaving differently, decreasing in fluorescence. Taken together, this demonstrates that MexJK-OprM/OpmH does not inhibit AHL synthesis via efflux of AHL precursors; however, there may be a role for MexAB-OprM. Further studies are required to evaluate whether or not MexAB-OprM or MexJK-OprM/OpmH play a role in active AHL efflux.

iii. The role of MexJK-OprM/OpmH in P. aeruginosa motility

Bacterial motility is typically achieved by one of two processes either swimming, or swarming. Swimming in *P. aeruginosa* is achieved with a single polar flagellum, and allows for population independent movement. In contrast, swarming is a community behavior and in *P. aeruginosa* typically requires multiple flagella.

Previous studies have demonstrated that in flagellar bacteria which produce biofilms, the flagellar genes are repressed during biofilm formation. This makes sense, because if embedded in a biofilm matrix, a bacterium would not require motility, and would therefore conserve resources by limiting production of unneeded motility genes. This motility-to-biofilm transition has been demonstrated for *Bacillus, Pseudomonas, Vibrio*, and E*scherichia* (Guttenplan and Kearns, 2013). In previous experiments reported in this thesis, we demonstrated that erythromycin could have profound effects on *P. aeruginosa* biofilm formation, and given the apparent link between motility and biofilm formation, we sought to evaluate the role of the RND efflux pump MexJK-OprM/OpmH on swimming and swarming.

All strains demonstrated swimming motility in the absence of erythromycin, although wild-type PA01 seemed to be somewhat limited in its capacity to swim compared to the other strains tested (Figure 13). With the addition of erythromycin, PA01 demonstrated sporadic growth which was not indicative of swimming, whereas PA050 (lacking OprM, MexAB-OprM, MexXY, MexCD-OprJ with mini-Tn7-lac), PA0702 (lacking OprM, MexAB-OprM, MexXY, MexCD-OprJ) and PA01172 (lacking MexAB-OprM, MexCD-OprJ, MexJK, MexEF-OprN, MexXY, OpmH, and TriABC) showed no obvious changes in swimming behaviour, aside from some changes in green pigment, which could be associated with modifications to the production of the toxin pyocyanin (Figure 13).

Swarming motility is an organized behaviour involving groups of bacteria traveling together. Like biofilm, swarming is prevalent during high cell density, and cells which participate in this particular type of mobility demonstrate increased antibiotic resistance compared to swimming cells, or cells which are immobile, although the mechanism remains unclear (Butler, *et al.*, 2009). The RND efflux pumps are notoriously involved in antibiotic resistance, and therefore, we sought to examine the role of efflux pump knock-outs on swarming motility grown with or without erythromycin, a potent biofilm inducer in some of our strains.

Curiously, the patterns of swarming were similar to the patterns seen with biofilm formation. Wild-type PA01 demonstrated limited swarming in the presence or absence of erthromycin (Figure 14). Whereas our pump knock-out strains, PA050 (lacking OprM, MexAB-OprM, MexXY, MexCD-OprJ with mini-Tn7-lac), PA0702 (lacking OprM, MexAB-OprM, MexXY, MexCD-OprJ) and PA01172 (lacking MexAB-OprM, MexCD-OprJ, MexJK, MexEF-OprN, MexXY, OpmH, and TriABC) demonstrated organized motility in the characteristic fractal pattern associated with swarming in the presence of erythromycin (Figure 14). In the absence of erythromycin, limited swarming was observed, particularly in strains PA0702 and PA01172. Erythromycin caused an enhancement of biofilm production as seen in Figure 11, as a result of this; we decided to further investigate virulence by examining motility in the presence of erythromycin.

Taken together, this data implicates erythromycin as an effector of community behaviour, given that it enhanced biofilm and swarming which are both considered organized community behaviours. Alternatively, erythromycin had no effect on swimming, an activity which is not organized community behaviour. Curiously, only strains lacking a sub-set of RND efflux pumps appear to be affected by erythromycin, as wild type PA01 demonstrated decreased biofilm production (Figure 11), and no real change in swarming activity when treated with erythromycin. It is therefore tempting to speculate that the loss of various RND-efflux components results in an increased stress response which facilitates organized community behaviours.

D. Expression Profile of Chimeras

The vast majority of RND efflux pumps have broad ranges of substrates they recognize and are able to efflux from the cell. The outer membrane proteins of these pumps generate a channel through the outer membrane which allows substrate to pass through it, and must therefore be capable of accommodating a variety of substrates. Unlike the majority of RND outer membrane proteins, OprM and OpmH have a very narrow range of substrates when in complex with MexJK. Both of these proteins are capable of forming the outer membrane channel for the MexJK efflux pump and share approximately 60% sequence similarity, however, they efflux different substrates. These characteristics of close relative homology, yet unique substrate specificity provide an opportunity to examine the particular regions within the outer membrane proteins which are responsible for substrate specificity. We decided to modify OprM by swapping various OpmH domains into OprM in an attempt to modify substrate specificity. These chimeric proteins were confirmed by nucleotide sequencing (Figure 16). The helix 1 and helix 3 regions of OprM were substituted with OpmH regions, because these are the regions thought to interact with the RND transporter protein, and have been shown to be important in MexAB-OprM substrate efflux (Nehme and Poole, 2007).

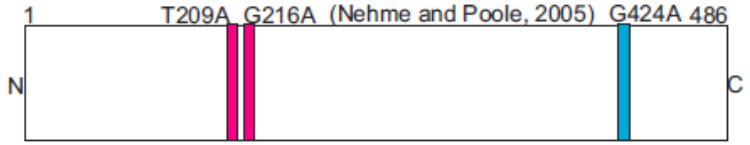
Hypothetical homology models were modeled using OprM (which has been crystalized) (Figure 17a) as a template for OpmH (which is yet to be crystalized) (Figure 17b). The regions of OprM and OpmH which may be swapped are highlighted (Figure 17

a and b respectively) and hypothetical homology models of each of the chimeric OprM proteins were generated with highlighted swapped regions (Figures 17d-g).

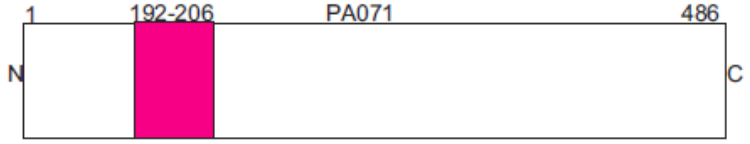
Despite relatively high sequence similarities between OprM and OpmH at the amino acid level (~60%); the two proteins have different substrate profiles. MexJK-OprM is capable of tetracycline and erythromycin efflux, whereas MexJK-OpmH is capable of triclosan efflux. This provided a unique opportunity to examine which regions within the proteins are critical to substrate efflux. Resistance to triclosan was not conferred to OprM in chimeras where helix 1, or helix 1 and 3, or helix 1 to 3 were swapped (Table 6). However, we found that when only helix 3 of OprM was replaced with OpmH there was an increase in resistance to triclosan (Table 6). This is a very interesting result because we have identified a region in OpmH (amino acids 425-440 located in helix 3) which confers the ability to efflux triclosan if inserted into OprM. Curiously, the ability to efflux triclosan comes at a cost; mainly the MexJK-chimericOprM lost the ability to efflux erythromycin, an effect which was observed in all chimeric OprM proteins. Erythromycin and triclosan are very different molecules chemically. Erythromycin is a relatively large (733 Da), relatively polar molecule, whereas triclosan is a relatively small (289 Da), relatively hydrophobic molecule. The differences between these two molecules may necessitate unique outer membrane proteins for their efflux; however, further studies are required to confirm this observation.

Protein analysis demonstrated the presence of a band at approximately 50 kDa for strains in PA058, PA072 and PA074 but not in PA071 and PA073 (Figure 19a). When probed with rabbit α-OprM antibodies, OprM expression was verified in PA058, PA072, and PA074 (Figure 19b). Others have shown that threonine 209 is critical to protein translation (Nehme and Poole, 2005) and here we show that amino acids 192-206 may also be critical for appropriate OprM translation (Figure 18b, 19). This could indicate that helix 1 contains residues necessary for OprM protein translation or folding. However, without performing site-directed mutagenesis studies it is difficult to pinpoint the exact amino acid residue(s) required for appropriate translation. Curiously, double chimeras with an additional mutation at AA225-240 rescued protein translation (Figure 18b, 19).

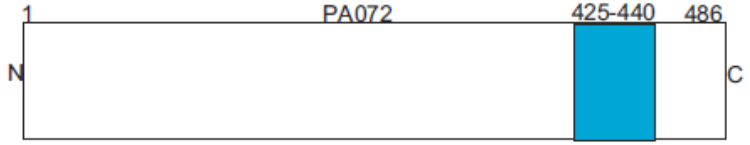
Figure 20. Summary of Domain Swaps made in *P. aeruginosa* OprM. A representative diagram highlighting the domains that were swapped from OpmH into OprM. Replaced sections of OprM with OpmH are represented by a change in colour; pink represents helix 1, blue represents helix 3 and yellow represents the swap encompassing helices 1 to 3.



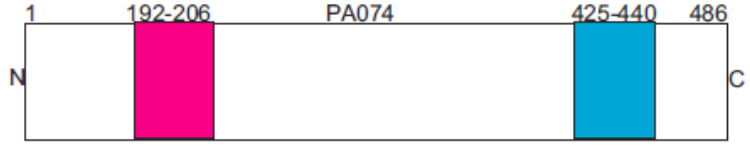
-T209A- change in resistance profile, no OprM protein detected
-G216A- change in resistance profile, OprM protein
-G424A- change in resistance profile, OprM protein



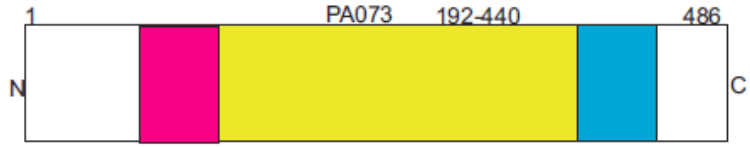
-Loss of Erythromycin Resistance
-No Triclosan Resistance
-No Protein Detected



- Loss of Erythromycin Resistance
- Gain of Triclosan Resistance
- OprM Protein Production



- Loss of Erythromycin Resistance
- No Triclosan Resistance
- OprM Protein Production



- Loss of Erythromycin Resistance
- No Triclosan Resistance
- No Protein Detected

E. Concluding Remarks

The MexJK-OprM/OpmH RND efflux pump is an important component in the repertoire of antimicrobial resistance mechanisms at the disposal of *P. aeruginosa*. The pump is capable of tetracycline and erythromycin efflux when in complex with OprM, and triclosan efflux when in complex with OpmH. This is the narrowest substrate specificity of any of the known *P. aeruginosa* RND efflux pumps, making it ideal for molecular characterization of substrate interaction. Initially, we designed single copy systems to study MexJK in the presence of either OprM, or OpmH. We confirmed these constructs using qRT, immunoblot, and MIC phenotype. Next, we examined the role of the MexJK-OprM/OpmH efflux pump on *P. aeruginosa* virulence by assessing biofilm formation, quorum sensing, and motility. We found that erythromycin plays an important role in organized community behaviour, enhancing biofilm formation, and swarming. Finally, we determine regions within the OprM/OpmH proteins which are critical to substrate specificity. We substituted 15 amino acids from OpmH into OprM generating a chimeric OprM protein which gained the ability to efflux triclosan. Together this data adds to our understanding of how the MexJK-OprM/OpmH efflux pump operates.

V.

FUTURE DIRECTIONS

While domains in OprM were replaced from those in OpmH, those in OpmH were not replaced by OprM. It would be interesting to see if helix three of OprM also modifies resistance in the same pattern as that from OpmH did. In order to do this, the same procedure would be adapted only this time taking regions from OprM and inserting them into OpmH. Next, phenotypic analysis in the form of MIC's would need to be performed to check the phenotype to see if the chimeric OpmH strains had gained the ability to efflux erythromycin; it would be interesting to see if the loss of native substrate specificity was also seen in these chimeras. We would also run SDS-PAGE and immunoblotting to ensure protein was being produced. Protein was not produced in all of the OprM chimeras, since the swaps would be taking place in the same regions of the protein, it would be interesting to see if the same possible destabilization effect and possible rescue was seen when both domains were swapped at the same time as was seem in the OprM chimeras (Figure 19b).

A region potentially responsible for substrate specificity was identified in OpmH and a very broad region in OprM was shown to effect erythromycin efflux and protein production. In order to narrow down the range of amino acids, site-directed mutagenesis studies on specific amino acids from helix three in OpmH should be performed to identify specific amino acids that facilitate the efflux of triclosan by the MexJK pump. As well, upon completion of the domain swapping experiments in OpmH, these same site-directed mutagenesis experiments should be carried out in OprM.

The mechanism of interaction between the MexJK-OprM/OpmH complex is currently unknown. Pull-down assays to investigate the interaction between the RND transporter protein and the OMP should be performed. Since both outer membranes have been his tagged, it would be fairly uncomplicated to check for an interaction with MexK in a nickel-NTA column, as described in the materials and methods. Additionally, if OprM and MexK are pulled down in the presence and absence of substrate, it should be obvious whether or not the complex is substrate dependent or constitutively expressed. If bands appear for MexK only when substrate is present it would indicate that the complex is substrate dependent, if bands for MexK appear in both variables, then it indicates the complex is constitutively expressed.

VI.

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VII.

APPENDICES

APPENDIX A. *Lactobacillus rhamnosus* R011 cell free supernatant sensitizes *P. aeruginosa* strains to various antibiotics in MIC checkerboard assay.(a) Summary table (b) -(l) Raw data from MIC plates, each set of two represents one 96-well plate. The top table contains the antibiotic concentrations used, while the bottom plate contains the amount of cell free supernatant added. Yellow colour represents a change in MIC and red colour represents the final MIC. Cell free supernatant from *Lactobacillus rhamnosus* R011 was grown overnight in deMan, Rogosa and Sharpe (MRS) medium, cells were removed by centrifugation and subsequent 0.22 µm filtration. 96-well plates were prepared and incubated for 18 hours at 37 °C.

(a)

Strain	Antibiotic	No Treatment MIC (µg/mL)	MIC after Cell Free Supernatant Treatment (µg/ml)	Fold Change	Cell Free Supernatant Added (µl)
PA01	Ciprofloxacin	0.6	0.3	2	6.25
PA01172	Ciprofloxacin	0.15	0.0000732	2049	0.09766
PA01	Trimethoprim	32	32	no change	25 (maximum assayed)
PA01172	Trimethoprim	4	0.0039063	1024	0.09766
PA01	Chloramphenicol	128	128	no change	6.25
PA01172	Chloramphenicol	4	0.025	160	0.78125
PA01	Carbenicillin	8	8	no change	1.5650
PA01172	Carbenicillin	64	0.125	512	0.0488
PA01	Tetracycline	32	32	no change	25 (maximum assayed)
PA01172	Tetracycline	2	0.03125	64	0.78125

(b) PA01

Ciprofloxacin µg/ml

0.00000	1.20000	0.60000	0.30000	0.15000	0.07500	0.03750	0.01875	0.00938	0.00469	0.00234	0.00117
	0.30000	0.15000	0.07500	0.03750	0.01875	0.00938	0.00469	0.00234	0.00117	0.00059	0.00029
	0.07500	0.03750	0.01875	0.00938	0.00469	0.00234	0.00117	0.00059	0.00029	0.00015	0.00007
	0.01875	0.00938	0.00469	0.00234	0.00117	0.00059	0.00029	0.00015	0.00007	0.00004	0.00002
	0.00469	0.00234	0.00117	0.00059	0.00029	0.00015	0.00007	0.00004	0.00002	0.00001	0.00000
	0.00117	0.00059	0.00029	0.00015	0.00007	0.00004	0.00002	0.00001	0.00000	0.00000	0.00000
	0.00029	0.00015	0.00007	0.00004	0.00002	0.00001	0.00000	0.00000	0.00000	0.00000	0.00000
	0.00007	0.00004	0.00002	0.00001	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000

Lactobacillus cell free supernatant µl

0.00000											
25.00000	6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001
12.50000	3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000
6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000
3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000
1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000
0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000	0.00000
0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000	0.00000

(c) PA01172

Ciprofloxacin µg/ml

0.000	1.2000	0.6000	0.3000	0.1500	0.0750	0.0375	0.0188	0.0094	0.0047	0.0023	0.0012
	0.3000	0.1500	0.0750	0.0375	0.0188	0.0094	0.0047	0.0023	0.0012	0.0006	0.0003
	0.0750	0.0375	0.0188	0.0094	0.0048	0.0023	0.0012	0.0006	0.0003	0.0002	0.0001
	0.0187	0.0093	0.0047	0.0023	0.0013	0.0006	0.0003	0.0002	0.0001	0.0001	0.0001
	0.0046	0.0023	0.0012	0.0006	0.0003	0.0002	0.0001	0.0001	0.0000	0.0000	0.0000
	0.0012	0.0006	0.0002	0.0002	0.0001	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0003	0.0002	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0001	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Lactobacillus cell free supernatant µl

0.00000											
25.0000	6.250000	1.562500	0.390625	0.097656	0.024414	0.006104	0.001526	0.000382	0.000095	0.000024	0.000006
12.5000	3.125000	0.781250	0.195313	0.048828	0.012207	0.003052	0.000763	0.000191	0.000048	0.000012	0.000003
6.25000	1.562500	0.390625	0.097656	0.024414	0.006104	0.001526	0.000382	0.000095	0.000024	0.000006	0.000002
3.12500	0.781250	0.195313	0.048828	0.012207	0.003052	0.000763	0.000191	0.000048	0.000012	0.000003	0.000001
1.56250	0.390625	0.097656	0.024414	0.006104	0.001526	0.000381	0.000095	0.000024	0.000006	0.000002	0.000000
0.78125	0.195313	0.048828	0.012207	0.003052	0.000763	0.000191	0.000048	0.000012	0.000003	0.000001	0.000000
0.39063	0.097656	0.024414	0.006104	0.001526	0.000381	0.000095	0.000024	0.000006	0.000002	0.000000	0.000000

(d) PA01

Trimethoprim µg/ml

0.00000	256.000	128.000	64.0000	32.0000	16.0000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000
	64.0000	32.0000	16.0000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250
	16.0000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563
	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391
	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098
	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024
	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006
	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002

Lactobacillus cell free supernatant µl

0.00000											
25.0000	6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001
12.5000	3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000
6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000
3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000
1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000
0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000	0.00000
0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00009	0.00002	0.00001	0.00000	0.00000	0.00000

(e) PA01172

Trimethoprim μg/ml

0.00000	64.0000	32.0000	16.0000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250
	16.0000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563
	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391
	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098
	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024
	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006
	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002
	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002	0.00001	0.00000

Lactobacillus cell free supernatant μl

0.00000											
25.0000	6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001
12.5000	3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000
6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000
3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000
1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000
0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000	0.00000
0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000	0.00000

(f) PA01

Chloramphenicol µg/ml

0.00000	128.0000	64.00000	32.00000	16.00000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500
	32.0000	16.0000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125
	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781
	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195
	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049
	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012
	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003
	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002	0.00001

Lactobacillus cell free supernatant µl

0.00000											
25.0000	6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001
12.5000	3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000
6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000
3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000
1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000
0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000	0.00000
0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000	0.00000

(g) PA01172

Chloramphenicol µg/ml

0.00000	16.0000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563
	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391
	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098
	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024
	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006
	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002
	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002	0.00001	0.00000
	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002	0.00001	0.00000	0.00000	0.00000

Lactobacillus cell free supernatant µl

0.00000											
25.0000	6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001
12.5000	3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000
6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000
3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000
1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000
0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000	0.00000
0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000	0.00000

(h) PA01

Carbenicillin µg/mL

0.0000	64.0000	32.0000	16.0000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250
	16.0000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563
	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391
	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098
	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024
	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006
	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002
	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002	0.00001	0.00000

Lactobacillus cell free supernatant µl

0.000											
25.00	6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001
12.50	3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000
6.250	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000
3.125	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000
1.562	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000
0.781	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000	0.00000
0.390	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000	0.00000

(i) PA01172

Carbenicillin μg/mL

0.00000	128.00000	64.00000	32.00000	16.00000	8.00000	4.00000	2.00000	1.00000	0.50000
	32.00000	16.00000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500
	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125
	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781
	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195
	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049
	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012
	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003

Lactobacillus cell free supernatant μl

0.00000											
25.0000	6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001
12.5000	3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000
6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000
3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000
1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000
0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000	0.00000
0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000	0.00000

(k) PA01

Tetracycline µg/ml

0.00000	32.00000	16.00000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125
	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781
	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195
	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049
	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012
	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003
	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002	0.00001
	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002	0.00001	0.00000	0.00000

Lactobacillus cell free supernatant µl

0.00000											
25.0000	6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001
12.5000	3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000
6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000
3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000
1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000
0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000	0.00000
0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000	0.00000

(l) PA01172

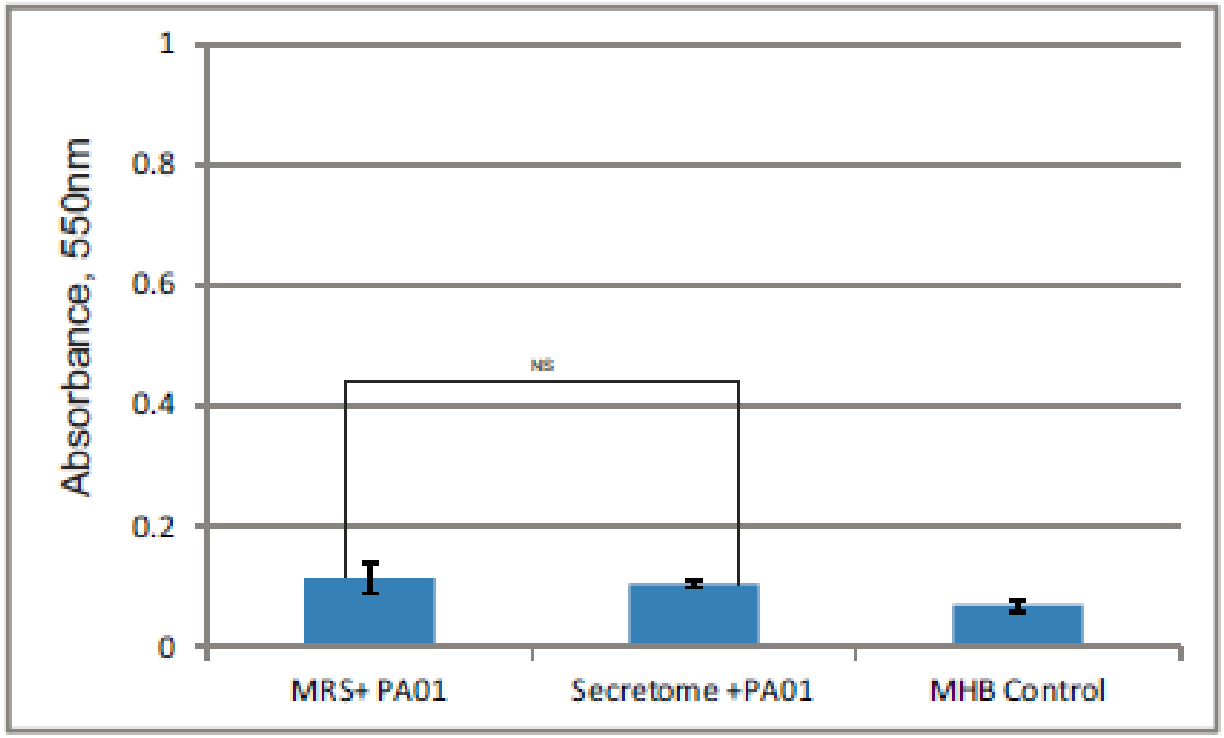
Tetracycline µg/ml

0.00000	8.00000	4.00000	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781
	2.00000	1.00000	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195
	0.50000	0.25000	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049
	0.12500	0.06250	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012
	0.03125	0.01563	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003
	0.00781	0.00391	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002	0.00001
	0.00195	0.00098	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002	0.00001	0.00000	0.00000
	0.00049	0.00024	0.00012	0.00006	0.00003	0.00002	0.00001	0.00000	0.00000	0.00000	0.00000

Lactobacillus cell free supernatant µl

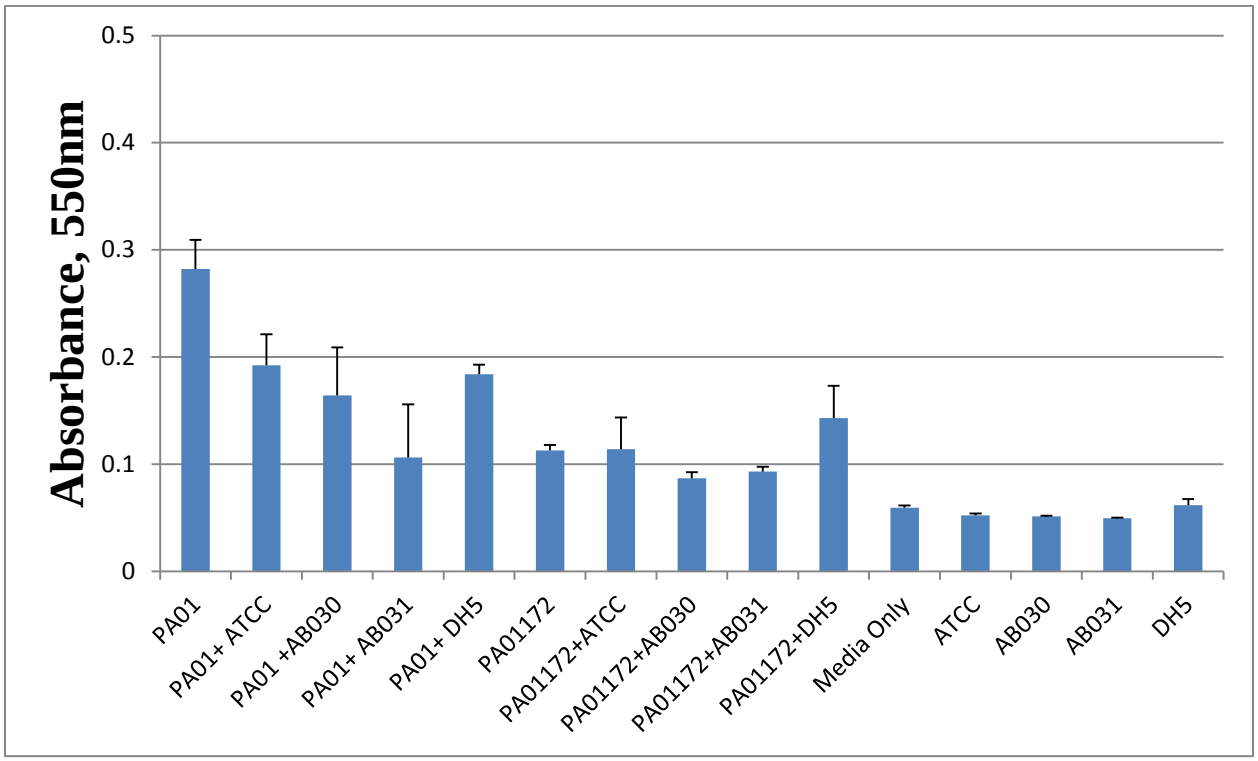
0.00000											
25.00000	6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001
12.50000	3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000
6.25000	1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000
3.12500	0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000
1.56250	0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000
0.78125	0.19531	0.04883	0.01221	0.00305	0.00076	0.00019	0.00005	0.00001	0.00000	0.00000	0.00000
0.39063	0.09766	0.02441	0.00610	0.00153	0.00038	0.00010	0.00002	0.00001	0.00000	0.00000	0.00000

APPENDIX B. The effect of cell free supernatant from *Lactobacillus rhamnosus* has on *P. aeruginosa* biofilm formation. Biofilm plates were prepared accoding to the materials and methods.



* p≤ 0.05
† p≤ 0.01
‡ p ≤ 0.001
NS not significant

APPENDIX C . The effect of cell free supernatant from *Acinetobacter baumanii* and *E. coli* strains on the production of biofilm in *P. aeruginosa*. Biofilm formation was measured with the addition of cell free supernatant from ATCC 19606, AB030, AB031 and DH5α. Data shown is representative of a minimum of two biological replicates.



APPENDIX D. Letter of permission from the Journal of Bacteriology to reprint Figure 17c from Nehme and Poole, 2007.

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Title: Assembly of the MexAB-OprM Multidrug Pump of Pseudomonas aeruginosa: Component Interactions Defined by the Study of Pump Mutant Suppressors

Author: Dominic Nehme, Keith Poole

Publication: Journal of Bacteriology

Publisher: American Society for Microbiology

Date: Sep 1, 2007

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APPENDIX E. Pull-down assay using Ni-NTA resin column chromatography and silver staining protocol.

Pull-Down Assay Using Ni-NTA Resin Column Chromatography

Pull down assays were performed using methods described by Pierce Biotechnologies, 2008 (Pierce Biotechnologies, 2008) and Cuenca *et al.* (Cuenca, *et al.*, 2003). Overnight cultures were subcultured 1:100 v/v into 1 L LB and grown to an A_{600nm}= 1.0, appropriate antibiotic and 1 mM IPTG was added at an A_{600nm}= 0.6 when required. Cultures were centrifuged at 4740 x *g* for 10 minutes at 4 °C and the resulting pellet was frozen overnight at -20 °C. Pellets were thawed and re-suspended in 100 mL cross-linking buffer (150 mM NaCl, 20 mM sodium phosphate, pH 7.2) followed by the addition of Dithiobis [succinimidylpropionate] (DSP) (0.2 mM). The solution was incubated for 30 mins at 37 °C before being quenched with 125 mL Tris-HCl (pH 7.5). Cells were harvested by centrifugation at 4740 x *g* for 10 mins at 4 °C, re-suspended in 150 mL 1 M Tris-HCl (pH 7.4), lysed by French Press (2000 psi) and ultra-centrifuged at 105000 x *g* for 1 hr at 4 °C. The ultra-centrifuge pellet was resuspended in 2 x 2 mL (4 mL total) solubilisation buffer (20 mM sodium phosphate, 0.5 M NaCl, 8 M Urea, 10 mM Imidazole, 0.1% w/v SDS, pH 7.4), and this material was designated "column starting material". Ni-NTA columns were prepared using 3 mL Ni-NTA resin (Fisher, Rockford, IL, USA), according to the manufacturers instruction, in brief, resin was degassed under vacuum in equilibration buffer (20 mM sodium phosphate, 300 mM NaCl, 10 mM Imidazole, 8 M Urea, pH7.4) and poured using a glass rod to limit gas within the matrix. The columns were equilibrated with no less than 10 column volumes of equilibration buffer. Column starting material was applied to the column and incubated for 1 hr at room temperature. Unbound protein was eluted with 2 column volumes equilibration buffer, and designated as column flow through. Loosely bound proteins were eluted with 10 mL of wash buffer (0.5 M NaCl, 20 mM Tris, 10 mM Imidazole, 0.1% w/v SDS, 8 M Urea, pH 8.0) and designated wash flow through. Bound protein was eluted with elution buffer (50 mM Tris, 8 M Urea, 2% w/v SDS, 0.4 M Imidazole, pH 6.8), in 0.5 mL fractions (10), and assessed for purity using SDS-PAGE with silver staining, or immunoblotting. Any protein still bound to the column was removed with 10

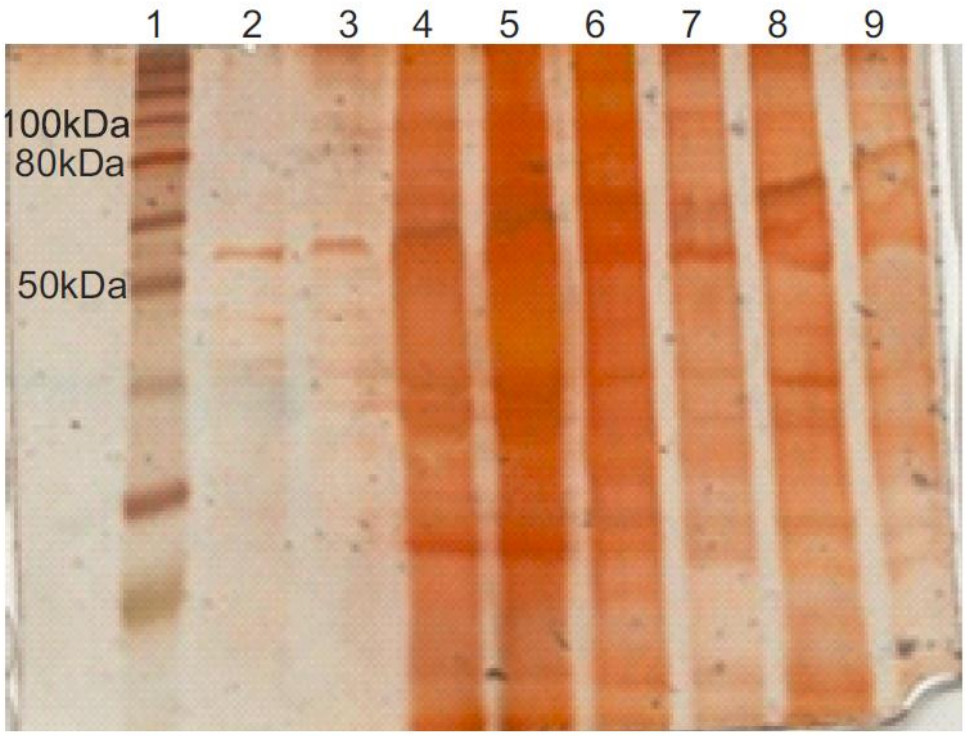
column volumes of MES buffer (20 mM 2-[N-morpholine]-ethanesulfonic acid, 0.1 M NaCl, pH 5.0), followed by 10 column volumes of distilled water. Columns were stored in two column volumes of column storage solution (20% (v/v) ethanol in distilled water). Columns were regenerated before use with 10 column volumes of MES buffer, followed by 10 column volumes distilled water, and were re-equilibrated as described above.

Silver Staining

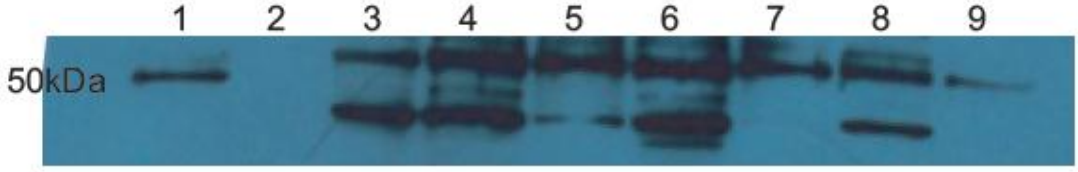
Silver staining was performed on polyacrylamide gels according to Merril *et al.* (Merril, *et al.*, 1981). Briefly, 200 mL of prefix solution (50% v/v methanol, 10% v/v ethanol, and 40% v/v distilled water) was added to the gel and heated in a microwave oven (Danby designer model) for 1.5 minutes at 50% power. The gel was removed from the microwave and shaken for approximately 10 seconds every 30 seconds. The gel was then incubated in the prefix solution for 2 mins at room temperature with shaking. Prefix solution was decanted and replaced with 200 mL of distilled water and microwaved for 2 minutes at 50% power. The gel was removed from the microwave and shaken for 10 secs every 30 secs. The gel was then incubated in distilled water for 2 ms at room temperature with shaking. Water was decanted and replaced with a 100 µM dithiothreitol (DTT) solution and heated in the microwave oven as above for 2 minutes. The gel was then incubated in the DTT solution for 2 mins at room temperature with shaking. DTT solution was decanted and replaced with a silver nitrate solution (0.1% w/v in 200 mL distilled water) and heated in a microwave oven for 1.5 minutes as described above. The silver nitrate was decanted, the gel was washed twice with 200 mL of distilled water and replaced with 200 mL of sodium carbonate (3% w/v sodium carbonate, 0.019% v/v formaldehyde) and incubated at room temperature with shaking until the protein bands reached desired intensity. Developer solution was decanted and replaced with 10 mL of 2.3 M citric acid solution for 1 minute with shaking to halt the reaction. Citric acid solution was decanted and the gel was washed with 200 mL of distilled water. The gel was stored in a 0.03% w/v sodium carbonate solution for no less than 1 hour prior to being photographed.

APPENDIX F. Pull down assay of induced OpmH in the presence and absence of triclosan**.** (a) Silver stain of SDS-PAGE with the following samples: 1. Ladder. 2. triclosan sample. 3. No triclosan sample. 4. pET1.6-OpmH with 1.0mM IPTG. 5. pET1.6 without 1.0mM IPTG. 6. Starting material with triclosan. 7. Starting material without triclosan. 8. Supernatant with triclosan. 9. Supernatant without triclosan. (b) Immunoblot were probed with a 1:10000 dilution of polyclonal Goat α-OpmH antibody, and subsequently probed with a 1:20000 dilution of (HRP)-conjugated mouse α-goat antibody. 1. Triclosan sample. 2. No triclosan sample. 3. pET1.6-OpmH with 1.0mM IPTG. 4. Starting material with triclosan. 5. Starting material without triclosan. 6. Supernatant with triclosan. 7. Supernatant without triclosan. 8. Pellet with triclosan. 9. Pellet without triclosan.

(a)



(b)



APPENDIX G. Additional triclosan MIC data for chimeric proteins**.**

Strain	**Efflux Components Expressed**	**Copy Number of OMP Gene**	**Triclosan (µg/mL)**	
			-IPTG	+IPTG
PA071	MexJ,MexK, OprM, Helix 1	Single	2	2
PA072	MexJ, MexK, OprM, Helix 3	Single	2	8
PA073	MexJ, MexK, OprM, Helix 1 to Helix 3	Single	2	2
PA074	MexJ, MexK, OprM, Helix 1 and Helix 3	Single	2	2
PA050	MexJ. MexK	None	2	2
PA051	MexJ,MexK, OpmH	Single	2	64
PA058	MexJ, MexK, OprM	Single	2	2