SINGLE COPY GENE EXPRESSION SYSTEM AS A TOOL FOR THE PURIFICATION OF MEMBRANE PROTEINS FROM *PSEUDOMONAS AERUGINOSA*

SUBMITTED BY

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ABSTRACT

Pseudomonas aeruginosa is a Gram-negative opportunistic human pathogen known to cause a variety of infections that are difficult to treat due to extremely high resistance to almost all antibiotics currently in clinical use. One of the major contributors to this resistance is the active efflux of antibiotics from the cell, primarily by action of the Resistance Nodulation Division (RND) family of efflux pumps. These pumps are composed of three proteins; an inner membrane RND pump, a periplasmic membrane fusion adaptor protein, and an outer membrane protein. The mechanism by which the three proteins interact to form a functional complex is largely unknown and the methods currently available for their study involves expression systems geared for high levels of expression. In the case of membrane proteins which play a role in clinically relevant activities, such as multidrug resistance, an expression system which does not always reflect biologically relevant levels of protein in the cell is not ideal for studying their interactions as correlation of conclusions from interaction studies to true interactions may not be possible. In this study a single copy gene expression system was designed and demonstrated to better reflect clinically relevant levels of overexpression compared to a multi-copy expression system. Quantitative-real time PCR analysis of C-terminally hexa-histidine tagged outermembrane protein, OpmH, expression shows approximately 100-fold and 20-fold overexpression from multi-copy and single-copy expression systems respectively. OpmH-H_6 was successfully purified from both multi copy and single copy expression systems with proportionate purification schemes indicating the feasibility of single copy expression systems for the study of membrane bound protein complexes.

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LIST OF ABBREVIATIONS

aacC1	acetyltransferase 3-1 encoding gene conferring Gm-resistance
Amp	ampicillin
bla	gene encoding *β*-lactamase
bp	base pair(s)
Cb	carbenicillin
CLSI	Clinical and Laboratory Standards Institute
dNTP	deoxyribonucleoside triphosphate(s)
Flp	*Saccharomyces cerevisiae* recombinase enzyme
FRT	Flp Recombinase Target
Gm	Gentamicin
IPTG	Isopropyl-β-D-thiogalactoside
H_6	Hexa-histidine tag
Kb	Kilobase(s) or 1000 bp
kDa	Kilodalton
LB	Luria–Bertani (medium)
$lacI^q$	*lacI* gene with q promoter-up mutation
MHB	Muller Hilton Broth
MIC	Minimum inhibitory concentration
MW	Molecular weight
NRT	No reverse transcriptase control

NTC	No template control
OD_{600}	Optical Density at 600nm
ori	origin of replication
oriT	origin of transfer
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate buffered saline – 0.1% Tween 20
PCR	Polymerase Chain Reaction
^{r}	Resistance/resistant
RND	Resistance Nodulation Division
RT	room temperature
sacB	*Bacillus subtilis* levansucrase-encoding gene
SDS	Sodium dodecyl sulphate
sec	second(s)
T7 Pol	T7 Polymerase
Tc	Tetracycline
v/v	Volume / volume
w/v	Weight / volume
Xg	x gravity
X-gal	5-bromo-4-chloro-3-indolyl-β-D -galactopyranoside

I

INTRODUCTION

A *PSEUDOMONAS AERUGINOSA*: CLINICAL RELEVANCE AND ANTIBIOTIC RESISTANCE

Pseudomonas aeruginosa is an opportunistic human pathogen that is most well known for its ability to infect immune-compromised individuals including Cystic Fibrosis (CF) patients. *P. aeruginosa* colonizes the respiratory tract and is often associated with high rates of mortality. (Vettoretti *et al*. 2009) In the case of critically ill patients, risk factors of nosocomial infection by *P. aeruginosa* include prolonged hospitalization, exposure to antimicrobial therapy and possibility of HIV infection (Obritsch *et al*. 2005).

It is widely agreed that the reason for the prevalence of this organism in hospitals and clinics is due to its intrinsic and acquired multidrug resistance. (Poole and Srikumar, 2001) Long-term monitoring and characterization of Gram-negative bacilli present in intensive care units across various health centers in the United States found *P. aeruginosa* to be the most frequently isolated organism, and that its multidrug resistance (defined as resistance to three or more different classes of antibiotics; in this case extended-spectrum cephalosporins, aminoglycosides and a fluoroquinolone) increased more than 5-fold within the 12-year period of the study - more than any of the other isolated organisms (Lockhart *et al*. 2007). A nation-wide study also found *P. aeruginosa* to be one of the most commonly isolated Gram-negative pathogens in Canadian hospitals, and again the only species displaying a multidrug resistant phenotype (Zhanel *et al*. 2008). The development and prevalence of multidrug resistance renders treatment options ineffective causing infections by these organisms more dangerous than before. Although difficult to track, many groups have reported a positive correlation between multidrug resistance in hospital acquired infections of multidrug resistant Gram-negative pathogens, including *Pseudomonas spp.*, and costs to health care (Lautenbach *et al*. 2010, Mauldin *et al*. 2010).

i. *Mechanisms of antibiotic resistance*

Antibiotic resistance in Gram-negative bacteria, including *P. aeruginosa*, is the direct result of multiple mechanisms operating in parallel including a) low permeability of outer membrane b) inactivation of antibiotics by various enzymes; ie. beta-lactamase, aminoglycoside modifying enzymes, and tetracycline and macrolide-inactivating enzymes c) alteration of target and d) active efflux of compound from the cell (Kumar and Schweizer. 2005). Of the above mentioned mechanisms, the most clinically relevant intrinsic antibiotic resistance can be attributed to synergism between active drug efflux and reduced drug uptake due to low permeability of the outer membrane (Li *et al*. 1994). Efflux of drugs has been widely studied in both Gram-negative and Gram-positive organisms, and has been found to be widespread throughout many bacterial species. To date there are five different types of efflux systems: a) major facilitator superfamily (MFS) (Pao *et al*. 1998) b) ATP-binding cassette superfamily (ABC) (van Veen and Konings. 1998) c) small multidrug resistance family (SMR) (Chung and Saier. 2001) d) Resistance Nodulation Division superfamily (RND) (Nikaido and Zgurskaya. 2001) and e) Multidrug And Toxic compound Extrusion family (MATE) (Miyame *et al*. 2001). Of these transporters, the RND superfamily is one of the best characterized due to its contribution to intrinsic antimicrobial resistance, in Gram-negative bacteria.

B RESISTANCE NODULATION DIVISION PUMPS

i. *Structure*

An RND efflux system spans the entire bacterial cell envelope and are composed of three proteins as depicted in Figure 1 below: an inner membrane RND pump, a periplasmic membrane

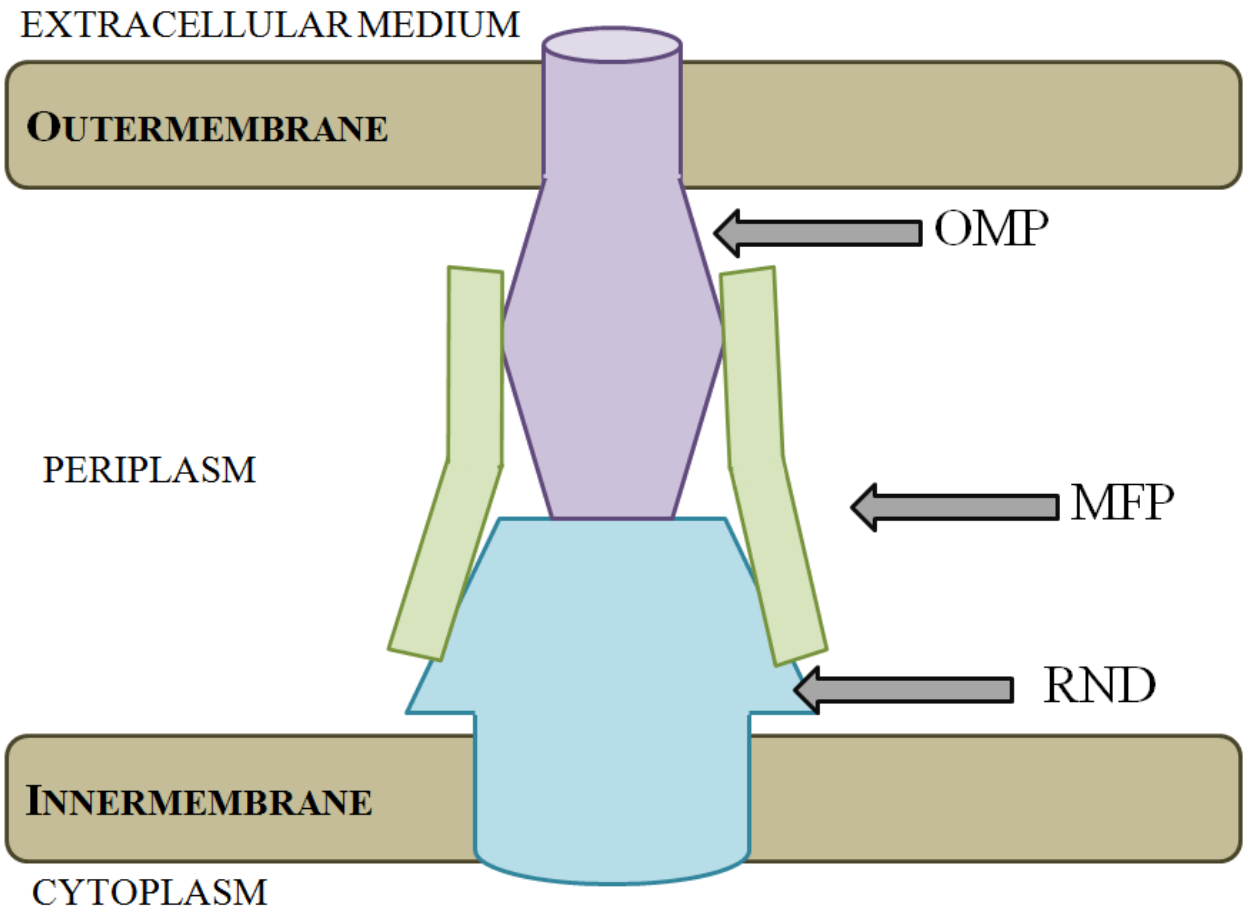
fusion protein (MFP) and an outer membrane protein (OMP) (Zgurskaya and Nikaido. 2000). These proteins are genetically encoded in operons where the MFP is encoded first, followed by the RND and finally the OMP; a regulator is sometimes encoded in the opposite direction, upstream of the MFP. An example of this is the MexAB-OprM RND efflux system of *P. aeruginosa* (Li *et al*. 1995), where *mexA*, *mexB* and *oprM* are the MFP, RND, and OMP respectively; and this system is negatively regulated by a transcriptional repressor *mexR*, encoded upstream of *mexA* (Srikumar *et al*. 2000). However, some operons consist of the MFP and RND alone, while some OMPs are found to be 'orphans', that is, they are not encoded in an operon with a cognate MFP and RND. An example of this is the AcrAB-TolC efflux complex of *E. coli*, a close homolog of the MexAB-OprM system, where *acrA* and *acrB* are encoded in an operon with their local repressor *acrR* (Ma *et al*. 1996), while the OMP found to complete the RND system, *tolC* (Fralick 1996) is found elsewhere in the *E. coli* genome as a standalone protein.

ii. *Mechanism*

Much insight into the pumping mechanism of RND efflux pumps in general; including substrate binding, tunnelling, and opening of the channel; has been derived from the crystal structures of the AcrAB-TolC complex of *E. coli* and presumed to function similarly in homologous RND pumps in various organisms. The crystal structure of AcrB, revealed the homo-trimeric nature of this protein, and that each trimer had a different confirmation differing primarily in the affinity of the substrate binding pocket for substrate and the orientation of the

Figure 1 Tri-partite composition of an RND-efflux system. The RND pump (blue), traversing the inner membrane is a drug-proton antiporter. The adaptor-like structure (green) is the periplasmic protein that serves to stabilize the interaction between the inner and outer membrane structures, known as the Membrane Fusion Protein (MFP). Finally, the channel-like structure forming a pore in the outer membrane (purple) is the Outer Membrane Protein (OMP).

Figure 1.


EXTRACELLULAR MEDIUM
OUTERMEMBRANE
OMP
PERIPLASM
MFP
RND
INNERMEMBRANE
CYTOPLASM

tunnel leading from the substrate binding pocket to the opening toward the channel formed by the OMP (Murakami *et al*. 2006). It is thought that each monomer cycles through each confirmation and in doing so will bind, channel and efflux a compound (Murakami *et al*. 2006). The crystal structure of Tol C (Koronakis *et al*. 2000), and OprM (Akama *et al*. 2004), and OMP from *P. aeruginosa* revealed that this structure was also composed of a homo-trimer, and more over the channel it forms is closed at the periplasmic end. Finally, the MFP is classically thought to serve as an adaptor, securing the interaction between the other two components of the complex, however recent studies indicate a role in opening of OMP channel (Janganan *et al*. 2010, Weeks *et al*. 2010).

Studies using chimeric RND proteins or heterologous efflux complexes have identified importance of the periplasmic loops of the RND transporter in substrate selectivity (Tikhonova *et al*. 2002, Elkins and Nikaido. 2002, Mao *et al*. 2002). Systematic mutagenesis studies have identified residues in AcrB for recognition and binding of substrate (Bohnert *et al*. 2008) and residues in TolC mediating interaction with AcrA (Lobedanz *et al*. 2007). Gain-of-function suppressor studies have identified residues in MexB that are important for mediating interaction with MexA (Nehme and Poole. 2005) and OprM (Middlemiss and Poole. 2004, 2007).

With the body of knowledge available, many models of the steps leading from substrate recognition to expulsion have been proposed (Murakami et a. 2006, Fernandez-Reico *et al*. 2004, Pietras *et al*. 2009), leading to the most recent model proposed by Weeks JW and colleagues, (Weeks *et al*. 2010) which describes a 5-step mechanism by which substrate binding leads to opening of the OMP via conformational changes communicated through the MFP. This model, while reasonable, would be strengthened with more evidence from a wider range of RND efflux complex models.

iii. *Clinical significance*

While AcrAB-TolC and MexAB-OprM of *E. coli* and *P. aeruginosa* have served as models to understand the mechanism and assembly of these RND efflux complexes, they are wide-spread amongst gram-negative organisms. The AdeABC (Magnet *et al*. 2001), AdeIJK (Damier-Piolle *et al*. 2008) and AdeFGH (Coyne *et al*. 2010) RND efflux pumps of *Acinetobacter baumannii* have been shown to mediate intrinsic and acquired multidrug resistance. The BpeAB-OprB (Chan et al. 2004), AmrAB-OprA (Moore et al. 1999), BpeEF-OprC (Kumar et al. 2006), BpeGH-OprD (as well as three other as yet un-characterized RND efflux systems of the Gram-negative pathogen *Burkholderia pseudomallei* were found to be expressed in 90% of isolates tested, with 82% of these isolates expressing more than one pump (Kumar *et al*. 2008). Efflux of drugs has been found to play a role in intrinsic multidrug resistance of *Klebsiella pneumoniae* (Aathithan and French. 2011); and some of this activity is attributed to the action of AcrAB efflux system (Bialek-Davenet *et al*. 2011). RND efflux systems, while mediating antimicrobial resistance in *Vibrio cholerae,* was also found to be required for production of virulence factors and colonization of infant mice (Bina *et al*. 2008).

iv. *Tools for drug discovery*

Due to their presence in the clinically relevant pathogen and role in multidrug resistance, these RND pumps are an attractive option for new therapeutic targets; particularly in the growing field of Efflux Pump Inhibitors (EPIs), a compound meant to be used in combination with an antibiotic to increase its efficacy (Misra and Bavro. 2009). The complex nature of these tri-partite pumps allow for a variety of mechanisms, as described in Figure 2, to inhibit their correct

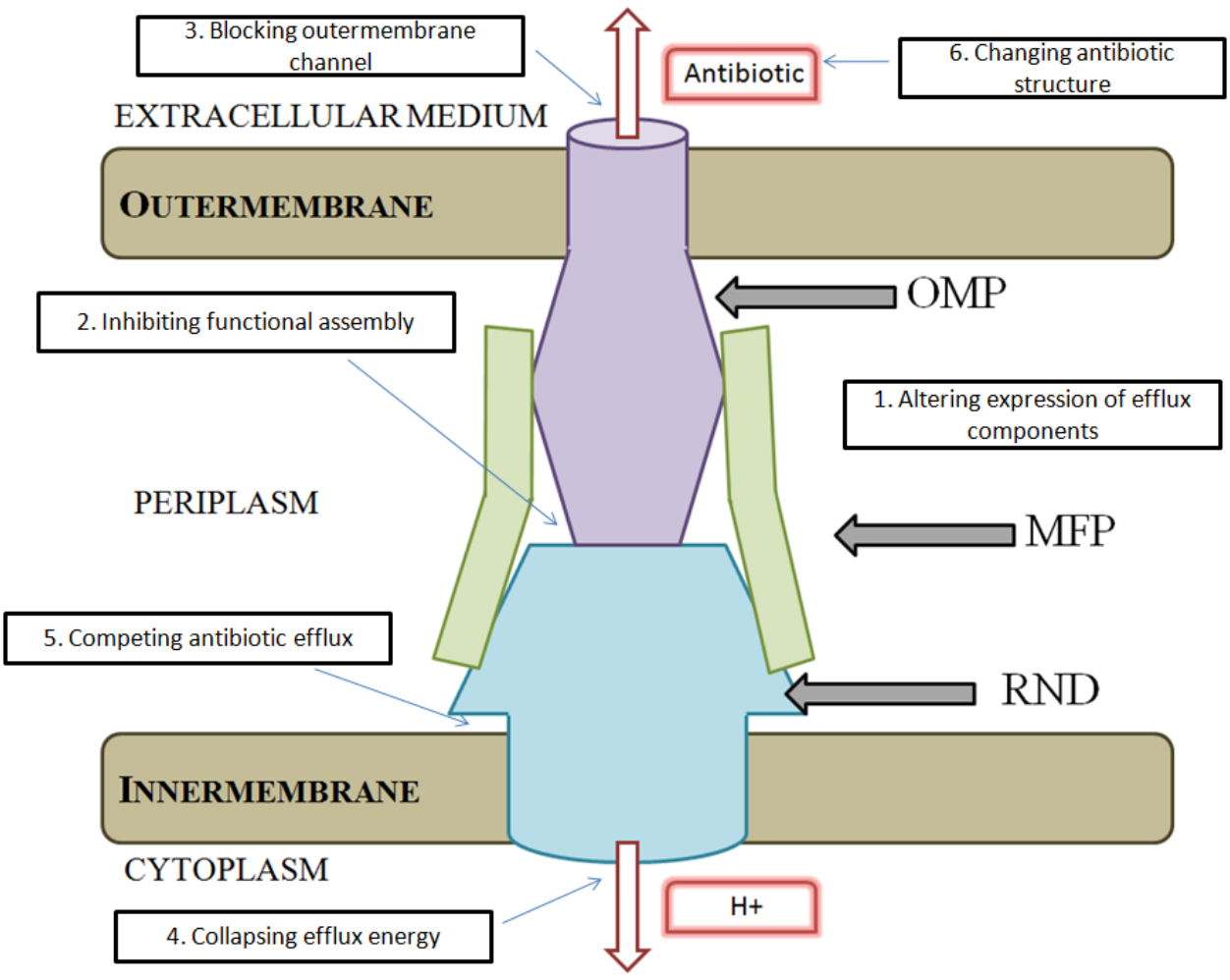
function in binding and extruding an antibiotic. Phe-Arg-β-Napthylamide (PAβN), discovered in a screen looking for compounds to poteintiate the activity of levofloxacin in *P. aeruginosa* (Renau *et al*. 1999) is an example of an EPI that works by competitively binding the substrate-binding pocket and being effluxed while allowing antibiotic to remain inside the cell and take effect on its target. While extremely potent as an EPI, it cannot be used clinically due to toxic effects (Olga Lomovskaya, personal communication). It does however provide a foundational structure from which to launch more studies into discovery of more effective and potent compounds for the use of efflux pump inhibition by way of substrate-binding competition. However, as with any system, much more needs to be understood about the way these pumps function in order to effectively inhibit their action.

v. *Knowledge gaps*

Despite the amount of information available on the structures of each component there remain many gaps and unanswered questions as to the *in vivo* functioning of these tri-partite complexes. Although some residues have been identified to mediate the proton relay network in AcrB (Takatsuka and Nikaido. 2006, Guan and Nakae. 2001), the sequential protonantion-deprotonation steps remain unclear, as well as the exact mechanism of drug/proton coupling. With respect to the drug transport mechanism a basic understanding of the kinetics of drug binding and expulsion and its relation to the energetic involved is lacking. Although the trimeric nature of the RND and OMP has been resolved, the stoichiometry with which the MFP fits in this complex has yet to be determined. The mechanism by which the components interact and assemble to form a functional complex, while several models have been postulated, remains

Figure 2. Mechanisms to target and inhibit the active efflux pump complex. (adapted from Pages and Amaral. 2009) 1. Change regulatory steps taken to express pump, particularly if pump has a characterized regulator. 2. Inhibit assembly of fully functional pump in bacterial cell envelope. 3. Plug the outer membrane channel to prevent escape of compound into extracellular matrix. 4. Breakdown or deplete energy source used for active efflux. 5. Out-compete the antibiotic binding sites with a non-antibiotic molecule to inhibit binding and subsequent efflux of antibiotic. 6. Altering chemistry of antibiotic such that it no longer has affinity for binding site.

Figure 2.


3. Blocking outermembrane channel
Antibiotic
6. Changing antibiotic structure
EXTRACELLULAR MEDIUM
OUTERMEMBRANE
OMP
2. Inhibiting functional assembly
1. Altering expression of efflux components
PERIPLASM
MFP
5. Competing antibiotic efflux
RND
INNERMEMBRANE
CYTOPLASM
H+
4. Collapsing efflux energy

relatively uncharacterized with very few studies examining the interactions between native complexes.

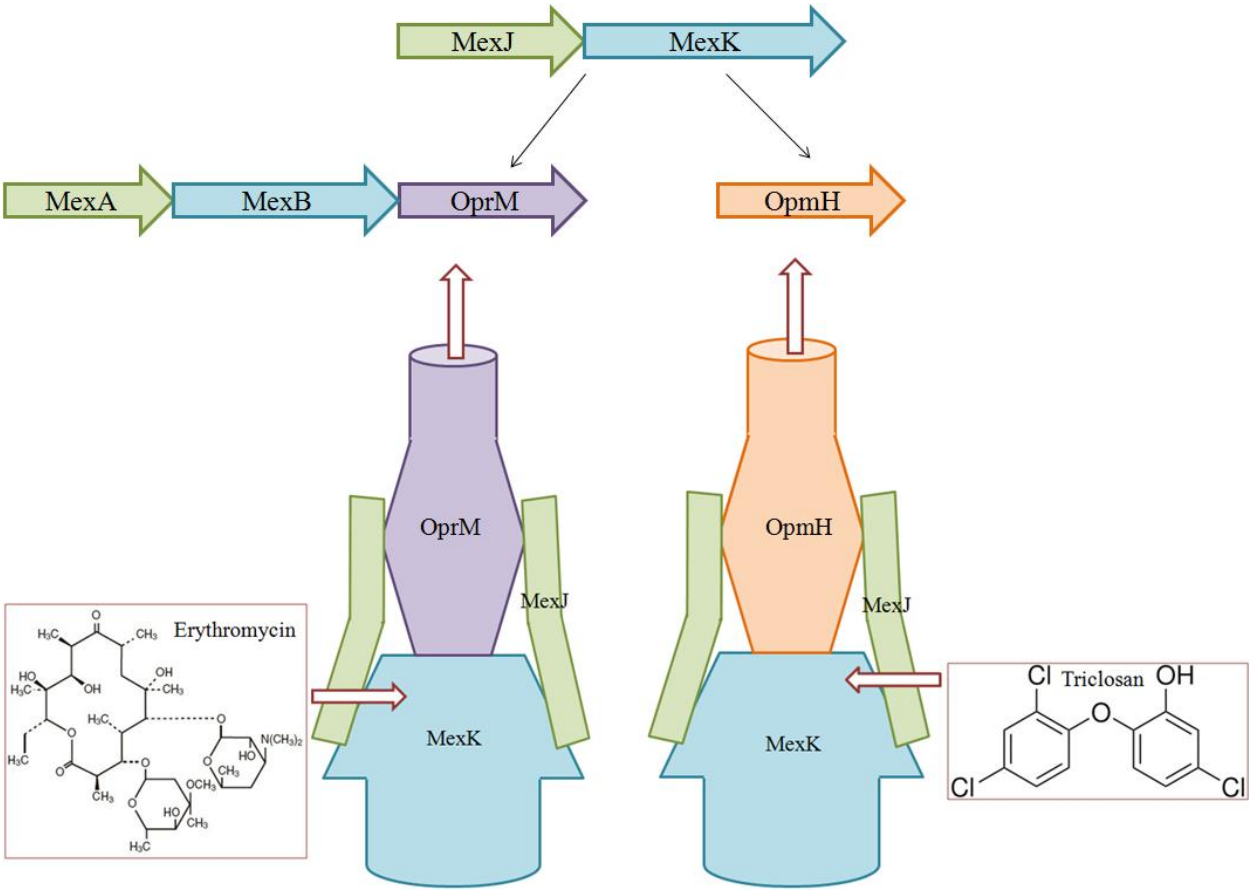
vi. *Suitability of MexJK-OpmH/OprM as model to study functional mechanism*

The MexJK efflux pump was identified by conferring triclosan resistance to a triclosan-sensitive strain of *P. aeruginosa*; which was deficient for the two main RND pumps responsible for triclosan efflux: MexAB-OprM and MexCD-OprJ (Chuanchuen *et al*. 2002). The reason this efflux complex is of interest is the specificity with which this interaction occurs; MexJK-OpmH complex expels triclosan while the MexJK-OprM complex expels erythromycin (Figure 3) (Chuanchuen *et al* 2005) and as seen by the antibiotic susceptibility data presented in this study. It is also notable that MexJK-OpmH does not expel erythromycin while MexJK-OprM is not capable of effluxing triclosan. The vast difference in chemical structure and size of these two antibiotics (Figure 3) indicate a difference in substrate binding and subsequent interaction between the components of the complex to efflux the compound.

Many OMPs are known to interact with multiple RND-MFP complexes. TolC, an OMP in *E. coli* is known to interact with various types of efflux pumps (Koronakis 2003), while OprM of *P. aeruginosa*, is known to interact with MexAB (Gotoh N *et al*. 1995) and MexXY (Mine et al. 1999). The MexJK complex is a novel example of an RND-MFP complex displaying promiscuity with respect to OMP partners. This raises the question of whether the two complementary complexes, MexJK-OpmH and MexJK-OprM, are formed constitutively but remain closed and open upon interaction with substrate or whether MexJK remains detached

Figure 3. Genetic organization of MexJK-OpmH/OprM efflux pump complex. The MexJK efflux pump is encoded in the *P. aeruginosa* as an operon lacking the outer membrane component. It has been found to form channels with o*pmH*, which is found elsewhere in the *P. aeruginosa* genome, not near any other RND gene; and with *oprM*, which is found in another RND operon.

Figure 3.


MexJ
MexK
MexA
MexB
OprM
OpmH
OprM
MexJ
MexK
OpmH
MexJ
MexK
Erythromycin
Cl
Triclosan
OH
O
Cl
Cl

from either OMP and presence of substrate induces the interaction. The selectivity of OMP for drug efflux, combined with the unlikely promiscuity displayed by the RND-MFP complex of this pump make MexJK-OpmH/OprM a prime model to study the influence of substrate binding on tri-partite pump formation.

As this type of study aims to understand whether an interaction will take place when two proteins are expressed in the same cell, it is important that conditions within that cell resemble the natural conditions of that cell. In spite of the plethora of genetic, biochemical and bio-informatic tools available to study these complexes, there remains a gap in the toolbox for studying interactions between these proteins at a biologically and clinically relevant level of expression.

vii. *Tools necessary to address knowledge gaps*

The study of bacterial membrane proteins, including that of RND efflux pumps in *P. aeruginosa*, is commonly carried out by tagging and expressing the recombinant protein from a plasmid based system in a host strain engineered for such expression. This system is generally geared for high levels of overexpression; ~800-fold increase in transcript levels with the classical pBluescriptII KS(-), a commonly used high-copy number cloning vector; (Choi *et al*. 2008) allowing for the purification and subsequent *in vitro* biochemical characterization of protein of interest. While popular due to the straightforward nature of this cloning and expression system, there exist a few inherent drawbacks that become significant when considering the study of membrane proteins.

The overexpression of membrane bound proteins has routinely proven to be more difficult due to effects on cell viability and low yield of protein (Wagner *et al*. 2009, Gubellini *et al*. 2011). Despite this, much knowledge has been gained by the expression of membrane proteins from medium to high-copy number vectors. Much of the work whereby one of the components of the RND efflux complex is mutagenized then assessed for ability to interact with cognate or non-cognate partner has been completed in this fashion (Tikhonova *et al*. 2002, Krishnamoorthy *et al* 2009). While appropriate for determination of ability to interact, this type of expression system may not be ideal for the study of native interactions between proteins.

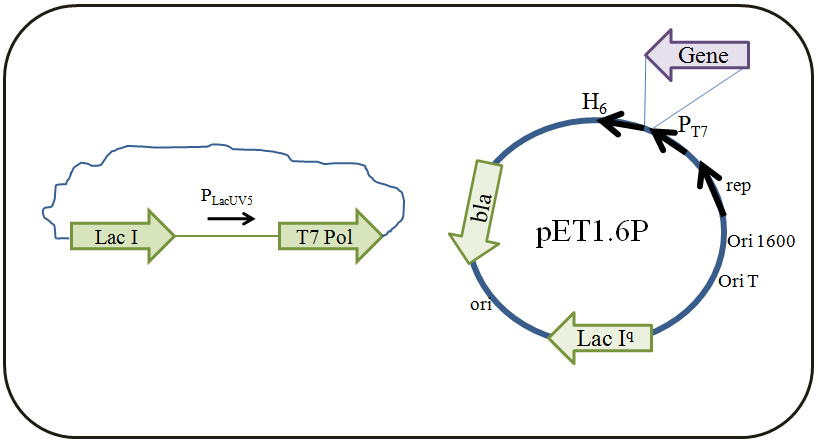
The levels of overexpression of resistance conferring proteins as detected in clinical isolates compared to wild type strains is approximately 2-10-fold as seen in *K. pneumoniae* strains over-expressing *acrB* in laboratory selected and clinical strains displaying resistance to fluoroquinolones and cefoxitin (Bialak-Davenent *et al*. 2011), as such a plasmid based system resulting in much higher levels of expression will not accurately represent biological conditions. This consideration is key for studying interactions between protein complexes as correlation of conclusions from interaction studies to true interactions may not be possible.

An appropriate alternative would be to integrate the recombinant protein of interest into the chromosome of the host strain allowing for native levels of expression. Such a system was described using Tn*7* transposition (Choi *et al*. 2005) whereby the gene of interest is transposed into the *att7* site of the bacterial chromosome at a genetically neutral locus (Figure 4).

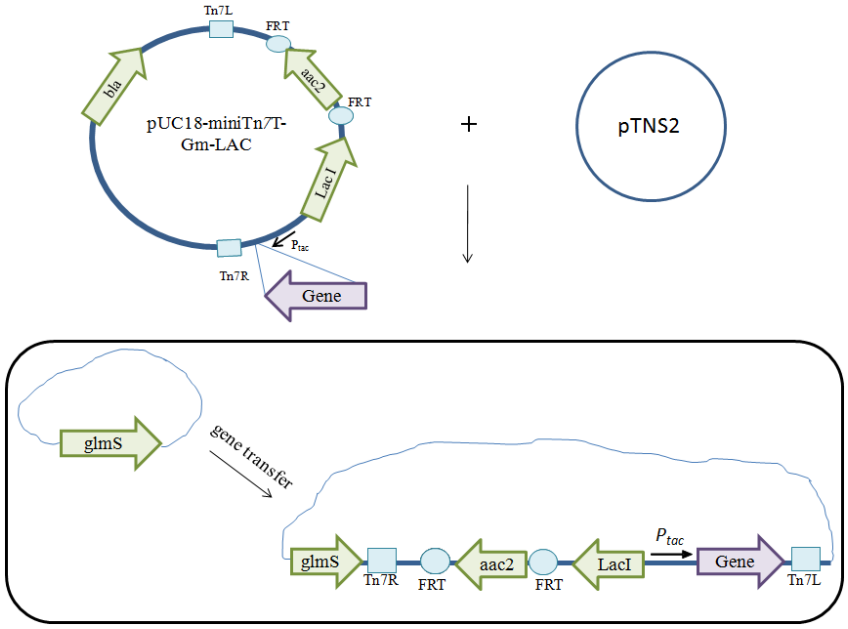
Figure 4. Systems for expression of recombinantly tagged proteins resulting in various levels of expression. (a) Components of pET expression system. Chromosomally encoded lacUV5 promoter and T7 RNA polymerase at the φCTX attachment site and plasmid encoded T7 promoter and His-tagged protein. LacI is natively transcribed, which will bind the lacUV5 promoter and repress T7 RNA polymerase expression. Upon addition of IPTG, LacI releases the promoter allowing for T7 polymerase expression, which subsequently recognizes the T7 promoter on the plasmid and transcribe the recombinant protein. It is advantageous that the lacUV5 promoter and T7 RNA polymerase are integrated into the chromosome, eliminating the need to utilize resources in the maintenance of an extra plasmid. However, the gene of interest must be maintained on a high-copy plasmid, thus requiring antibiotic selection in the medium. H_6 – hexa-histidine tag; P_{T7} – T7 promoter to which T7 RNA polymerase binds and starts transcription; ori_{1600} – origin of replication recognized by *P. aeruginosa*, *bla* – B-lactamase gene confers resistance to ampicillin (*E. coli)* and carbenicillin (*P. aeruginosa)*. **(b) MiniTn7 expression system.** MiniTn7 vector is incorporated into the att7 site just downstream of glmS gene, of which there is only one copy in *P. aeruginosa*. Transposase A, B, C, and D allow for insertion at this location specifically. Insertion events can be verified by PCR using primers that attach to the glmS gene and miniTn7 vector at the Tn7R site. LacI encoded lac repressor binds the P_{tac} promoter until addition of IPTG, whereby the promoter will be released allowing for native RNA polymerase to bind and transcribe the recombinant protein. Tn7R and Tn7L: attachment sites. FRT: FLP recombinase target sequences; to allow FLP-mediated excision of aacC1 gene, encoding gentamycin resistance. MCS: multiple cloning site. Ori: origin of replication not recognized by *P. aeruginosa*, making it a suicide vector in this organism.

Figure 4.

(a)


Gene
H6
PT7
rep
Ori 1600
Ori T
Lac Iq
ori
bla
pET1.6P
PLacUV5
Lac I
T7 Pol

(b)


Tn7L
FRT
aac2
FRT
bla
pUC18-miniTn7T-
Gm-LAC
LacI
Ptac
Tn7R
Gene
+
pTNS2
glmS
gene transfer
glmS
Tn7R
FRT
aac2
FRT
LacI
Ptac
Gene
Tn7L

C RESEARCH AIMS

HYPOTHESIS:

Single-copy gene expression system in *P. aeruginosa* will result in a lower yield of target protein, but better approximate biological and clinical levels of expression compared to a multi-copy gene expression system and therefore allow better means to study protein-protein interactions.

Therefore the objectives of this thesis are:

- Create two systems for expression of proteins in *P. aeruginosa*
- Validate and compare expression of proteins utilizing multi-copy and single-copy systems based on mRNA transcript levels, protein expression and phenotypic analysis
    - Multi-copy - pET1.6P vector
    - Single-copy - miniTn*7*T vector

II

MATERIALS AND METHODS

A BACTERIAL STRAINS, GROWTH AND CULTURE CONDITIONS

Bacterial strains used in this study is listed in Table 1. Bacteria were routinely cultured in Luria-Bertani medium (Biobasic Inc., Markham, ON, Canada) supplemented with appropriate antibiotics where necessary to maintain plasmids at the following concentrations: 100 μg/mL Ampicillin (Bioshop Canada Inc., Burlington, ON, Canada), 30 μg/mL Gentamycin (Bioshop Canada Inc., Burlington, ON, Canada), 200 μg/mL Carbenicillin (Bioshop Canada Inc. Burlington, ON, Canada). Counterselection for curing plasmids via the *sacB* gene was carried out in VBMM media (10mM Citric acid, 12mM Sodium citrate, 57mM Potassium phosphate dibasic, 17mM Sodium ammonium hydrogen phosphate tetrahydrate, pH 7.0, 0.1mM Calcium chloride, and 1mM Magnesium sulfate) supplemented with 10% sucrose (Bioshop Canada Inc., Burlington, ON, Canada) and induction of gene expression was accomplished by supplementing the growth medium with 0.5mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Biobasic Inc., Markham, ON, Canada). Antibiotic susceptibility assays were carried out in Mueller-Hinton media (Becton, Dickinson and Company, Sparks, MD, USA).

B DNA MANIPULATIONS

Plasmids and oligonucleotides used in this study are listed in Table 2 and Table 3 respectively. Plasmid DNA was extracted using EZ-10 Spin Column Plasmid Miniprep Kit (Biobasic Inc., Markham, ON, Canada) according to manufacturer's instructions. DNA amplified by polymerase chain reaction (PCR) or digested with restriction enzymes were purified by separation by 1.0% agarose gel electrophoresis and subsequent purification using EZ-10 Spin

Table 1. List of bacterial strains used in this study

Strain	Relevant Characteristics	Reference
***E. coli* Strains**		
DH5α	F- φ80d *lacZ*ΔM15 Δ(*lacZYA-argF) U169 deoR recA1endA1 hsdR17* (r_{k-}, m_{k+}) *phoA supE44 λ- thi-1 gyrA96 relA1*	Laboratory collection
JM109	*recA1, endA1, gyrA96, thi,hsd*R17(rK-, mK+), relA1,supE44, Δ(lac-proAB), [F',traD36, proAB, lacIqZΔM15]	Promega
***P. aeruginosa* Strains**		
PAO1	*P. aeruginosa* prototroph	Holloway and Zhang 1990
PAO238-1	PAO1: Δ*mexAB-oprM*, Δ*mexCD-OprJ* ↑MexJK	Chuanchuen *et al* 2002
PAO367	PAO238-1: Δ*mexXY*, Δ*opmH*	Chuanchuen *et al* 2005
PAO702	PAO367:miniCTX-T7polymerase	Kumar lab collection
PA048	PAO702:miniTn*7*T-Gm-Lac-*opmH*-H_6	This study
PA049	PAO702:miniTn7T-lac-*opmH*-H_6	This study
PA050	PAO702:miniTn*7*T-Gm-Lac	This study
PA051	PAO702:miniTn7T-Lac	This study

Table 2. List of plasmids used in this study

Plasmid	Relevant Characteristics	Reference
pCR2.1	Amp^r TA cloning vector	Invitrogen
pET21b	C-terminal his-tag, T7 polymerase	EMD Biosciences
pET1.6P	*P. aeruginosa* compatible pET21b	Choi *et al* 2008
pPS1667	pET1.6P-*opmH*-H_6	Choi *et al* 2008
pPS1668	pET1.6P-*oprM*-H_6	Kumar lab collection
pGEMT-easy	Amp^r TA cloning vector	Promega
pPLS032	pGEMT-easy-opmH-H_6	This study
pPLS037	pGEMT-easy-oprM-H_6	This study
pUC18-miniTn*7*T-Gm-Lac	Amp^r, removable Gm^r flanked by FRT sites, lacIq multiple cloning site downstream of P_{tac}	Choi *et al* 2005
pPLS040	miniTn*7*T-*opmH*-H_6	This study
pPLS038	miniTn*7*T-*oprM*-H_6	This study
miniCTX-T7pol-lacIq	Tc^r, T7 RNA polymerase delivery at φCTX attachment site	
pTNS2	Amp^r, R6K replicon, helper plasmid encoding site-specific TnsABCD Tn*7* transposition pathway	Choi and Schweizer 2005
pFLP2	Cb^r, source of *Flp* recombinase, *sacB* allows for removal of plasmid by sucrose counterselection	Hoang *et al* 1998

Table 3. List of oligonucleotides used in this study

Target Gene	Primer #	Name	Sequence (5' – 3')	Reference
Tn7R	72	Tn7R_Fwd	CACAGCATAACTGGACTGATTTC	Choi *et al* 2005
***glmS* (*P. aeruginosa*)**	73	Pa_glmS_Rev	GCACATCGGCGACGTGCTCTC	Choi *et al* 2005
pET1.6P	128	pET_Fwd-SpeI	GTTTAACTAGTAGAAGGAGATATAC	This Study
	129	pET_Rev-PstI	CTGCAGCTTCCTTTCGGGCTTTG	This Study
opmH	142	OpmH_Fwd_RT	AGTACCAGAAGGGCGACAAC	This Study
	143	OpmH_Rev_RT	ATCGGGATGTTCAGTTCCAG	This Study
rpsL	148	RpsL_Fwd_RT	GCAACTATCAACCAGCTGGTG	Mima *et al* 2009
	149	RpsL_Rev_RT	GCTGTGCTCTTGCAGGTTGTG	Mima *et al* 2009

Column Gel Extraction kit (Biobasic Inc., Markham, ON, Canada) according to the manufacturer's instructions. Concentration and quality of extracted DNA was measured by spectrophotometry using Eppendorf BioPhotometer model AG (Barkhausenweg, Hamburg, Germany) and agarose gel electrophoresis respectively.

i. *Making chemically competent E. coli DH5α cells*

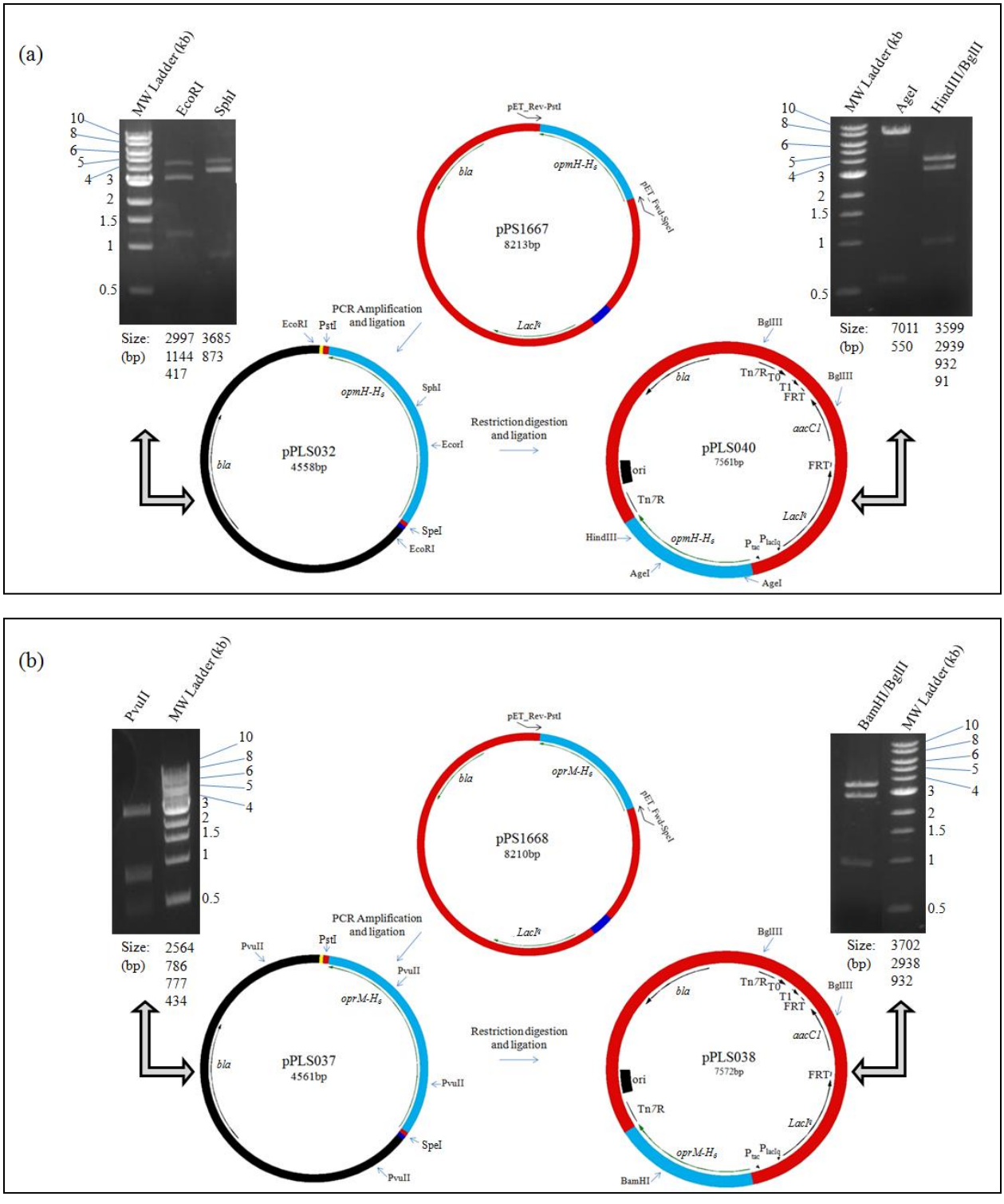
E. coli DH5α were cultured with a 1/50 inoculum from overnight culture and grown at 37˚C until mid-log phase (OD_{600} 0.4-0.6). Cells were harvested in a pre-chilled rotor at 3800xg, 5 min at 4˚C and re-suspended in 0.4x original culture volume of ice cold Transformation Buffer I (TFBI – 100mM rubidium chloride, 50mM manganese chloride, 30mM potassium acetate, 10mM calcium chloride, 15% w/v glycerol, pH 5.8). Mixture was incubated on ice for exactly 5 minutes, cells were harvested as before and re-suspended in 0.04x original culture volume of ice cold Transformation Buffer II (TFBII – 10mM 3-(N-morpholino) propanesulfonic acid, 10mM rubidium chloride, 75mM calcium chloride, 15% w/v glycerol, pH 6.5). Cells were incubated in TFBII for 30 min -1 hour then 100 µl aliquots were made and frozen immediately on dry ice and stored long-term at -80˚C.

ii. *Cloning opmH and oprM into pUC18-miniTn7T-Gm-Lac*

Hexa-histidine tagged genes were PCR amplified from previously constructed pET1.6P constructs using pET1.6_Fwd_SpeI and pET1.6_Rev_PstI primers (Fig 5a and 5b) with Taq DNA Polymerase (New England Biolabs, Pickering, ON, Canada) according to manufacturer's

Figure 5. Cloning His-tagged OMP into suicide vector miniTn*7*T. His-tagged genes **(a) OpmH-H_6 (b) OprM-H_6** were PCR amplified from respective pET1.6P vectors using primers that bind to the vector backbone and contain *Spe*I and *Pst*I restriction sites on the forward and reverse primers respectively. PCR products were gel cleaned and ligated into pGEM-T Easy vector and transformed into *E. coli* JM109 competent cells. After screening for confirmation of correct insert by restriction digest, plasmids were cut at indicated restriction sites and ligated to appropriately digested pUC18-miniTn*7*T-Gm-lac vector and transformed into *E. coli* DH5α competent cells. Plasmids were once again verified for correct insert by restriction digestion.

Figure 5.


(a)
MW Ladder (kb)
EcoRI
SphI
Size: (bp)
2997
1144
417
3685
873
pET_Rev-PstI
pPS1667
8213bp
opmH-Hs
pET_Fwd-SpeI
bla
LacIq
PCR Amplification and ligation
EcoRI
PstI
SphI
EcoRI
SpeI
pPLS032
4558bp
Restriction digestion and ligation
MW Ladder (kb)
AgeI
HindIII/BglII
Size: (bp)
7011
550
3599
2939
932
91
BglIII
Tn7R
T0
T1
FRT
aacC1
ori
Tn7R
HindIII
AgeI
Ptac
PlacIq
pPLS040
7561bp
(b)
PvuII
MW Ladder (kb)
Size: (bp)
2564
786
777
434
pPS1668
8210bp
oprM-Hs
PstI
PvuII
SpeI
pPLS037
4561bp
BamHI/BglII
3702
2938
932
pPLS038
7572bp
BamHI

instructions. Products were cloned using pGEMT-Easy® vector systems (Promega, Madison, WI, USA) according to manufacturer's protocol. Briefly, purified DNA was ligated into linearized pGEM-T Easy vector using T4 DNA Ligase, the reaction was incubated overnight at room temperature, the mixture transformed into competent *E. coli* JM109 cells and plated on LB medium containing 100µg/mL ampicillin and 40µg/mL X-Gal. Transformants were screened by plasmid extraction and digestion using *EcoR*I (pPLS032) and *Pvu*II (pPLS037).

The His-tag containing pGEM-T easy constructs and pUC18-miniTn7T-Gm-LAC were digested in a 20µl volume with the restriction enzymes *Spe*I and *Pst*I (New England Biolabs, Pickering, ON, Canada), and appropriate ratios of vector and insert were ligated using T4 DNA Ligase (New England Biolabs, Pickering, ON, Canada) and the reaction was incubated overnight at room temperature. The ligation mixture was added to an aliquot of competent *E. coli* DH5α cells (created as described above) and incubated on ice for 20-30 min. Cells were heat shocked by incubation at 42˚C for 90 sec, immediately placed on ice for 2 min and room temperature LB broth was added to a total volume of 1mL. The cells were recovered at 37˚C with shaking for 1 hour before plating on LB media containing 100 µg/mL ampicillin. Transformants were screened by plasmid extraction and digestion using *BamH*I and *Bgl*II (pPLS038) and *Age*I, *Hind*III and *Bgl*II (pPLS040).

iii. *Delivery of vectors into P. aeruginosa*

All plasmids were delivered into *P. aeruginosa* strains by electroporation as described previously (Choi *et al*. 2006). Briefly, recipient *P. aeruginosa* cells were grown overnight and washed twice with 300mM room temperature sucrose and concentrated to a 100µl volume,

appropriate amount of plasmid DNA was added to the cells and the mixture transferred to a 2mm electroporation cuvette (Fisher Scientific Canada, Ottawa, ON, Canada). Cells were shocked with a 2500V electrical pulse for 5 seconds in the Electroporator 2510 (Eppendorf, Westbury, NY, USA), diluted immediately in 1mL of room temperature LB broth and allowed to recover for 1 hour at 37°C with shaking before plating on LB agar containing appropriate antibiotic.

1. Multi-copy plasmid-based expression system

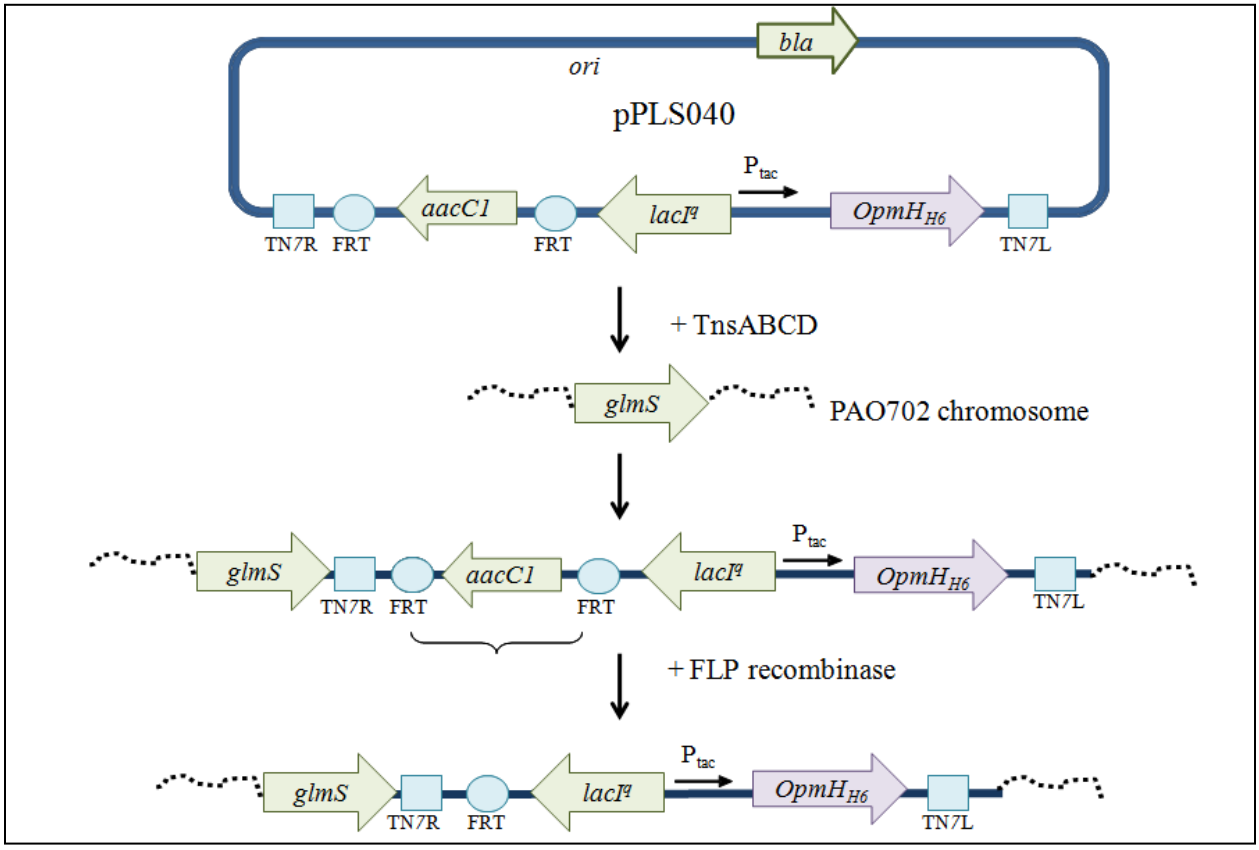
Three millilitres of overnight culture of recipient *P. aeruginosa* PAO702 cells was washed with 300mM sucrose and concentrated to a 100µL volume, to which ~50 - 75ng of either pET1.6-*opmH*-H_6 or pET1.6-*oprM*-H_6 plasmid DNA was added. Cells were shocked, allowed to recover and plated on LB medium containing 200µg/mL carbenicillin. Transformants were verified by plasmid extraction and digestion with *Age*I (New England Biolabs, Pickering, ON, Canada) for pET1.6P-*opmH*-H_6 and *Xho*I (New England Biolabs, Pickering, ON, Canada), *Pst*I (New England Biolabs, Pickering, ON, Canada) for pET1.6P-*oprM*-H_6.

2. MiniTn7 based single-copy gene delivery System

Six millilitres of recipient *P. aeruginosa* PAO702 overnight culture was electroporated as described above with ~600ng of the suicide plasmid pPLS040 and ~200ng of transposase encoding vector pTNS2 (Figure 6). Transformants were selected on medium containing 30µg/mL gentamycin, and insertion of vector into the *P. aeruginosa* chromosome was verified with Tn7R_Fwd and Pa_glmS_Rev primers that bind the vector backbone and *P. aeruginosa glmS* gene respectively and generate a 292bp PCR product. Once cells were verified for Gm^r

Figure 6. Schematic of creation of single copy system for expression of OpmH-H_6. MiniTn7T-based vector pPLS040 and site-specific transposase providing vector pTNS2 was delivered into *P. aeruginosa* PAO702 cells. FLP recombinase providing vector pFLP2 was delivered into appropriate transformants to remove gentamicin resistance marker, *aacC1*, transformants were screened for sensitivity to gentamicin then cured of pFLP2 plasmid by exposure to 10%sucrose. Sucrose tolerant colonies were screened once again for sensitivity to gentamicin and carbenicillin (antibiotic resistance marker on pFLP2). Appropriate colonies were further screened by PCR for insertion of vector backbone and presence of *opmH* gene with specific primers.

Figure 6.


bla
ori
pPLS040
P tac
aacC1
lacIq
OpmH H6
TN7R
FRT
FRT
TN7L
+ TnsABCD
glmS
PAO702 chromosome
glmS
TN7R
FRT
aacC1
FRT
lacIq
P tac
OpmH H6
TN7L
+ FLP recombinase
glmS
TN7R
FRT
lacIq
P tac
OpmH H6
TN7L

marked insertion of o*pmH-H*$_6$ (*P. aeruginosa* PA048), the gentamycin resistance marker *aacC1* was removed by Flp-FRT system as described previously (Hoang *et al*. 1998). Briefly, *P. aeruginosa* PA048 was electroporated with 25ng-50ng of Flp-recombinase providing vector pFLP2 and transformants were selected on medium containing 200μg/mL carbenicillin and screened for sensitivity to gentamicin by patching. The pFLP2 plasmid was then cured by exposure to 10% sucrose and screened for sensitivity to carbenicillin. These cells containing the unmarked insertion of His-tagged genes were once again verified for presence of the miniTn*7*T vector backbone and the *opmH* gene using specific primers.

C QUANTITATIVE – REVERSE TRANSCRIPTASE PCR

i. *RNA Extraction*

RNeasy RNA isolation kit (Qiagen, Mississauga, ON, Canada) was used to extract total RNA from cells with or without induction by addition of 0.5mM IPTG according to manufacturer's instructions. Briefly, cells were grown by sub-culturing overnight culture 1:50 into fresh LB broth, induction of the target gene was achieved by adding 0.5mM IPTG to cells at OD_{600} ~ 0.4-0.6, 1mL of cells were harvested at OD_{600}~0.6 – 0.8 by centrifugation at 17 000xg for 3min. Cell pellets were flash frozen at -80°C for 15min – 40min, thawed out at room temperature, re-suspended in buffer containing 400μg/mL egg-white lysozyme (Bioshop Canada Inc., Burlington, ON, Canada) and disrupted using a guanidine-thiocyanate containing lysis buffer. Total RNA was precipitated by adding 96%-100% ethanol and the mix was loaded onto the RNeasy silica membrane, which was then washed with an ethanol containing wash buffer.

RNA was eluted off the column using RNAse-free water, quality and quantity of sample were assessed by agarose gel electrophoresis and spectrophotometry respectively.

ii. *Complementary-DNA Synthesis*

Genomic DNA was removal was carried out using RNase-free DNAse kit (Qiagen, Mississauga, ON, Canada) according to manufacturer's instructions. Briefly, 2µg of total RNA was incubated with 0.5 units of DNase for 30 minutes at 37°C, DNase was inactivated at 65°C for 5 minutes and 800ng of RNA was reverse transcribed with GO Script Reverse Transcriptase (Promega Corp., Madison, WI, USA).

iii. *Real-time PCR*

Real-time PCR was carried out in CFX-96 Thermal Cycler (BioRad Laboratories, Hercules, CA, USA Laboratories, Hercules, CA, USA) using Eva green Sso-fast PCR supermix (BioRad Laboratories, Hercules, CA, USA). Cycling conditions used were: 95°C 3 minutes; 95°C 10 seconds, 60°C 30 seconds repeat 39x; while 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 *opmH* (110 bp product) and reference gene *rpsL* (242bp product), were designed using OligoPerfect™ Designer program on http://tools.invitrogen.com and are listed in Table 3. Efficiency of each primer set was determined by constructing standards using pooled cDNA samples. No Reverse Transcriptase (NRT) controls which were included for each cDNA template, and to rule out contamination by genomic DNA. No Template Controls (NTC) were

also included for each primer set. Expression of target genes under induced and un-induced conditions normalized to reference gene was analyzed using the CFX Manager software (BioRad Laboratories, Hercules, CA, USA) for gene expression analysis. Raw cycle threshold (CT) values for are provided in Appendix A.

D. PURIFICATION OF HIS_{6X} TAGGED GENES AND IMMUNODETECTION

i. *Culture growth and expression of proteins*

Protein expression, extraction and purifications protocols were followed as described in Touze *et al*. 2004 with some minor changes. For expression of tagged protein from multi-copy vector overnight cultures of appropriate strains were sub-cultured 1/100 into a 500mL volume of LB supplemented with 200μg/mL carbenicillin and grown at 37°C with shaking at 200 rpm. Cultures were induced (0.5mM IPTG) at OD_{600} 0.5 – 0.6 then transferred to 30°C with shaking at 135 rpm, as this was determined to be optimal for expression of proteins, until OD_{600} ~ 0.8-1.0. *P. aeruginosa* PAO702 cells harbouring pET1.6P and pET1.6-*oprM*-H_6 were induced only until OD_{600} ~ 0.65-0.7 as these cells would not grow further. Cells were harvested by centrifugation at 7440xg for 10 minutes at 4°C and cell pellets frozen overnight at -20°C.

For expression of tagged protein from single-copy expression system cultures were prepared in same manner as described above with exception of no antibiotic in medium and 2.5L volume. Cultures were induced (0.5mM IPTG) at OD_{600} ~0.5-0.7 and grown at 37°C with shaking at 200 rpm until OD_{600} 1.2-1.5. Cultures were not transferred to lower temperature as no marked difference was observed for target protein production, and cultures were grown until

early stationary phase as cultures grew readily with no detrimental effects as seen in multi-copy system as described above.

ii. *Preparation of Whole Cell Lysate for crude protein expression analysis*

Whole cell lysates were prepared by two different methods. To analyze proteins from the multi-copy system, cells from 1mL of appropriately grown culture (as described above) were harvested by centrifugation at 17 000xg for 3 minutes and re-suspended in 2x SDS Sample Buffer (SB) (0.2% w/v Bromophenol blue, 20% v/v glycerol, 0.6% v/v β-mercaptoethanol, 4% w/v SDS dissolved in a 62.5mM Tris buffer pH 6.8). Volume of sample buffer to dissolve cell pellet was determined by the following formula: mL of 2x SB = $0.08 \times OD_{600}$ of culture. Mixtures were boiled for 4 -5 minutes, vortexed and resolved by 10% SDS-PAGE.

In the case of single-copy expression system, cultures were grown (100mL volume) with varying parameters as indicated, cells were harvested by centrifugation at 7440xg, 10min, 4°C and cell pellets frozen overnight at -20°C. Pellets were then re-suspended in 4mL of lysis buffer (50mM Tris 2mM EDTA), and cells were sonicated on ice using 15:40 pulse: rest cycles until cell lysis as determined by visual inspection. Cell debris was removed by centrifugation at 15300xg for 10min. Samples were denatured by addition of appropriate volume of 2x SB (described above) and resolved by 10% SDS-PAGE.

iii. *Extraction of membrane proteins*

Cell pellets from cultures grown as described above were thawed at room temperature for 30 minutes and re-suspended in 1/10 original culture volume of cold lysis buffer (50mM Tris, 2mM EDTA). The cell suspension was lysed by sonication in Sonic dismembrator model 500 (Fisher Scientific Canada, Ottawa, ON, Canada). Cell debris was collected by centrifugation at 15300xg for 10 minutes at 4°C. Membrane fraction was collected by centrifugation at 105 000xg in Sorvall® Discovery 100SE (Mandel Scientific Company Inc. Guelph, ON, Canada), for 1 hour at 4°C. Pellets were solubilized in PUTTS buffer (100mM Sodium phosphate, 8M Urea, 1% Triton X-100, 50mM Tris, 1% N-lauroyl sarcosine) and incubated overnight at 4°C with 0.8 - 1mL of Ni-NTA resin (Pierce Biotechnology, Rockford, IL, USA) equilibrated with 10 – 15 column volumes of the same buffer.

iv. *Purification of tagged protein on Ni-NTA column*

Membrane fractions collected as described above were incubated overnight at 4°C with 0.8 - 1mL of Ni-NTA resin (Pierce Biotechnology, Rockford, IL, USA) equilibrated with 10 – 15 column volumes of the same buffer. Resin was washed with 7-10 bed volumes of PUTTS buffer containing 10mM imidazole in 500µL fractions. Proteins were eluted in a step-wise manner with 1mL each of PUTTS buffer containing 200mM, 300mM, 400mM and 500mM imidazole. Fractions were collected in a 250µL volume. Fractions of interest were mixed with buffer containing β-mercaptoethanol and resolved by sodium-dodecyl-sulfate – 10% polyacrylamide gel electrophoresis (SDS-PAGE) run at 50V through the 4% stacking gel (0.5M Tris buffer pH 6.8 with 0.4% SDS) and 100V through the 10% resolving gel (1.5M Tris buffer pH 9.2 with

0.4% SDS). Proteins were visualized by staining with 0.001% Coomassie Blue R-250 solution containing 45% Methanol and 10% glacial acetic acid. Occasionally proteins were visualized by staining with silver nitrate. This was carried out by microwaving the gel for 2 minutes with shaking every 30 seconds in a prefix solution (50% Methanol, 10% Ethanol), shaking for two minutes, replacing the prefix solution with ~100mM Dithiothreitol (DTT) solution, and microwaving as before. The DTT solution was replaced with 0.1% silver nitrate, heated for 90 seconds, and then washed thoroughly with water before adding developer solution (0.05% v/v formaldehyde, 3% w/v sodium carbonate. Development was stopped by addition of 2.3M citric acid, washed thoroughly with water and stored in a 0.03% sodium carbonate solution.

v. *Immunodetection of purified proteins*

Fractions of interest, as determined by SDS-PAGE, were once again resolved by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Thermo Scientific, Mississauga, ON, Canada) in a pre-chilled 119mM Tris-4M Glycine-0.1% SDS buffer containing 20% Methanol at 4°C, 25V for approximately 16 hours. Membranes were then blocked for ~2h – 3h with shaking at 60 rpm, with Phosphate Buffered Saline pH 7.2 containing 0.1% Tween 20 (PBST) and 10% skim milk, and then washed twice for 10 minutes with 50mL PBST. Membranes were then exposed to primary antibody for 1.5 hours with shaking at 60rpm as follows: 1:2500 rabbit α-opmH diluted in 10mL PBST + 3% bovine serum albumin (BSA), α-OpmH (Chuanchuen *et al.* 2005) was occasionally stored in 25% glycerol, 5µL goat α-OprM (a gift from Dr. Herbert Schweizer, Colorado State University, Fort Collins, CO, USA) diluted in 10mL PBST + 3% BSA, 5µL rabbit α-MexK diluted in 10mL PBST + 3% BSA. This was

followed once again by washing twice for 10 minutes with 50mL PBST and exposure to secondary antibody for 1 hour with shaking at 60rpm as follows: 2µL of either 2mg/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 10mL of PBST + 3 % BSA. Membranes were then washed twice for 10 minutes with 50mL PBST and developed by addition of 1:10 diluted Pierce ECL detection reagent 1 and 2 (Pierce Biotechnology, Rockford, IL, USA) and exposure to CL-Xposure Film (Pierce Biotechnology, Rockford, IL, USA) for 2 – 5 minutes.

E. ANTIBIOTIC SUSCEPTIBILITY TESTING

i. *Agar Dilution Method*

Overnight cultures of appropriate strains were sub-cultured 1:50 into fresh LB, grown to OD_{600} ~ 1 and standardized to 0.5 McFarland 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 in Mueller-Hinton Broth (MHB) and a 5µl volume was spotted onto Mueller-Hinton Agar (MHA) plates in duplicate. Agar plates were made by adding triclosan to a final concentration ranging from 0.5µg/mL – 256µg/mL in serial increments of 2. MHA medium was supplemented with 0.5mM 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 triclosan that inhibited the growth of cells.

ii. *Broth Dilution Method*

Bacterial cultures of interest were standardized in the same manner as for *Agar Dilution Method* described above. Antibiotic dilutions were prepared in 96-well plates in triplicate for each condition tested (ie. +/- 0.5mM IPTG) whereby two-fold serial dilutions of erythromycin (Biobasic Inc. Markham, ON, Canada) were made. Cells were added to the wells, cultures were induced where indicated with 0.5mM IPTG at time of plating. Plates were incubated at 37°C for 18 hours and MIC was determined to be the lowest concentration of erythromycin which inhibited bacterial growth.

F. PROTEIN MODELING AND SEQUENCE ALIGNMENT

OpmH and OprM were modeled using online modeling program Phyre at http://www.sbg.bio.ic.ac.uk/~phyre/. Sequences used for modeling are provided in Appendix C. PDB files were downloaded and visualized using the modeling program USCF Chimera version 1.5.2. Alignment of OpmH to OprM was done using ClustalW2 on the European Bioinformatics Institute website at http://www.ebi.ac.uk/Tools/msa/clustalw2/, and was used to identify corresponding residues of interest in OpmH. Alignment is provided in Appendix D.

III

RESULTS

A CREATION OF EXPRESSION SYSTEMS

i. *Multi copy expression system – pET1.6P vectors*

The multi-copy expression system is comprised of the chromosomally encoded T7 polymerase and the plasmid encoded His-tagged gene. Expression of this system was confirmed by SDS-PAGE and immunoblotting as described in later sections.

ii. *Single copy expression system – miniTn7T vectors*

Construction of the single copy expression system was carried out by inserting the His-tagged genes into the chromosome of *P. aeruginosa* PAO702 cells, a process comprised of several steps, as described in the Materials and Methods section (Figure 6). Insertion of the vector backbone and *opmH* gene was confirmed by PCR (Figure 7a and 7b).. Expression of this system was confirmed by SDS-PAGE and immunoblotting as described in later sections.

B VERIFICATION OF EXPRESSION SYSTEMS

i. *Quantitative-Real Time (Q-RT PCR) analysis of mRNA expression*

Induction of expression from the multi-copy system resulted in approximately 107-fold overexpression of *opmH-*H_6 mRNA compared to un-induced cells (Figure 8a). Expression from

Figure 7. Verification of *P. aeruginosa* PA049 by PCR. (a) Insertion of miniTn*7* vector and (b) Presence of *opmH* gene. PCR was carried out using primers Tn7R_Fwd and Pa_glmS_Rev (listed in Table 3) for confirmation of miniTn*7* insertion, yielding a 292bp product. *OpmH* gene was confirmed by PCR using primers OpmH_RT_Fwd and OpmH_RT_Rev (listed in Table 3) which bind within the *opmH* gene yielding a 110bp product.

Figure 7.

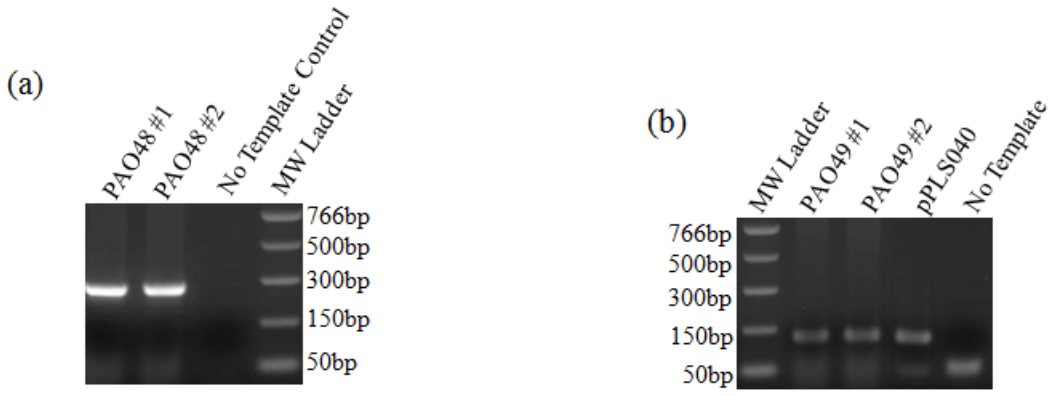
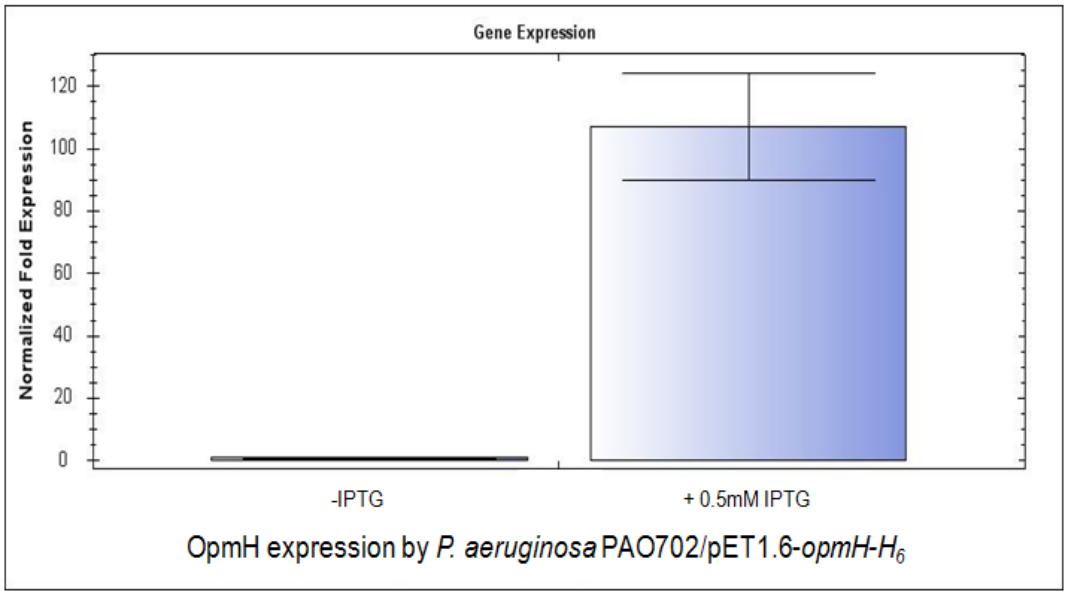

(a)
PAO48 #1
PAO48 #2
No Template Control
MW Ladder
766bp
500bp
300bp
150bp
50bp
(b)
MW Ladder
PAO49 #1
PAO49 #2
pPLS040
No Template
766bp
500bp
300bp
150bp
50bp

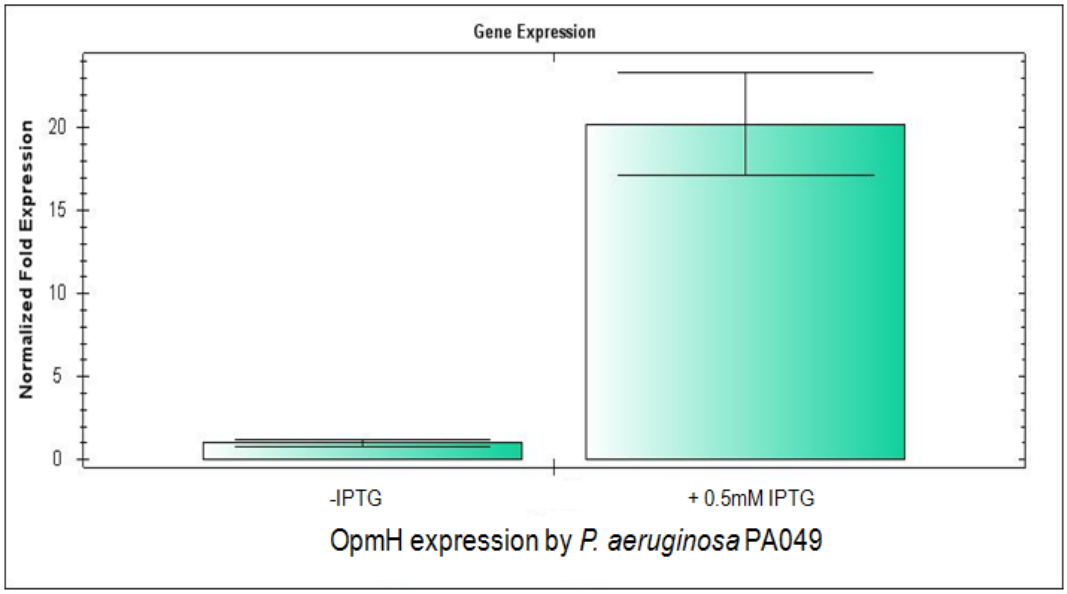
Figure 8. Quantitative-Real Time analysis of OpmH-H_6 mRNA expression under inducing and non-inducing conditions. Complementary DNA was synthesized and analyzed by amplification of a 110bp fragment of *opmH* using 30S ribosomal subunit *rpsl* as internal control. **(a) Multi-copy system.** Total RNA was extracted from un-induced and induced (0.5mM IPTG) mid-log phase *P. aeruginosa* PAO702/pET1.6P-*opmH*-H_6 cells. Normalized expression analysis indicates approximately 107-fold overexpression of *opmH* mRNA when induced compared to un-induced cells. **(b) Single-copy system.** Total RNA was extracted from un-induced and induced (0.5mM IPTG) mid-log phase *P. aeruginosa* PAO702:miniTn*7*T-*opmH*-H_6 cells. Normalized expression analysis shows a 20-fold overexpression under inducing conditions compared to un-induced cells.

Figure 8.

(a)


Gene Expression
Normalized Fold Expression
120
100
80
60
40
20
0
-IPTG
+ 0.5mM IPTG
OpmH expression by P. aeruginosa PAO702/pET1.6-opmH-H6

(b)


Gene Expression
Normalized Fold Expression
20
15
10
5
0
-IPTG
+ 0.5mM IPTG
OpmH expression by P. aeruginosa PA049

single-copy system resulted in approximately 20-fold overexpression of *opmH-H*$_6$ mRNA compared to un-induced cells (Figure 8b).

ii. *Protein expression analysis of whole cell extracts*

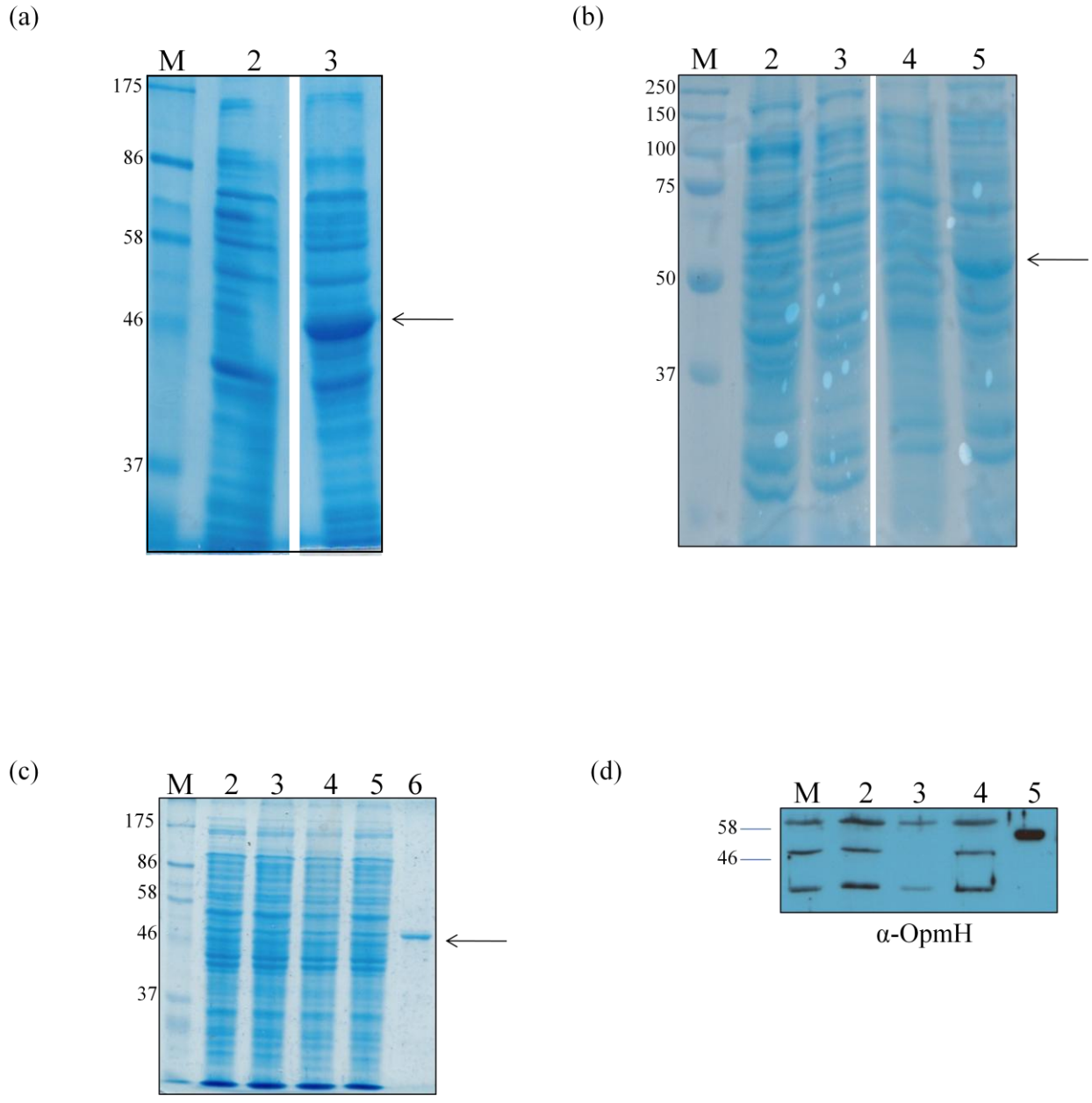
Upon induction of target protein from multi-copy vector pET1.6P, a prominent band weighing approximately 50kDA, the approximate weight of both outer membrane proteins OpmH and OprM, was observed (Figure 9a and 9b). Induction of the single-copy expression system did not yield a visibly detectable over-expressed protein in the range of the expected molecular weight by this method (Figure 9c). When these cell lysates were probed with polyclonal rabbit α-OpmH, no signal corresponding to OpmH was detected (Fig 9d). The bands that are observed in the whole cell extracts are expected to be non-specific targets that cross-react with the α-OpmH, no cross-reaction was observed with the secondary goat- α-rabbit (Data not shown).

C. ANTIBIOTIC SUSCEPTIBILITY ANALYSIS OF PHENOTYPE

Phenotypic verification of constructs was analyzed by antibiotic susceptibility assays where the MIC was determined for each strain by agar dilution (Triclosan) or broth dilution (Erythromycin) method according to CLSI guidelines. As the MexJK-OpmH complex has been shown to efflux triclosan, resistance to this biocide was used as indication of functional complex formation. Alternatively, resistance to erythromycin was used as evidence of functional MexJK-OprM complex formation.

Figure 9. Protein expression analysis by whole cell extracts. (a) OpmH-H_6 expression from multi-copy system. Lane M-molecular weight marker, lane 2-Whole Cell Lysate (WCL) from *P. aeruginosa* PAO702/pET1.6 + 0.5mM IPTG, lane 3-WCL from *P. aeruginosa* PAO702/pET1.6-opmH-H_6 + 0.5mM IPTG. SDS-PAGE analysis of *P. aeruginosa* PAO702 cells harbouring pET1.6 or pET1.6-opmH-H_6 were grown at 37°C until mid-log phase, induced (0.5mM IPTG) and grown for 2 hours at 30°C. **(b) OprM-H_6 expression from multi-copy system.** Lane M-molecular weight marker, lane 2-WCL from *P. aeruginosa* PAO702 cells without any plasmids, lane 3-WCL from *P. aeruginosa* PAO702/pET1.6 + 0.5mM IPTG, lane 4-WCL from un-induced *P. aeruginosa* PAO702/pET1.6-oprM-H_6, lane 5-WCL from *P. aeruginosa* PAO702/pET1.6-oprM-H_6 + 0.5mM IPTG. SDS-PAGE analysis of *P. aeruginosa* PAO702 cells harbouring pET1.6 and pET1.6-oprM-H_6 were grown at 37°C, and induced with 0.5mM IPTG and grown at 30°C and until mid-log phase. *P. aeruginosa* PAO702 cells without any plasmids were grown at 37°C and harvested at OD_{600} 1.1. **(c) OpmH-H_6 expression from single-copy system, *P. aeruginosa* PA049.** Lane M-molecular weight marker, lane 2-WCL from un-induced cells, lane 3-WCL from cells induced (0.5mM IPTG) at time of inoculum and grown at 37°C, lane 4-WCL from cells induced (0.5mM IPTG) at time of inoculums and grown at 30°C, lane 5-WCL from cells induced (0.5mM IPTG) at mid-log phase and grown at 37°C, lane 6-purified OpmH-H_6 from multi-copy vector. *P. aeruginosa* PAO702:miniTn*7*T-opmH-H6 cells grown in 100mL LB broth were harvested and whole cell lysate prepared as described in Materials and Methods. Cell lysates were analysed by reducing 10% SDS-PAGE. Purified OpmH-H_6 from multi copy was included as positive control for comparison purpose. Western detection of OpmH-H_6 **(d)** (same order of sample as (c)) was carried out using polyclonal α-opmH antibodies.

Figure 9.


(a)
M 2 3
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46
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(b)
M 2 3 4 5
250
150
100
75
50
37
(c)
M 2 3 4 5 6
175
86
58
46
37
(d)
M 2 3 4 5
58
46
α-OpmH

Table 4. Antibiotic susceptibility profile of *P. aeruginosa* PAO702 constructs. Minimum inhibitory concentrations for Triclosan, a substrate for the MexJK-OpmH efflux complex, were determined according to CLSI standards and as described in Materials and Methods. MIC reflects the lowest concentration of antibiotic or compound to inhibit growth of bacteria. **(a) Triclosan.** Agar dilution method was used to determine triclosan MIC. **(b) Erythromycin.** Broth dilution method was used to determine erythromycin MIC. Data shown is representative of at least two independent assays.

Table 4.

(a)

Strain	Efflux components expressed	Copy number of OMP gene	Triclosan MIC (µg/mL)		Fold change in MIC compared to PAO702	
			-IPTG	+IPTG	-IPTG	+IPTG
PAO702	MexJ, MexK	0	4	4	0	0
PAO702/pET1.6P	MexJ, MexK	0	8	16	2	4
PAO702/pET1.6P-*opmH*-H$_6$	MexJ, MexK, OpmH	Multiple	256	4	64	0
PA051	MexJ, MexK	0	4	4	0	0
PA049	MexJ, MexK, OpmH	Single	16	128	4	32

(b)

Strain	Efflux components expressed	Copy number of OMP gene	Erythromycin MIC (µg/mL)		Fold change in MIC compared to PAO702	
			-IPTG	+IPTG	-IPTG	+IPTG
PAO702	MexJ, MexK	0	31.25	31.25	0	0
PAO702/pET1.6P	MexJ, MexK	0	31.25	<0.122	0	n/a
PAO702/pET1.6P-*oprM*-H$_6$	MexJ, MexK, OprM	Multiple	125	<0.122	16	n/a
PAO702/pET1.6P-*opmH*-H$_6$	MexJ, MexK, OpmH	Multiple	62.5	31.25	2	0
PA051	MexJ, MexK	0	62.5	125	2	4

P. aeruginosa PAO702/pET1.6P-*opmH*-H_6 cells displayed a high level of resistance under non inducing conditions with 64-fold change in MIC compared to parental strain *P. aeruginosa* PAO702 (Table 4). However, induction of pET1.6P-*opmH*-H_6 caused a significant decrease in resistance with MIC comparable to that of the parental strain. This high-level of resistance was not observed in *P. aeruginosa* PAO702/pET1.6P-*oprM*-H_6 cells (appendix B), indicating that the increase in resistance observed is due to presence of *opmH* gene specifically.

In contrast, *P. aeruginosa* PA049 cells displayed high-level of resistance upon induction with a 32-fold change in MIC compared to the parental strain (Table 4). Although an increase in resistance is observed upon induction of empty vector, it is only two-fold, an insignificant change as outlined by the CLSI standards (Clinical Laboratory Standards Institute, 2011).

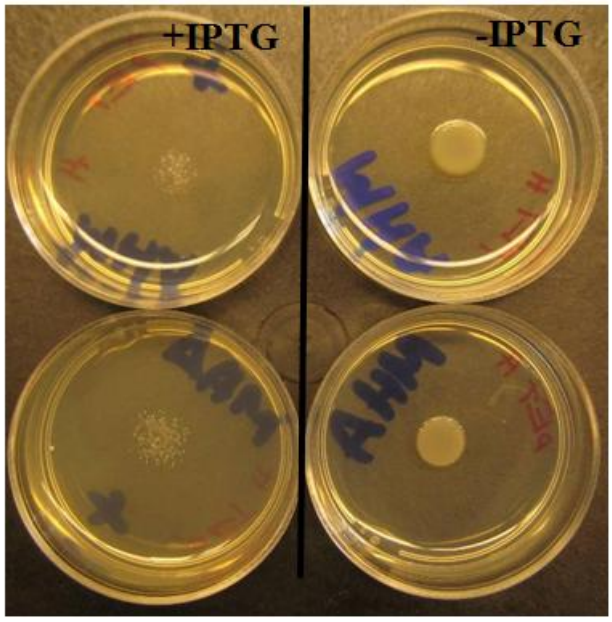
P. aeruginosa PAO702/pET1.6P-*oprM*-H_6 displays a high level of resistance to erythromycin under non-inducing conditions compared to strain containing empty vector and parental strain (Table 4). This is consistent with the trends observed with *P. aeruginosa* PAO702/pET1.6P-*opmH*-H_6 and triclosan susceptibility levels.

The PAO702/pET1.6-*opmH*-H_6 colonies on the MHA + IPTG plates were considerably smaller than their growth on the uninduced MHA plates (Figure 10a), indicative of the effect of IPTG on the cell's fitness and viability. This same effect was not observed for the colonies of PA051 (Figure 10b)

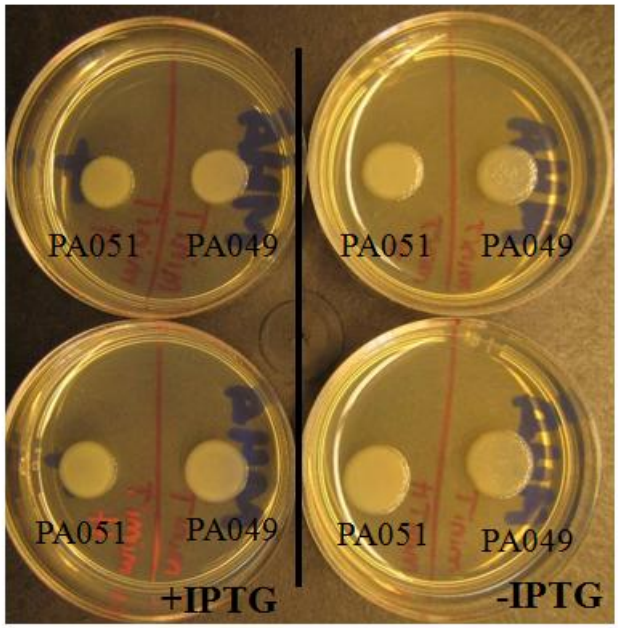
Figure 10. Colony morphology of strains expressing OpmH-H_6 from multi- and single-copy. *P. aeruginosa* **(a)** PAO702/pET1.6-*opmH*-H_6 and **(b)** PA051 colonies were grown, standardized and diluted as described in Materials and Methods for Agar Dilution MIC. Two representative plates of colony morphology under inducing (indicated) and non-inducing conditions are shown.

Figure 10.

(a) PAO702/pET1.6-*opmH*-H_6



(b) PA049/PA051



D. PROTEIN PURIFICATION AND DETECTION

i. *Purification of tagged proteins from multi-copy expression system*

The first three elution fractions from *P. aeruginosa* PAO702/pET1.6P-*opmH*-H_6 cells induced to express OpmH-H_6 contained a strong band weighing approximately 50kDa, as seen in the protein expression studies (Figure 11a indicated by arrow). A similar observation was made for *P. aeruginosa* PAO702/pET1.6P-*oprM*-H_6 cells (Figure 11b indicated by arrow). Absence of this band from *P. aeruginosa* PAO702/pET1.6 cells subjected to the same purification scheme (Figure 11c), indicates 50kDa band to be the respective purified His-tagged protein.

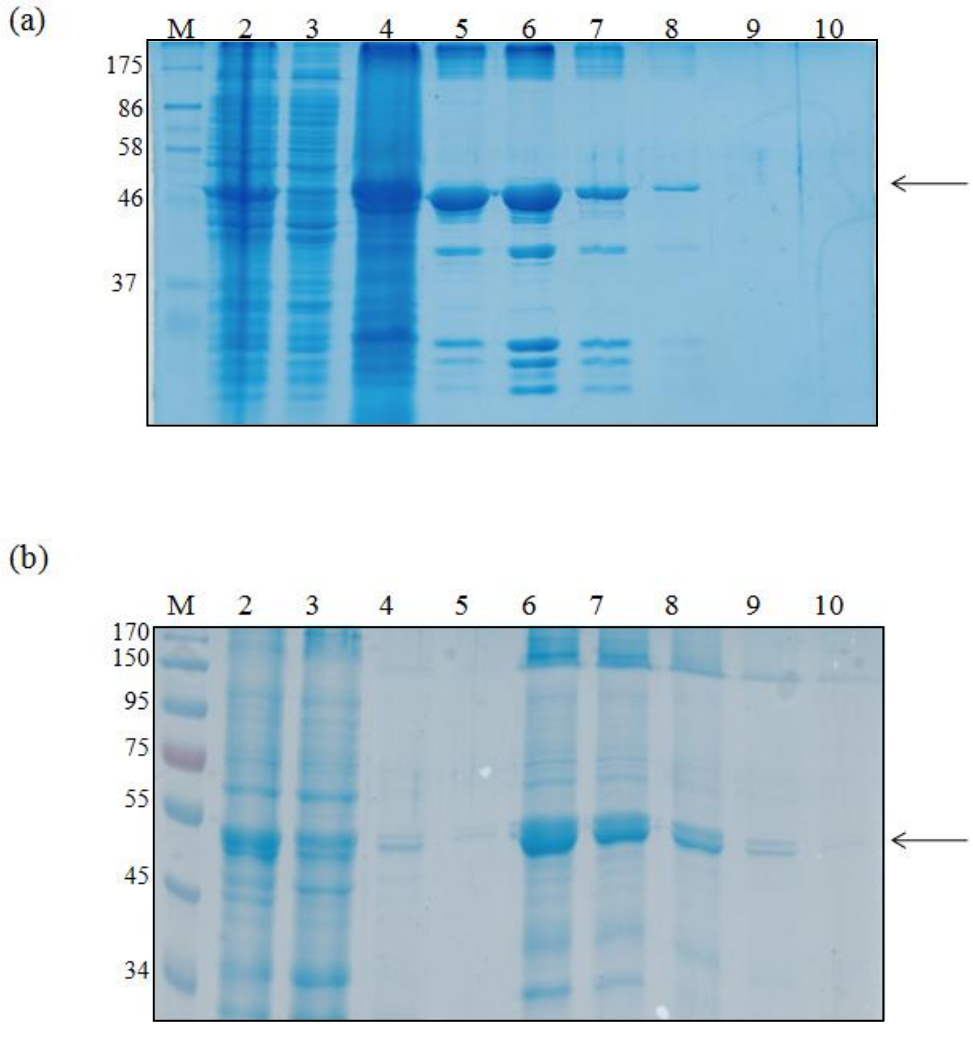
Purification of protein of interest was further confirmed by probing the fractions of interest with antibodies raised against OpmH or OprM. The prominent 50kDa bands present in the fractions from cells containing pET1.6P-*opmH*-H_6 were detected by the α-opmH antibodies while no signal was detected in the fractions originating from cells harbouring the empty pET1.6P vector (Figure 11d top panel). Similarly, the prominent 50 kDa bands present in the fractions from cells containing pET1.6P-*oprM*-H_6 were detected by the α-OprM (Figure 11d bottom panel) antibodies while no signal was detected in the fractions originating from the empty vector. There was also no cross reaction between purified OpmH-H_6 and α-OprM antibodies or vice versa, indicating that while the α-OpmH has cross-reacting material within the whole cell lysate, it does not detect OprM (Fig11d).

ii. *Purification of OpmH-H_6 from single-copy expression system*

The first three elution fractions from *P. aeruginosa* PAO702:miniTn*7*T-opmH-H6 cells induced to express the tagged protein once again did not contain a visibly prominent band as

Figure 11. Purification of His-tagged proteins expressed from multi-copy system on by affinity chromatography. (a) OpmH-H_6 from *P. aeruginosa* PAO702/pET1.6-opmH-H_6. Lane M-molecular weight marker, lane 2-cell debris, lane 3-supernatant from ultracentrifugation step, lane 4-material loaded onto column, lane 5- 10 - 1^{st} – 6^{th} elution fractions. **(b) OprM-H_6 from *P. aeruginosa* PAO702/pET1.6-oprM-H_6.** Lane M-molecular weight marker, lane 2-flow through, lane 3-1^{st} wash fraction, lane 4-6^{th} wash fraction, lane 5-10^{th} wash fraction, lane 6-10 - 1^{st} – 5^{th} elution fractions **(c) Purification from *P. aeruginosa* PAO702/pET1.6.** Lane M-molecular weight marker, lane 2-material loaded onto column, lane 3-flow through, lane 4-1^{st} wash fraction, lane 5-7^{th} wash fraction, lane 6-purified OpmH-H_6 from multi-copy vector, lane 7-10 - 1^{st} – 4^{th} elution fractions. The column was washed with 8-10 bed volumes of PUTTS buffer containing 10mM imidazole, and eluted with 1mL each of PUTTS buffer containing 200mM – 500mM imidazole, increasing in 100mM increments. Fractions were resolved by 10% SDS-PAGE stained with Coommassie blue R-250. **(d) Detection of purified His-tagged proteins with respective antibodies.** First elution fraction from each purification as well as membrane proteins from *P. aeruginosa* PAO750, a strain deficient for both OprM and OpmH was resolved by SDS-PAGE, transferred onto a nitrocellulose membrane and probed with polyclonal rabbit-α-OpmH antibodies or goat-α-OprM antibodies. Lane M-molecular weight marker, lane 2-first eluate from *P. aeruginosa* PAO702/pET1.6-*oprM-H_6*, lane 3-first eluate from *P. aeruginosa* PAO702/pET1.6, lane 4-first eluate from *P. aeruginosa* PAO702/pET1.6-*opmH-H_6*, lane 5-membrane proteins from *P. aeruginosa* PAO750.

Figure 11.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(b)
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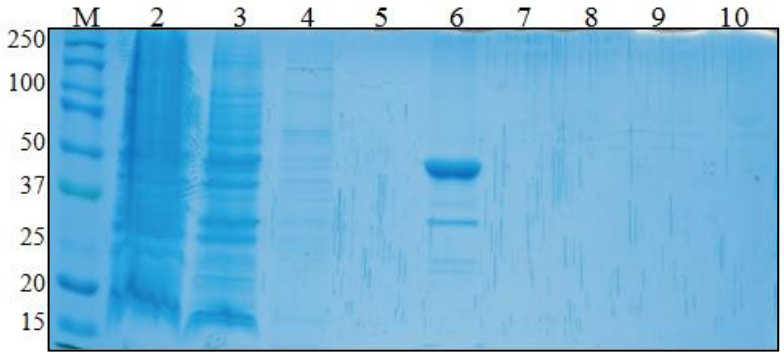
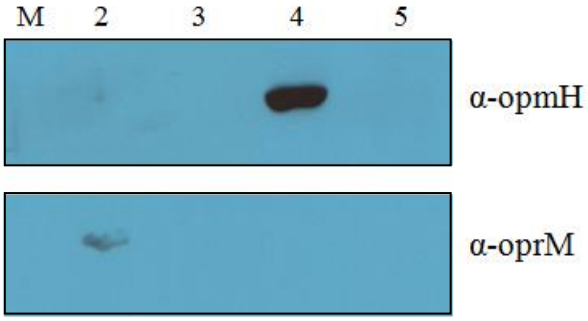
(c)



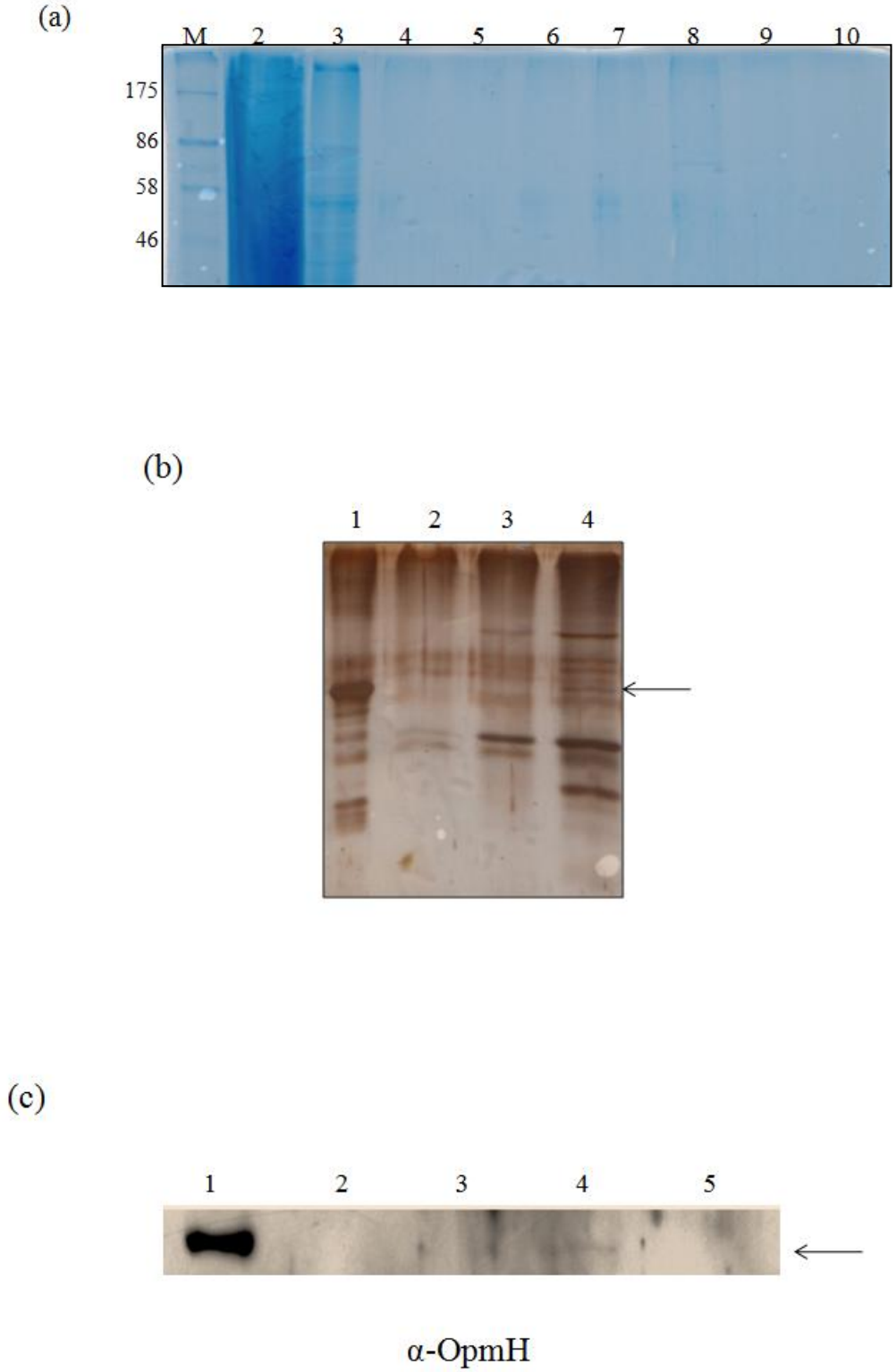
(d)



seen with purification from the multi-copy vector (Figure 12a). Upon closer inspection however, one of the fractions did contain a band of same molecular weight as that purified from multi-copy. The first three elution fractions along with purified OpmH-H_6 from multi copy system were subjected to SDS-PAGE stained with the more sensitive silver nitrate, which has a limit of detection of ~0.1ng protein/band (Dunn and Crisp, 1994) thus allowing for a better picture of the proteins present within these samples (Figure 12b). The third elution fraction contained a faint band which lined up with the prominent one in the lane containing purified OpmH-H_6 sample (Figure 12b, indicated by arrow). Immunodetection using α-OpmH antibody, confirmed the band to be OpmH-H_6 (Figure 12c).

Figure 12. Purification of OpmH-H_6 from single copy expression system. (a) Coommassie stain of OpmH-H6 purification fractions. Lane M-molecular weight ladder, lane 1-column flow through, lane 2-1st wash fraction, lane 3-7th wash fraction, lane 4-10th wash fraction, lane 5-10 - 1st – 5th elution fractions. *P. aeruginosa* PAO702:miniTn*7*T-*opmH*-H_6 cells were grown in a total of 2.5L volume at 37°C induced (0.5mM IPTG) at mid-log phase and grown further at 37°C until early stationary phase, at which time they were harvested. Cell lysis, isolation of membrane fraction, purification of OpmH-H_6 and analysis of fractions was carried out as described above. **(b)Silver nitrate stain of OpmH-H_6 purification fractions.** Lane 1-purified OpmH-H_6 from multi-copy vector, lane 2-4 - 1st – 3rd elution fractions. Fractions of interest were analyzed by staining with silver nitrate (as described in materials and methods section). **(c) immunodetection of OpmH-H_6 purification fractions.** Lane 1-purified opmH-H_6 from multi-copy vector, lane 2-4 - 1st – 3rd elution fractions, lane 5-membrane proteins from *P. aeruginosa* PAO750. Fractions of interest were transferred onto a nitrocellulose membrane and probed with polyclonal rabbit α-opmH. OpmH-H_6 purified from multi-copy system was used as positive control and membrane fraction isolated from *P. aeruginosa* PAO750, a strain deficient for *opmH*, was used as negative control.

Figure 12.



IV

DISCUSSION

A MULTI-COPY EXPRESSION SYSTEM

i. *Protein expression and purification*

The prevalence of RND efflux pumps and their ability to expel a wide range of substrates including antibiotics confers intrinsic and acquired multidrug resistance to important pathogens such as *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *A. baumannii*, rendering antibiotic treatments ineffective. This makes these protein complexes an appealing target for discovery of new drugs and the focus of much research aimed at understanding the various aspects of the proper functioning of RND efflux pumps. Crystal structures of the individual components have provided insight as to the pumping mechanism of the RND transporter. Genetic and biochemical studies have allowed the characterization of the regions and specific residues that mediate interaction between components, binding of substrate and transduction of energy necessary to expel drug. Nevertheless, a basic understanding of the mechanism of functional assembly of the tri-partite complex is still lacking. One approach to elucidating these mechanisms is the study of native interactions between the components of the RND pump complex, however the membrane bound nature of these proteins makes this type of characterization more challenging.

The array of techniques used to examine the interactions between membrane proteins begin with cloning the gene of interest into a high-copy number plasmid to allow high enough levels of protein production for subsequent purification and characterization. This has proven successful in much of the characterization of RND efflux pump complexes. Vectors pSPORTI (Takatsuka and Nikaido. 2006), pUC18 (Tikhonova and Zgurskaya. 2004), pTrc99a (Hussain *et al*. 2004) and various derivatives are commonly used to express the various efflux components to purify and further characterize them.

A *P. aeruginosa* compatible derivative of the pET vector was used to examine multi-copy, plasmid-based expression systems in this study and it is clear that with minimal optimization purification of protein under these conditions is readily attainable, as seen previously (Mokhonov *et al*. 2004). Although not quantified, it is easy to see by 10% SDS-PAGE with coommassie staining that upon induction, tagged protein is produced by the cell in very high quantities such that it is detectable in whole cell lysate from 1mL of culture (Figure 9a and 9b) and only a small amount of culture is required for their purification, 0.5 L in this study (Figure 10a and 10b). Furthermore, as the amount of target protein produced inside the cell is so high, purification schemes become forgiving to loss of yield during the various steps. For example, the cell lysis step in the purification of OpmH-H_6 from *P. aeruginosa* PAO702/pET1.6-*opmH*-H_6 resulted in some loss of target protein seen by the presence of a thick 50kDa band in the cell debris (Figure 10a), and loss of protein in column flow through as seen in the flow through while purifying OprM-H_6 from *P. aeruginosa* PAO702/pET1.6-*oprM*-H_6 (Figure 10b). For these reasons multi-copy vectors are the choice method for purifying these proteins.

However, to study and elucidate native interactions between proteins, a purification scheme utilizing native protein must be devised, or a recombinant system for purification of protein that mimics native conditions must be developed. Analysis of mRNA transcript levels of a His-tagged OMP from *P. aeruginosa, opmH-H_6* shows a 107-fold increase in expression upon induction from a pET-based vector made compatible for expression in this bacterium (Figure 8a). This is not consistent with what is observed in either laboratory-derived mutants or clinical isolates displaying a multidrug resistant phenotype attributed to up-regulation of efflux pump expression. *K. pneumoniae* mutants isolated in laboratory settings displaying resistance to clinically relevant compounds, such as fluoroquinolones, up regulate expression of efflux pumps

by only 2-10-fold (Bialek-Davenet *et al*. 2011). Components of RND efflux pumps were found to be expressed ~2-5-fold more in multidrug resistant clinical isolates of *A. baumannii* compared to a susceptible reference strain (Coyne *et al*. 2010), and ~2-39-fold in multidrug resistant clinical isolates of *P. aeruginosa* compared to wild-type laboratory strain *P. aeruginosa* PAO1 (Hocquet *et al*. 2006). As the ultimate goal of studying RND efflux pumps is to eventually inhibit their action in clinical settings, it is necessary to adapt an expression system that better approximates the levels of expression observed in clinical isolates.

ii. *Phenotypic analysis*

While an abundance of target protein was observed upon induction of multi-copy expression system, a severe decrease in resistance and overall cell-viability is also observed under these conditions. When induced *P. aeruginosa* PAO702 cells harbouring either pET1.6-*opmH*-H_6 or pET1.6-*oprM*-H_6 display a severe drop in triclosan (Table 4a) and erythromycin (Table 4b) MIC respectively. The *P. aeruginosa* PAO702/pET1.6-*opmH*-H_6+IPTG colonies observed on triclosan containing MHA plates were significantly smaller and seemingly less fit than their un-induced counterparts (Figure 10). This observation raises the question of whether the vast amount of protein produced upon induction is functional in its efflux capabilities.

Particles resembling aggregates, similar to that observed by Wagner S. and associates during the over-expression of GST fusions of membrane proteins YidC, YedZ and LepI in *E. coli* (Wagner *et al*. 2007) were observed during the purification of OprM-H_6 after the culture had been induced It is of note that these aggregates were reported to contain the overexpressed proteins, chaperones, soluble proteases, and many precursors of periplasmic and outermembrane

proteins; and were not observed when the soluble GST-GFP fusion was over expressed (Wagner *et al*. 2007). This may provide some insight as to the fate of the excess protein produced upon induction, indicating that the cell does not use these proteins for efflux purposes. The hyper-sensitivity to substrate and low fitness of culture upon induction illustrates the toxicity associated with over-expression of membrane proteins and the need for a system of protein expression that upon induction can achieve conditions that better reflect biological conditions.

B SINGLE-COPY EXPRESSION SYSTEM

i. *Protein expression and purification*

A system that stably incorporates and expresses the target gene from the bacterial chromosome in single-copy has the potential to more accurately mimic biologically relevant conditions. Such a system was described by Choi and associates (Choi *et al*. 2005) utilizing the bacterial transposon Tn*7* and its well characterized transposition into the bacterial chromosome at the Tn*7* attachment site (*attTn7*). The strengths of this expression system lies in the predictable and well characterized Tn*7* transposition mechanism (Choi *et al*. 2005). This transposon, while capable of high-frequency non-specific transposition into conjugal plasmids using the TnsABC+E proteins, it is also capable of specific and polar insertion into the bacterial chromosome at the *attTn7* site located downstream of the *glmS* gene encoding a highly conserved bacterial glutamine synthetase (Peters and Craig. 2001). The specificity of the second mode of transposition is directed by the sequence specific DNA binding protein, TnsD of the Tn*7* transposition system. The specificity, polarity and high frequency of insertion into the bacterial chromosome characteristics of this system were harnessed to design a single-copy gene

expression system whereby any gene of interest can be cloned and expressed in a wide variety of bacterial hosts (Choi *et al*. 2005).

This single-copy gene expression system was successfully shown to replicate up-regulation of RND efflux pumps to the same levels as that of a mutant promoter (Mima *et al*. 2007). The TriABC-OpmH RND efflux pump of *P. aeruginosa* was discovered by screening triclosan-sensitive mutants for increased resistance to this biocide (Mima *et al*. 2007). The resistance was attributed to a promoter-up mutation in the promoter of the TriABC pump which resulted in a >7-fold increase in MIC and ~10-fold increase of this pump compared to the triclosan-sensitive strain. Upon cloning this pump into the single-copy gene expression vector, a >8-fold increase in MIC compared to strain not expressing the pump and ~9-fold increase in TriABC pump expression compared to triclosan-sensitive strain was achieved.

In this study, the single-copy system was adapted for the expression of the efflux component *opmH-H_6*. Induction of the system results in a 20-fold increase in expression of target mRNA transcript compared to un-induced cells (Figure 8b), which as seen previously (Mima *et al*. 2007) is a more accurate replication of biological systems than a multi-copy system.

While such a system is more appropriate for the study of native interactions between efflux complex components, it is less favoured due to the lowered levels of expression making purification of target protein proportionately more troublesome. The classic obstacles faced when purifying membrane proteins surface when implementing this system of expression. Culture volume, induction conditions, cell lysis and affinity purification are some parameters that may be standardized to determine the optimal conditions for the purification of target protein. As expression of protein is not easily detected, optimization is not possible with a mere 1mL culture

as was attained with multi-copy expression system. In the case of *P. aeruginosa* PA049, a larger culture volume (2.5L), and longer induction was required to purify OpmH-H_6. Elution fractions from the purification scheme had to be subjected to the more sensitive stain silver nitrate to visualize the faint band corresponding to putatively purified OpmH-H_6 (Figure 11b), which was finally detected by immunoblotting (Figure 11c). Taken together, these studies show that while multi copy expression systems drive strong protein expression, allowing for straightforward purification of target protein in high yields; expression and purification from single-copy expression systems is both achievable and detectable.

ii. *Phenotypic analysis*

Despite the low levels of protein production upon induction of the single-copy expression system, antibiotic susceptibility data also indicates that this system is more reflective of a clinical strain (Coyne *et al*. 2010, Sobel *et al*. 2005) than the multi-copy system as a 32-fold increase in triclosan MIC upon induction of *opmH-H_6* compared to strains not expressing the OMP (Table 4a). This phenotype is indicative of an interaction of the induced OpmH-H_6 with MexJK to form a functional efflux complex capable of triclosan efflux. This data is comparable to the previously published report (Chuanchuen *et al.* 2002) where the MexJK pump was discovered to efflux triclosan due to a promoter-up mutation; in the absence of MexAB-OprM and MexCD-OprJ. The mutant strain capable of effluxing triclosan had an MIC for this compound of 128μg/mL whereas the parental strain had a triclosan MIC of 20μg/mL. Thus the single-copy expression system successfully replicates a biological system, while allowing for further study of this system as the genes may be recombinantly tagged.

VI

FUTURE DIRECTIONS

A PURIFICATION OF OPRM-H_6 FROM SINGLE COPY EXPRESSION SYSTEM

While OpmH-H_6 has been successfully purified and detected from the single-copy expression system, OprM-H_6 has yet to be inserted into *P. aeruginosa* PAO702 in single copy. Once this construct has been made, characterization of its expression by q-RT PCR and antibiotic susceptibility assays will have to be completed. Although both are outer membrane proteins, conditions used to purify OpmH-H_6 may not translate directly to the purification of OprM-H_6, but nevertheless the purification scheme outlined in this study is an acceptable place to start.

B CO-PURIFICATION STUDIES OF MEXJK-OPRM/OPMH EFFLUX COMPLEX

The applications of a single-copy gene expression system transferrable to any bacterial species containing *att7* sites are numerous. One such application utilizing the constructs made in this study is the investigation of the interactions between the RND-MFP complex MexJK and the two outer membrane proteins it is known to interact with, OpmH and OprM.

Pull down studies where by the OMP is purified and fractions of interest are probed for presence of MFP or RND under various conditions, whereby substrate is present and/or absent will give further insight about the nature of this interaction. For this reason, the single-copy gene expression system constructed in this study is both ideal and required for this type of assay.

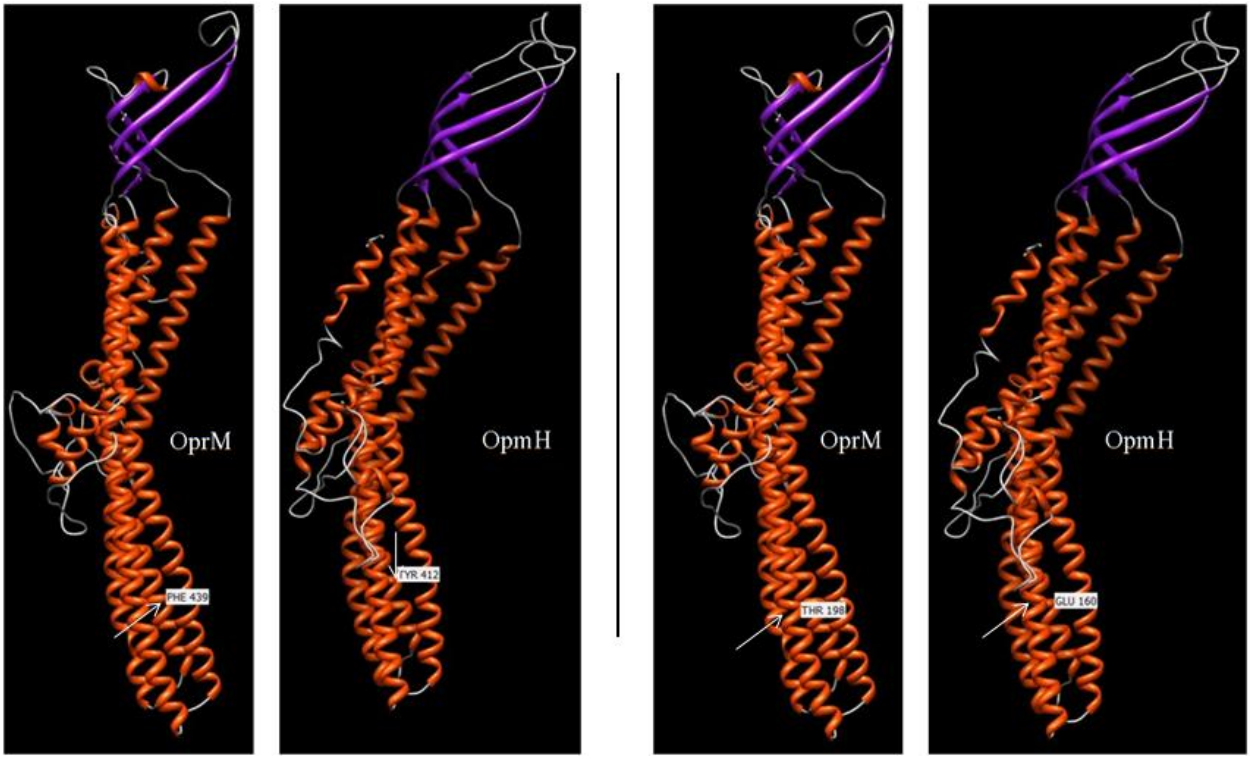
C MODELLING AND SITE-DIRECTED MUTAGENESIS STUDIES OF OPRM AND OPMH

The substrate specificity with which the MexJK pump interacts with the OMPs OprM and OpmH can be exploited to better understand the interaction between the RND-MFP complex and the OMP. In particular site-directed mutagenesis of residues in the OMP known to mediate interaction with the MFP to elucidate whether the substrate specificity can be reversed. Previous mutagenesis studies in the OprM homolog TolC have shown helices 3 and 8 of the OMP to mediate interaction with its MFP, AcrA (Lobedanz *et al*. 2007). Further mutagenesis and suppressor studies in MexAB-OprM of *P. aeruginosa* identified residues T198 and F439 in OprM to suppress the hypersensitive phenotype of a mutation in the MFP, implicating that these mutated residues restored interaction with the mutant MFP (Nehme and Poole. 2007).

While a crystal structure is not available for OpmH, its structure, as well as OprM were modeled using an online modelling program, Phyre, visualized using UCSF Chimera v.1.5.2., and OpmH sequence was aligned to that of OprM using online multiple sequence alignment program ClustalW2 on the European Bioinformatics Institute website. The residues corresponding to OprM T198 and F439 in OpmH are E160 and Y412 respectively (Figure 13). As the corresponding residues have significantly different biochemical properties, it would be interesting to examine whether T198E and F439Y mutations in OprM, either independently or in tandem, has an effect on the effluxing capabilities of the MexJK-OprM complex. Particularly to see if substrate specificity would be altered from Erythromycin to Triclosan. Alternatively, the MexJK-OpmH complex effluxing capabilities can be examined with E160T and Y412F mutations in OpmH.

Figure 13. Models of OprM and OpmH. OprM and OpmH were modeled using an online program, Phyre, with sequences retrieved from NCBI (Appendix 3). Residues of interest in OprM as previously determined and the corresponding residues in OpmH, as determined by sequence alignment performed by ClustalW2, are indicated on the models.

Figure 12.



Further mutagenesis may be done on these mutants to isolate suppressors that alter the phenotype conferred by the proposed mutants T198E and F439Y in OprM, and E160T and Y412F in OpmH. It is likely that such suppressors map to the C-terminal domain of MexJ, as this region of the MFP has been characterized to mediate interaction with the OMP, and more specifically play a role in aperture opening (Weeks *et al*. 2010).

The novelty of the MexJK-OprM/OpmH RND efflux complex and its substrate specificity provides a good model with which to study and elucidate inter-component interactions between RND efflux pumps. Co-purification studies in the presence of various substrates will give insight into the native interactions between these proteins. Mutagenesis studies with this model will give insight as to the role in substrate selectivity of the OMP-MFP interaction.

VI

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APPENDIX A. Raw data from quantitative-real-time analysis of OpmH-H_6 expression

Target	Sample	Threshold Cycle (C(t))	C(t) Mean	C(t) Std. Dev
rpsl	PAO702/pET1.6P-opmH	18.70	18.79	0.129
rpsl	PAO702/pET1.6P-opmH	18.74	18.79	0.129
rpsl	PAO702/pET1.6P-opmH	18.94	18.79	0.129
rpsl	PAO702/pET1.6P-opmH + IPTG	18.32	18.48	0.196
rpsl	PAO702/pET1.6P-opmH + IPTG	18.41	18.48	0.196
rpsl	PAO702/pET1.6P-opmH + IPTG	18.70	18.48	0.196
rpsl	PA049	18.59	18.61	0.138
rpsl	PA049	18.48	18.61	0.138
rpsl	PA049	18.75	18.61	0.138
rpsl	PA049 + IPTG	17.94	18.14	0.190
rpsl	PA049 + IPTG	18.32	18.14	0.190
rpsl	PA049 + IPTG	18.16	18.14	0.190
rpsl	NTC	30.12	30.12	0.000
rpsl	NTC	30.15	30.15	0.000
opmH	PAO702/pET1.6P-opmH	22.67	22.91	0.330
opmH	PAO702/pET1.6P-opmH	23.14	22.91	0.330
opmH	PAO702/pET1.6P-opmH + IPTG	16.01	15.86	0.128
opmH	PAO702/pET1.6P-opmH + IPTG	15.77	15.86	0.128
opmH	PAO702/pET1.6P-opmH + IPTG	15.81	15.86	0.128
opmH	NTC		0.00	0.000
opmH	NTC	34.17	34.17	0.000
opmH	PA049	23.85	23.85	0.228
opmH	PA049	23.62	23.85	0.228
opmH	PA049	24.07	23.85	0.228
opmH	PA049 + IPTG	18.91	19.03	0.125
opmH	PA049 + IPTG	19.03	19.03	0.125
opmH	PA049 + IPTG	19.16	19.03	0.125

APPENDIX B. Broth dilution MICs of Triclosan for *P. aeruginosa* PAO702 constructs

Strain	**Efflux components expressed**	**Copy number of OMP gene**	**Triclosan MIC (µg/mL)**		**Fold change in MIC compared to PAO702**	
			-IPTG	+IPTG	-IPTG	+IPTG
PAO702	MexJ, MexK	0	1	1	0	0
PAO702/pET1.6P	MexJ, MexK	0	2	0	1	N/A
PAO702/pET1.6-opmH-H_6	MexJ, MexK, OpmH	Multiple	256	32	8	5
PAO702/pET1.6-oprM-H_6	MexJ, MexK, OprM	Multiple	1	1	0	0
PA051	MexJ, MexK	0	1	1	0	0
PA049	MexJ, MexK, OpmH	Single	2	32	2	5

APPENDIX C. Sequences of OpmH and OprM used for modelling and alignment

>OprM

MKRSFLSLAVAAVVLSGCSLIPDYQRPEAPVAAAYPQGQAYGQNTGAAAVPAADIGWREFFRDPQLQQLI
GVALENNRDLRVAALNVEAFRAQYRIQRADLFPRIGVDGSGTRQRLPGDLSTTGSPAISSQYGVTLGTTA
WELDLFGRLRSLRDQALEQYLATEQAQRSAQTTLVASVATAYLTLKADQAQLQLTKDTLGTYQKSFDLTQ
RSYDVGVASALDLRQAQTAVEGARATLAQYTRLVAQDQNALVLLLGSGIPANLPQGLGLDQTLLTEVPAG
LPSDLLQRRPDILEAEHQLMAANASIGAARAAFFPSISLTANAGTMSRQLSGLFDAGSGSWLFQPSINLP
IFTAGSLRASLDYAKIQKDINVAQYEKAIQTAFQEVADGLAARGTFTEQLQAQRDLVKASDEYYQLADKR
YRTGVDNYLTLLDAQRSLFTAQQQLITDRLNQLTSEVNLYKALGGGWNQQTVTQQQTAKKEDPQA

>OpmH

MLRRLSLAAAVAAATGVAWAAQPTPLPTKTDLISVYKEAVDNNADLAAAQADYLARKEVVPQARAGLLPQ
LGAGARVGDTRIAFDERPATVKRNSQVVQATLSQPLFRADRWFQWQAAKETSDQARLEFSATQQDLILRS
AETYFTVLRAQDNLATSKAEEAAFKRQLDQANERFDVGLSDKTDVLEAQASYDTARANRLIAEQRVDDAF
QALVTLTNRDYSAIEGMRHTLPVVPPAPNDAKAWVDTAVQQNLRLLASNYAVNAAEETLRQRKAGHLPTL
DAVAQYQKGDNDALGFANSAANPLVHYGKYVDERSIGLELNIPIYSGGLTSSQVRESYQRLNQSEQSREG
QRRQVVQDTRNLHRAVNTDVEQVQARRQAIISNQSSLEATEIGYQVGTRNIVDVLNAQRQLYAAVRDYNN
SRYDYILDTLRLKQAAGTLSPADLEALSAYLKQDYDPDKDFLPPDLAKAAAEQLQSKPRQQY

APPENDIX D. Multiple sequence alignment of OpmH with OprM

```
CLUSTAL 2.0.12 multiple sequence alignment


OprM            MKRSFLSLAVAAVVLSGCSLIPDYQRPEAPVAAAYPQGQAYGQNTGAAAVPAADIGWREF
60
OpmH            ----------------------MLRRLSLAAAVAAATGVAWAAQP------------TPL
26
                                       :* . ..*.* . * *:. :.             :

OprM            FRDPQLQQLIGVALENNRDLRVAALNVEAFRAQYRIQRADLFPRIGVDGSGTRQRLPGDL
120
OpmH            PTKTDLISVYKEAVDNNADLAAAQADYLARKEVVPQARAGLLPQLGAGARVGDTRIAFDE
86
                  ..:* .:   *::** ** .*  :  * :      **.*:*::*...     *:. *

OprM            STTGSPAISSQYGVTLGTTAWELDLFGRLRSLRDQALEQYLATEQAQRSAQTTLVASVAT
180
OpmH            RPATVKRNSQVVQATLSQPLFRADRWFQWQAAKETSDQARLEFS----ATQQDLILRSAE
142
                 .:     *.   .**. . :. * : : :: :: : :  *  .    ::*  *:   *

OprM            AYLTLKADQAQLQLTKDTLGTYQKSFDLTQRSYDVGVASALDLRQAQTAVEGARATLAQY
240
OpmH            TYFTVLRAQDNLATSKAEEAAFKRQLDQANERFDVGLSDKTDVLEAQASYDTARANRLIA
202
                :*:*:   * :*  :*   .:::::.:* ::. :***::.  *: :**:: : ***.

OprM            TRLVAQDQNALVLLLGSGIPANLPQGLGLDQTLLTEVP---AGLPSDLLQRRPDILEAEH
297
OpmH            EQRVDDAFQALVTLTNRDYSA--IEGMRHTLPVVPPAPNDAKAWVDTAVQQNLRLLASNY
260
                 : * :  :*** * . . .*   :*:    .::. .*    .  .  :*:.  :* :::

OprM            QLMAANASIGAARAAFFPSISLTANAGTMSRQLSGLFDAGS------GSWLFQP----SI
347
OpmH            AVNAAEETLRQRKAGHLPTLDAVAQYQKGDNDALGFANSAANPLVHYGKYVDERSIGLEL
320
                 : **: ::   :*..:*::. .*:  . ..:  *: ::.:      *.:: :     .:

OprM            NLPIFTAGSLRASLDYAKIQKDINVAQYEKAIQTAFQEVADGLAARGTFTEQLQAQRDLV
407
OpmH            NIPIYSGGLTSSQVRESYQRLNQSEQSREGQRRQVVQDTRNLHRAVNTDVEQVQARRQAI
380
                *:**::.*   :.:  :  : : .  . *   : ..*:. :   * .* .**:**:*: :

OprM            KASDEYYQLADKRYRTGVDNYLTLLDAQRSLFTAQQQLITDRLNQLTSEVNLYKALGGGW
467
OpmH            ISNQSSLEATEIGYQVGTRNIVDVLNAQRQLYAAVRDYNNSRYDYILDTLRLKQAAGTLS
440
                 :.:.  : ::  *:.*. * : :*:***.*::* ::  ..* : : . :.* :* *

OprM            NQQTVTQQQTAKKE-DPQA---------------------- 485
OpmH            PADLEALSAYLKQDYDPDKDFLPPDLAKAAAEQLQSKPRQQY 482
                  :  : .   *:: **:
```