Influence of membrane-damaging agents and the sigma factor AlgU on the induction of the MexCD-OprJ efflux system of Pseudomonas aeruginosa

by

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Abstract

The MexCD-OprJ multidrug efflux pump of *Pseudomonas aeruginosa* confers resistance to a range of antimicrobials. Although not expressed under normal laboratory conditions, exposure to the membrane-active biocides, chlorhexidine or benzalkonium chloride, results in *mexCD-oprJ* expression. This suggests that membrane disruption provides the inducing signal. Consistent with this, increased *mexCD-oprJ* expression was demonstrated in the presence of additional membrane-damaging agents including polymyxin B, ethanol, SDS, EDTA, the organic solvents *n*-hexane and *p*-xylene, and the antimicrobial peptides melittin, V8 and V681. MexCD-OprJ expression was initially verified through increased resistance to known MexCD-OprJ antimicrobial substrates and subsequently using a *mexC-lacZ* transcriptional fusion and RT-PCR. Since the *P. aeruginosa* sigma factor AlgU is responsive to envelope stress, it was of interest to ascertain whether AlgU is capable of mediating this increased *mexCD-oprJ* expression. Thus, the impact of AlgU loss on *mexCD-oprJ* expression in response to membrane-damaging agents was assessed in a Δ*algU* strain. In contrast with above, little or no *mexCD-oprJ* expression (assessed using resistance to MexCD-OprJ antimicrobial substrates, the *mexC-lacZ* transcriptional fusion and RT-PCR) occurred in response to membrane-damaging agents in the Δ*algU* strain, consistent with AlgU playing a role in the envelope stress inducibility of *mexCD-oprJ*. Overall, envelope stress, and the ability to react to this stress through AlgU, appears to play an important role in *mexCD-oprJ* induction. This suggests an important role for MexCD-OprJ in alleviating envelope stress, independent of its ability to export and provide resistance to antimicrobials. A gene, PA4596, whose product shows substantial homology to the NfxB repressor of
mexCD-oprJ expression, occurs downstream of mexCD-oprJ and shows AlgU-dependence and chlorhexidine inducibility, suggesting a role in the chlorhexidine-induced, AlgU-mediated expression of mexCD-oprJ. Thus, the impact of PA4596 loss on mexCD-oprJ expression was assessed. Paradoxically, the loss of PA4596 increases mexCD-oprJ expression in wild-type cells in response to chlorhexidine treatment (as assessed through RT-PCR), while its loss compromises mexCD-oprJ expression in an nfxB mutant. Nonetheless, this suggests that PA4596 is involved in the induction of mexCD-oprJ and that its ability to induce mexCD-oprJ differs depending on the state of nfxB.
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Table of Contents

Abstract................................................................................................................................i
Acknowledgements............................................................................................................iii
Table of Contents...............................................................................................................iv
List of Tables.....................................................................................................................vii
List of Figures...................................................................................................................viii
List of Abbreviations.........................................................................................................ix

CHAPTER 1: Literature Review .................................................................................. 1
1.1 Introduction........................................................................................................... 1
1.2 Antimicrobial agents’ mechanism of action ......................................................... 2
1.3 Bacterial antimicrobial resistance mechanisms .................................................... 5
  1.3.1 Target site modification ................................................................................ 5
  1.3.2 Antibiotic inactivation ................................................................................ 7
  1.3.3 Efflux mediated resistance ............................................................................ 8
1.4 Organization of non-RND efflux systems .......................................................... 10
1.5 Structure of RND-type efflux pumps ................................................................. 13
  1.5.1 The RND-type efflux systems of P. aeruginosa............................................... 17
  1.5.2 Genetic control and expression of RND-type efflux systems in P. aeruginosa................................................................................................... 19
1.6 Bacterial envelope structure and function .......................................................... 22
1.7 Membrane-damaging antimicrobials .................................................................. 27
  1.7.1 Cationic membrane-damaging agents ......................................................... 28
  1.7.2 Other membrane-damaging agents ............................................................. 31
1.8 The bacterial envelope stress response ............................................................... 33
  1.8.1 Induction of the $\sigma^E$ stress response in E. coli ............................................. 34
  1.8.2 Regulation of AlgU in P. aeruginosa ............................................................. 37
1.9 Experimental focus ............................................................................................. 39

CHAPTER 2: Materials and methods......................................................................... 41
2.1 Bacterial strains, plasmids, and growth conditions ............................................ 41
2.2 DNA isolation......................................................................................................... 41
2.3 Restriction digests and ligation of DNA fragments ............................................ 47
2.4 PCR amplification and nucleotide sequencing ................................................... 48
2.5 DNA transformation ........................................................................................... 49
  2.5.1 Calcium chloride competent E. coli ............................................................ 49
  2.5.2 Electrocompetent P. aeruginosa ................................................................. 49
2.6 Construction of gene deletions ........................................................................... 50
  2.6.1 Construction of a P. aeruginosa K1536 ΔalgU mutant .............................. 50
  2.6.2 Construction of a P. aeruginosa K1542 ΔmexCD-oprJ mutant ................. 51
  2.6.3 Construction of P. aeruginosa ΔPA4596 mutants ...................................... 52
2.7 Complementation of gene deletions ................................................................... 53
  2.7.1 Complementation of ΔalgU mutants .......................................................... 53
  2.7.2 Complementation of ΔPA4596 mutants ..................................................... 54
2.8 Antimicrobial susceptibility testing .................................................................... 54
2.9 Construction of lacZ fusion reporter strains ................................................... 55
2.10 β-galactosidase assay ....................................................................................... 57
2.11 Quantification of mexCD-oprJ expression using RT-PCR .............................. 59
  2.11.1 RNA isolation ............................................................................................. 59
  2.11.2 RT-PCR ....................................................................................................... 61

CHAPTER 3: Results ........................................................................................................... 62
  3.1 Influence of membrane-damaging agents on MexCD-OprJ-mediated antibiotic resistance in P. aeruginosa ................................................................. 62
  3.2 Influence of membrane-damaging agents on mexCD-oprJ expression in P. aeruginosa ........................................................................................................ 65
  3.3 Involvement of AlgU in MexCD-OprJ inducibility by membrane-damaging agents ................................................................................................................ 68
  3.4 The influence of a 2-hour exposure to membrane-damaging agents and the loss of AlgU on mexCD-oprJ promoter activity and expression ............................................ 69
  3.5 The effect of membrane-damaging agents and AlgU on mexCD-oprJ expression in wild-type P. aeruginosa ................................................................. 73
  3.6 Importance of AlgU for mexCD-oprJ hyperexpression in nfxB mutants ........ 77
3.7 Effect of cell wall-damaging agents and LPS mutations on mexCD-oprJ expression ................................................................. 79

3.8 Influence of PA4596 on mexCD-oprJ expression in P. aeruginosa ............... 81

3.9 Contribution of PA4596 to mexCD-oprJ hyperexpression in an nfxB-mutant strain of P. aeruginosa ................................................................. 84

CHAPTER 4: Discussion .................................................................................................................. 87

CHAPTER 5: Summary and conclusion ......................................................................................... 100

References .................................................................................................................................................. 101
List of Tables

Table 1. Substrates exported by RND-type efflux pumps in *P. aeruginosa*.............................18

Table 2. Bacterial strains used in this study........................................................................42

Table 3. Plasmids used in this study..................................................................................44

Table 4. Antibiotic concentrations used to maintain plasmids........................................45

Table 5. Sub-inhibitory concentrations of membrane-damaging agents used to determine antibiotic MICs in *P. aeruginosa*.................................................................56

Table 6. Sub-inhibitory concentrations of membrane-damaging agents used to determine *mexCD-oprJ* promoter activity in *P. aeruginosa* K1542 and K2861................58

Table 7. Sub-inhibitory concentrations of membrane-damaging agents used in the RNA isolation from *P. aeruginosa*..................................................................................60

Table 8. Susceptibility of *P. aeruginosa* K1542-derived strains to membrane-damaging agents.........................................................................................................................63

Table 9. Membrane-damaging agents induce *mexCD-oprJ* in an AlgU-dependent manner.................................................................................................................................64

Table 10. Susceptibility of *P. aeruginosa* K767 derived strains to membrane-damaging agents.................................................................................................................................74
# List of Figures

Figure 1. Organization of antibiotic efflux pump families in gram-negative bacteria......9

Figure 2. Genetic organization of select RND-type efflux pumps in *P. aeruginosa*........14

Figure 3. Structure of the gram-negative cell envelope..............................................23

Figure 4. Structure of lipopolysaccharide..................................................................26

Figure 5. Induction of the $\sigma^E$ pathway in *E. coli* in response to envelope stress........36

Figure 6. Proposed model of MexCD-OprJ induction following membrane damage.....40

Figure 7. Chlorhexidine induces *mexCD-oprJ* expression in an AlgU-dependent manner in $\Delta$mexB-$\Delta$mexXY strains of *P. aeruginosa*..............................................................66

Figure 8. Influence of membrane-damaging agents and AlgU on *mexCD-oprJ* expression in $\Delta$mexB-$\Delta$mexXY strains of *P. aeruginosa*...........................................................................67

Figure 9. Promoter activity of *mexCD-oprJ* after a 2-hour exposure to membrane-damaging agents in *P. aeruginosa* strains K1542 (A) and K2861 (B).............71

Figure 10. Induction of *mexCD-oprJ* expression following a 2-hour exposure to chlorhexidine is AlgU-dependent in a $\Delta$mexB-$\Delta$mexXY strain of *P. aeruginosa*....................................................................................................................72

Figure 11. Chlorhexidine induces *mexCD-oprJ* expression in an AlgU-dependent manner in wild-type *P. aeruginosa*................................................................................75

Figure 12. Influence of membrane-damaging agents and AlgU on *mexCD-oprJ* expression in wild-type *P. aeruginosa*..............................................................................76

Figure 13. Influence of AlgU on *mexCD-oprJ* expression in wild-type and *nfxB*-mutant strains of *P. aeruginosa*.................................................................78

Figure 14. Influence of fosfomycin on *mexCD-oprJ* expression in a $\Delta$mexB-$\Delta$mexXY strain of *P. aeruginosa*.....................................................................................80

Figure 15. Influence of LPS mutations on *mexCD-oprJ* expression in *P. aeruginosa*.....82

Figure 16. Genetic organization of PA4596 and the *mexCD-oprJ* operon...................83

Figure 17. Influence of PA4596 on *mexCD-oprJ* expression in response to chlorhexidine treatment in wild-type *P. aeruginosa*.....................................................85

Figure 18. Influence of PA4596 on *mexCD-oprJ* expression in an *nfxB*-mutant strain of *P. aeruginosa*..............................................................................................86
List of Abbreviations

AAC  aminoglycoside acetyltransferase
ABC  ATP-binding cassette
ANT  aminoglycoside nucleotidyltransferase
APH  aminoglycoside phosphotransferase
ATP  adenosine 5’-triphosphate
ALA  alanine
AMP  ampicillin
bp  base pair
BSA  bovine serum albumin
CAM  chloramphenicol
CAP  cationic antimicrobial peptide
CAR  carbenicillin
CAT  chloramphenicol acyltransferase
CHX  chlorhexidine
DEPC  diethylpyrocarbonate
DHPS  dihydropteroate synthetase
DMSO  dimethyl sulfoxide
DNA  deoxyribonucleic acid
dNTP  deoxyribonucleotide triphosphate
ECF  extracytoplasmic function
EDTA  ethylenediamine tetraacetic acid
erm  erythromycin ribosome methylation
ERY  erythromycin
EtOH  ethanol
FOS  fosfomycin
GlcN  D-glucosamine
HEX  n-hexane
KAM  kanamycin
kb  kilobase
KDO  2-keto-3-deoxy-octonic acid
LAC  lactone
LB  Luria Bertani
LPS  lipopolysaccharide
MATE  multidrug and toxic compound extrusion
MCS  multicloning site
MDR  multidrug resistance
MEL  melittin
MFP  membrane fusion protein
MF  major facilitator
MIC  minimal inhibitory concentration
NAG  N- acetylglucosamine
NOR  norfloxacin
O.D.  optical density
OMF  outer membrane factor
OMP, outer membrane protein
ONPG, o-nitrophenyl-β-D-galactopyranoside
PBP, penicillin binding protein
PCR, polymerase chain reaction
PMF, proton motive force
PxB, polymyxin B
RNA, ribonucleic acid
RNase, ribonuclease
RND, resistance-nodulation-division
rpm, revolutions per minute
rRNA, ribosomal ribonucleic acid
RT-PCR, reverse transcriptase-polymerase chain reaction
SMR, small multidrug resistance
SDS, sodium dodecyl sulfate
TAE, tris-acetate-EDTA
TE, tris-EDTA
TET, tetracycline
X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XYL, p-xylene
CHAPTER 1: Literature Review

1.1 Introduction

*Pseudomonas aeruginosa* is a common opportunistic human pathogen acquired in both the hospital and community setting. It is the most prevalent cause of nosocomial infections when a gram-negative bacterium has been determined to be the causative agent (Driscoll *et al.*, 2007). In total, *P. aeruginosa* is responsible for 11 – 13.8% of all microbiologically-associated nosocomial infections, being only second to *Staphylococcus aureus* (van Veen *et al.*, 1996; Driscoll *et al.*, 2007). Nosocomial infections typical of *P. aeruginosa* include pneumonia, urinary tract infections, bacteraemia, surgery-associated infections, and skin infections in burn patients. Hospital-acquired pneumonia caused by *P. aeruginosa* is the leading cause of death attributed to a hospital-acquired infection, while in burn patients, *P. aeruginosa* is the etiological agent in 57% of the associated infections (Estahbanati *et al.*, 2002; Driscoll *et al.*, 2007; Siempos *et al.*, 2007). In addition to nosocomial infections, *P. aeruginosa* plays an important pathogenic role in immunocompromised and in cystic fibrosis patients. Over 50% of respiratory samples from cystic fibrosis patients contain *P. aeruginosa* while serological samples of 97.5% of these patients indicate past or present infection with *P. aeruginosa*, thereby demonstrating the large impact the organism has on this patient population (Burns *et al.*, 2001).

Pathogenicity of the bacterium is due to a range of virulence factors. A single flagellum allows for motility within a host, while pili permit adherence to cell surfaces, particularly within the respiratory tract (O'Toole and Kolter, 1998). Once adherence
occurs, production of the extracellular polysaccharide alginate protects the bacterium from clearance (Ramsey and Wozniak, 2005; Alkawash et al., 2006). The production of bacterial biofilms is another barrier protecting cells from clearance and environmental stresses (bdi-Ali et al., 2006). Next, secreted virulence factors, including four exoenzymes and exotoxin A, alter the immune response in favour of colonization (Lanotte et al., 2003; Shaver and Hauser, 2004; Lau et al., 2005). Finally, P. aeruginosa has an intrinsically high resistance to many antimicrobial compounds. As is common with many gram-negative bacteria, a low outer membrane permeability is the first antimicrobial barrier, while expression of a chromosomally encoded β-lactamase provides resistance to many β-lactam compounds (Ciofu, 2003). A final important resistance mechanism is the active extrusion of antimicrobial compounds by efflux pumps; it is these systems that often provide a high level of resistance to a variety of antimicrobial compounds (Hancock and Speert, 2000; Poole, 2005b).

1.2 Antimicrobial agents’ mechanism of action

Antimicrobial compounds typically use one of three unique mechanisms to prevent bacterial growth or survival, each targeting a specific cellular process, such as bacterial cell wall synthesis, protein synthesis, or nucleic acid synthesis (Neu, 1992); other less common antimicrobial targets are bacterial metabolic pathways or the disruption of membrane structures. Both the β-lactam antibiotics, which include penicillins, cephalosporins, and carbapenems, and the glycopeptides, which include vancomycin, inhibit synthesis of the peptidoglycan layer found in the bacterial cell wall. This inhibition occurs through two distinct mechanisms. β-lactams bind to, and interfere
with, peptidoglycan binding proteins (PBPs), which are responsible for the transpeptidation reaction that links peptidoglycan subunits, while glycopeptides bind to the terminal D-alanine residue found on peptidoglycan subunits, thereby preventing cross-linking (McManus, 1997; Prescott et al., 2002). Both mechanisms result in a weakened peptidoglycan structure and cell lysis due to osmotic pressure.

Protein synthesis is inhibited by antibacterial agents, such as macrolides, aminoglycosides, tetracyclines, and chloramphenicol, which interfere with bacterial ribosomes (Neu, 1992). Macrolides and chloramphenicol bind to the 50S bacterial ribosomal subunit preventing protein synthesis, while aminoglycoside and tetracycline antibiotics interfere with protein synthesis through an interaction with the 30S ribosomal subunit. These agents are able to specifically target bacterial cells because of structural differences between prokaryotic and eukaryotic ribosomes (Prescott et al., 2002). Nucleic acid synthesis is prevented by fluoroquinolone antibiotics through the inhibition of DNA synthesis. This inhibition occurs with fluoroquinolone binding to either the DNA gyrase, in gram-negative organisms, or the DNA topoisomerase IV enzyme, in gram-positive organisms, although some exceptions do exist (Drlica and Zhao, 1997; Prescott et al., 2002). DNA gyrases are enzymes that are required to introduce negative supercoils into a replicating DNA strand, thereby preventing the accumulation of positive superhelical twists that block replication, while DNA topoisomerase IV is required for the separation of DNA strands following replication (Drlica and Zhao, 1997). Generally, both enzymes introduce DNA breaks, manipulate the DNA backbone structure, rejoin the separated strands, and finally release the DNA backbone. The binding of fluoroquinolones to these enzymes, stabilizes the DNA-DNA gyrase or DNA-DNA
topoisomerase IV complex, thereby preventing enzyme dissociation (Hooper, 2000). These complexes block the forward movement of DNA replication and result in double-stranded DNA breaks; both harmful occurrences within the bacterial cell that are able to cause cell death (Drlica and Zhao, 1997). Rifampin also prevents nucleic acid synthesis, although instead of inhibiting DNA synthesis, rifampin prevents the synthesis of RNA (Quinlivan et al., 2000). In this instance, disruption of transcription by rifampin occurs through an interaction of the antimicrobial agent with the bacterial DNA-dependent RNA polymerase.

The disruption of bacterial metabolic pathways or membrane structures may occur following exposure to agents such as sulphonamides and polymyxin B, respectively. Sulphonamide antimicrobial agents disrupt the bacterial metabolic process that is responsible for the production of folic acid, an essential product required for the synthesis of nucleic acids; they act as competitive inhibitors of the enzyme dihydropteroate synthetase (DHPS) required in folic acid production (Prescott et al., 2002). This agent does not affect eukaryotic cells, since eukaryotic cells do not manufacture folic acid but rather require dietary folic acid. Finally, polymyxin B, which disrupts bacterial membranes, is a cationic polypeptide that interacts with the anionic components found in the inner and outer membrane of gram-negative bacteria by displacing magnesium (Mg$^{2+}$) and calcium (Ca$^{2+}$) ions that are required for the maintenance of membrane integrity (Schindler and Osborn, 1979; Falagas and Kasiakou, 2005); this results in the increased permeability of these membranes and ultimately cell death.
1.3 **Bacterial antimicrobial resistance mechanisms**

Bacteria possess a variety of mechanisms able to provide resistance to antimicrobial agents and the presence of these mechanisms often limit the number of available treatment options for bacterial infections. While some resistance mechanisms are chromosomally encoded, others are present on mobile genetic elements, including plasmids and transposons, thus allowing for the transfer of the resistance mechanism to initially susceptible bacterial populations (Harbottle *et al.*, 2006). Still other resistance mechanisms occur as a result of mutations (Alekshun and Levy, 2007). The spread of both the mobile resistance mechanisms or the acquisition of beneficial mutations often requires a selective pressure for maintenance of the resistant population. The principle mechanisms of antimicrobial resistance are the modification of an antimicrobial target site, the inactivation of the drug, and the reduced concentration of an antimicrobial within a bacterium by either a decreased penetration of drug into a bacterium or the export of the drug from the bacterium (Simonsen *et al.*, 2004).

1.3.1 **Target site modification**

Modification of the bacterial ribosome is able to provide resistance to macrolides, such as erythromycin, while the production of proteins that compete with tetracycline for its ribosomal target site confer tetracycline resistance (Walsh, 2000; Connell *et al.*, 2003). Resistance in both gram-negative and gram-positive organisms to macrolides, such as erythromycin, may be mediated by the *erm* (erythromycin ribosome methylation) genes, which are methylases commonly located on mobile elements (Walsh, 2000; Roberts *et al.*, 2002). Erm proteins modify an adenine residue on the 23S rRNA, which is part of
the 50S ribosomal subunit found in bacterial cells, resulting in a target site modification that makes erythromycin, as well as other macrolide antibiotics, unable to bind the ribosome (Arthur et al., 1987). This modification may only occur during ribosome synthesis since the modified residue is inaccessible once the ribosome is assembled (Gaynor and Mankin, 2003).

In addition to the direct modification of the ribosomal target site, the production of proteins that physically compete with, and displace, the antibiotic from a target site confer resistance. The tetO and tetM genes, which are normally plasmid-encoded, mediate tetracycline resistance in this manner (Connell et al., 2003). These proteins are able to release tetracycline from the tetracycline-ribosome complex, thereby allowing the ribosome to continue translation, thus, conferring tetracycline resistance. This mechanism is present in both gram-negative and gram-positive organisms, having first been identified in the gram-negative organism Campylobacter jejuni (Chopra and Roberts, 2001). Although not yet identified in any clinical P. aeruginosa isolates, if introduced into P. aeruginosa, the TetO protein is active and able to contribute to tetracycline resistance (Jeannot et al., 2005).

Mutations within the DNA gyrase or DNA topoisomerase IV enzymes are also common mediators of resistance to fluoroquinolone antibiotics (Jalal and Wretlind, 1998; Roberts et al., 2002). In P. aeruginosa, mutations within the gyrA gene of DNA gyrase are most common (Jalal and Wretlind, 1998); additional DNA gyrase mutations, however, may also occur in gyrB or the parC gene of DNA topoisomerase IV (Hancock, 1998). Regardless of which mutation is present, they all provide resistance to fluoroquinolones, such as ciprofloxacin, and are often found in clinical strains that were
treated with ciprofloxacin (Jalal and Wretlind, 1998). Again, these mutations reduce the affinity of the fluoroquinolone antibiotic for their target while still allowing the enzyme to function normally.

1.3.2 Antibiotic inactivation

Resistance to β-lactam antibiotics is most commonly associated with the presence of β-lactamase enzymes (Babic et al., 2006). β-lactamases may be encoded by genes found within a bacterium’s chromosome or on plasmids and transposons. These enzymes hydrolyze the β-lactam ring present in β-lactam antibiotics, thereby inactivating the antibiotic before it is able to bind and sequester PBPs. β-lactamases may be categorized based on amino acid similarity and grouped into one of four categories, namely the Ambler classes A to D (Babic et al., 2006). In P. aeruginosa, classes B to D are most prevalent. Class B enzymes are metallo-β-lactamases that require zinc (Zn$^{2+}$) to function. They are often encoded on mobile elements, such as plasmids and integrons, and present in nearly 20% of imipenem resistant P. aeruginosa clinical isolates (Walsh et al., 2005; Babic et al., 2006). AmpC, a member of the Ambler class C family, is the most common β-lactamase in gram-negative bacteria, including P. aeruginosa, and is normally chromosomally-encoded, although ampC may be present on plasmids in bacterial species that lack the gene on the chromosome (Jacoby and Munoz-Price, 2005). It is hyperexpressed in response to an increased number of uncrosslinked peptidoglycan subunits, a phenomenon that occurs in the presence of β-lactam antibiotics (Jacobs et al., 1997). Finally, the class D of β-lactamases are grouped due to the use of a serine residue
in their active site. Many of the newly discovered β-lactamases of *P. aeruginosa* belong to this rapidly expanding family (Walther-Rasmussen and Hoiby, 2006).

Resistance to aminoglycosides is also mediated by antibacterial inactivation in *P. aeruginosa*. Three different classes of aminoglycoside modifying enzymes are known, namely aminoglycoside phosphoryltransferase (APH), aminoglycoside acetyltransferase (AAC), and aminoglycoside nucleotidylyltransferase (ANT), which phosphorylate, acetylate, or adenylate aminoglycosides, respectively (Poole, 2005a). These resistance conferring enzymes are often located on mobile elements with multiple enzymes found in a single *P. aeruginosa* strain, thus complicating aminoglycoside therapy.

### 1.3.3 Efflux mediated resistance

Efflux systems within bacteria are used to export a range of different natural compounds out of a cell under normal growth states. It is when these systems mediate resistance to antimicrobial agents that the presence of these systems is most problematic (Poole, 2005b). The exportation of antibiotic compounds decreases the intracellular antibiotic concentration resulting in increased bacterial survival. Expression of efflux pumps may confer resistance towards a single substrate or towards multiple structurally unrelated compounds. Efflux pumps that export a variety of substrates are often associated with multidrug resistance (MDR). Bacterial efflux pumps may be categorized into one of five different families consisting of the major facilitator (MF), ATP-binding cassette (ABC), resistance-nodulation-division (RND), small multidrug resistance (SMR), and multidrug and toxic compound extrusion (MATE) family (Figure 1)
Figure 1. Organization of antibiotic efflux pump families in gram-negative bacteria.

Representation of the major facilitator (MF), ATP-binding cassette (ABC), resistance-nodulation-division (RND), small multidrug resistance (SMR), and multidrug and toxic compound extrusion (MATE) family of antibiotic efflux pumps in gram-negative bacteria. The structural components of these systems include the outer membrane factor (OMF) and the membrane fusion protein (MFP). Also indicated is the system’s energy source (H⁺, Na⁺, or ATP). Adapted from (Poole, 2005b).
The driving force to export compounds by these pumps is normally a proton motive force (PMF); members of each efflux family are capable of using the energy from importing a proton to drive drug expulsion, with the exception being the ABC family that only utilizes ATP hydrolysis to drive drug export (Paulsen et al., 1996). While pumps exist in each family that confer antibiotic resistance, the RND family is often most associated with a high level of resistance to multiple compounds in clinical strains, often conferring resistance to clinically relevant compounds (Poole, 2002).

1.4 **Organization of non-RND efflux systems**

The efflux pump proteins that belong to the MF, ABC, SMR, and MATE families are normally located solely within the bacterial inner membrane. An exception to this rule occurs in some gram-negative bacteria where the ABC family may function as a tripartite system with proteins in the inner membrane, outer membrane, and periplasm; this organization is used by the MacAB-TolC transporter associated with macrolide resistance in *Escherichia coli* (Kobayashi et al., 2001). Generally, however, in gram-negative organisms these systems export substrates into the periplasm, unlike in gram-positive cells, where these systems export the substrate directly out of the cell, since they do not possess an outer membrane (Piddock, 2006).

The first recognized MF-type efflux pump belongs to the Tet family and mediates resistance to tetracycline and related compounds. This family is found in both gram-negative and gram-positive bacteria, however, it is more prevalent in gram-negative
organisms (Poole, 2005b; Poole, 2007). In both cases, these efflux genes are commonly found on plasmids and transposons, therefore allowing transfer to occur between bacterial populations (Poole, 2005b). The *tet* genes that are commonly associated with gram-negative bacteria are *tetA, tetB, tetC, tetD,* and *tetE,* where resistance in *P. aeruginosa* is typically mediated by TetB; each of these genes are controlled by the repressor protein TetR (Poole, 2001; Butaye *et al.*, 2003; Roberts, 2005). In contrast, the two prevalent *tet* genes in gram-positive bacteria, *tetK* and *tetL,* are inducible in the presence of tetracycline through a translational attenuation mechanism. Under non-inducing conditions a leader peptide is translated, which alters the conformation of the *tet* gene promoter region to a state incompatible with translation, while the presence of tetracycline binding to the ribosome, stalls leader peptide translation, which allows an alternative promoter conformation to form that is conducive to translation (Roberts, 1996; Butaye *et al.*, 2003).

As opposed to the proton gradient utilized by other systems, the principal feature of the ABC family of transporters is their use of ATP to drive extrusion of various compounds. Generally, these transporters are associated with resistance to a single antimicrobial agent. The LmrA protein is the one identified exception to this rule since it confers MDR, as opposed to resistance to a single compound, within *Lactococcus lactis* (van Veen *et al.*, 1996; Poelarends *et al.*, 2002; Poole, 2005b). MsrA is the best characterized ABC-type efflux protein and is found in *Staphylococcus* species, where it is able to export macrolide compounds (Ross *et al.*, 1990). Similar proteins to MsrA are found in other gram-positive organisms and, more recently, gram-negative organisms including *P. aeruginosa,* suggesting that *msrA*-type efflux systems are widespread among
bacterial species (Ojo et al., 2006). Although msrA was identified in gram-negative organisms, it still consists of only an inner membrane protein, in contrast to the MacAB-TolC system of E. coli, which possesses a unique construction for an ABC-type pump (Kobayashi et al., 2001). MacAB-TolC is part of a tripartite system that allows export through both membranes of the gram-negative envelope.

While there are two subfamilies of SMR-type efflux systems, only one is involved in antimicrobial resistance within bacteria (van Veen et al., 1996; Saier, Jr. et al., 1998; Putman et al., 2000). Even within this subfamily, antimicrobial resistance is not common, but exceptions do exist in both gram-positive and gram-negative bacteria. The smr gene, found in S. aureus, encodes a SMR-type efflux pump whose expression confers resistance to quaternary ammonium compounds (e.g. benzalkonium chloride) found in antiseptics and disinfectants, while the EmrE protein is an example of an SMR-type system that confers antimicrobial resistance in E. coli, a gram-negative organism, to tetracycline (Yerushalmi et al., 1995; Bjorland et al., 2001). A homologue of EmrE has been identified in P. aeruginosa, EmrE_{Pae}, and provides resistance to ethidium bromide, acriflavine and some aminoglycosides (Li et al., 2003a). Finally, there are examples of efflux pumps that belong to the MATE family, capable of providing antimicrobial resistance, found in many bacteria including Vibrio parahemolyticus (NorM), Haemophilus influenza (HmrM), Clostridium difficile (CdeA) and P. aeruginosa (PmpM) (Poole, 2005b; Piddock, 2006); fluoroquinolones are the often the target compounds exported by these pumps. A unique feature of these systems is the use of different energy sources; some systems use the PMF typical of the other efflux families
(e.g. PmpM of *P. aeruginosa*), while most utilize a Na\(^+\) gradient to drive substrate export (e.g. NorM of *V. parahemolyticus*) (Morita *et al.*, 1998; He *et al.*, 2004).

### 1.5 Structure of RND-type efflux pumps

The RND family of efflux pumps are organized as tripartite efflux systems in gram-negative bacteria, while in gram-positive organisms they are composed of a single protein; their presence in gram-positive bacteria, however, is limited (Poole, 2001). The three proteins that make up these systems are the RND transporter protein, located within the inner membrane (e.g. AcrB in *E. coli*/MexB in *P. aeruginosa*), a periplasmic membrane fusion protein (MFP) (e.g. AcrA in *E. coli*/MexA in *P. aeruginosa*), and an outer membrane protein, called the outer membrane factor (OMF) (e.g. TolC in *E. coli*/OprM in *P. aeruginosa*). The genomic organization is fairly conserved among different bacterial species where efflux genes are organized into operons. The MFP gene is normally located first in the operon, with the gene encoding the RND component located adjacent to the MFP gene. Finally, a gene encoding a regulatory protein is located upstream of gene for the RND component. The OMF may be encoded within the operon (e.g. OprM of the MexAB-OprM efflux system) or may be found elsewhere in the chromosome (e.g. TolC of the AcrAB-TolC efflux system) (Ma *et al.*, 1993; Li *et al.*, 1995; Gotoh *et al.*, 1995). The genetic organization of major RND-type efflux systems in *P. aeruginosa* is shown in Figure 2.

Crystal structures are available for all proteins of the AcrAB-TolC system in *E. coli* (Koronakis *et al.*, 2000; Murakami *et al.*, 2002; Mikolosko *et al.*, 2006), while in *P.*
Figure 2. Genetic organization of select RND-type efflux pumps in *P. aeruginosa*.

The organization of RND-type efflux pumps that are clinically relevant to antimicrobial resistance. Genes that encode the membrane fusion protein (red arrow), RND transporter (blue arrow), outer membrane factor (orange arrow), and regulatory proteins (green arrows) are shown. Adapted from (Schweizer, 2003).
*P. aeruginosa* the structures of MexA and OprM from the MexAB-OprM system have been solved (Akama *et al.*, 2004a; Akama *et al.*, 2004b). These structures assist in explaining how the components might be assembled, as well as how the components may function. The structure of the *E. coli* RND component AcrB suggests that it exists as a trimer with three domains: a 50 Å (length) by 100 Å (width) transmembrane domain, a 40 Å (length) pore domain, and a 30 Å (length) ‘TolC docking’ domain (Murakami *et al.*, 2002; Eswaran *et al.*, 2004). The periplasmic domains of the trimer form a central cavity that is accessible from the periplasm by three vestibules on the structure’s side, while the funnel-like top of the periplasmic domain is in close contact with the OMF (Tamura *et al.*, 2005). This protein is responsible for substrate recognition and uptake into the export apparatus from either the cytoplasm or periplasm, where substrate recognition by this component has been demonstrated by at least two different means (Mao *et al.*, 2002; Eswaran *et al.*, 2004). First, in *P. aeruginosa*, alterations in amino acid sequences of the large periplasmic loops, results in changes to the substrate recognition profile of the RND pump (Mao *et al.*, 2002). This demonstrates that the RND component is responsible for substrate recognition and that recognition may occur in the periplasm. Substrate recognition by this component is supplemented by studies examining the functional replacement of the RND protein of one efflux system (e.g. MexB) with the RND protein from another system (e.g. MexC) and examining the resistance profile. The resistance profile for these chimeric systems mimic that of the RND component’s original system, and is independent of the MFP and OMF present, again demonstrating the significance of this protein in determining the resistance profile (Murata *et al.*, 2002).
While the RND component determines resistance patterns, the OMF forms an exit duct through the outer membrane for substrates. The crystal structure of the TolC (*E. coli*) and OprM (*P. aeruginosa*) proteins have been solved (Koronakis *et al.*, 2000; Akama *et al.*, 2004a). These structures indicate that the proteins have two general domains: a membrane spanning β-barrel domain and a long periplasmic cavity-forming α-helical domain. Like the RND components, these proteins also function as trimers within the outer membrane. Although both TolC and OprM are functionally homologous, a major difference between the two protein structures lies in the size of the membrane pore. TolC forms a pore diameter of approximately 19.8 Å, which is constitutively open to the exterior, while the pore size of OprM is 6 – 8 Å and too small to allow for the passage of antibiotics, thus, suggesting the presence of a pore opening mechanism (Akama *et al.*, 2004a). Both proteins, however, have tightly closed periplasmic domains to control the movement of substrates; this is the only conformational opening required to allow movement of a substrate out of TolC.

Stabilizing the RND component and OMF protein interaction is the MFP. In both *E. coli* (AcrA) and *P. aeruginosa* (MexA), a MFP monomer is composed of a β-barrel, a lipoyl, and an α-helical hairpin region (Akama *et al.*, 2004b; Mikolosko *et al.*, 2006). A functional MFP structure is proposed to be oligomeric, either consisting of a joined hexamer and heptamer for MexA or a trimer for AcrA, where monomers interact through the α-helical domains of the proteins. The interaction of MFP oligomers, the RND component, and the OMF is essential in the formation of a functional efflux system. The interaction between these components may be demonstrated by the recovery of both the AcrAB-TolC and MexAB-OprM protein complexes without prior cross-linking.
Additionally, genetic second-site suppressor experiments, where loss of function mutations in one protein may be complemented with mutations in another protein, have also suggested these components interact in a functional efflux system (Gerken and Misra, 2004; Nehme and Poole, 2005).

1.5.1 The RND-type efflux systems of *P. aeruginosa*

Within *P. aeruginosa*, there are a number of potential RND efflux systems based on genome analysis (Stover *et al.*, 2000), and nine have been characterized: MexAB-OprM (Poole *et al.*, 1993), MexCD-OprJ (Poole *et al.*, 1996), MexEF-OprN (Kohler *et al.*, 1997), MexXY (Mine *et al.*, 1999), MexJK (Chuanchuen *et al.*, 2002), MexGHI-OpmD (Aendekerk *et al.*, 2002), MexVW (Li *et al.*, 2003b), MexPQ-OpmE (Mima *et al.*, 2005) and MexMN (Mima *et al.*, 2005). Each system is part of an operon as previously described. The four systems listed above that do not have an associated OMF, namely MexXY, MexJK, MexVW, and MexMN, use the OMF OprM, which is located in the constitutively expressed *mexAB-oprM* operon. Each of the nine systems does not contribute equally to the overall antimicrobial resistance of *P. aeruginosa* with resistance being principally mediated by the MexAB-OprM, MexCD-OprJ, MexXY, MexEF-OprN systems. Often it is only after cloning, over-expression, or the use of deletion strains (strains deficient in the 4 major systems) that antimicrobial resistance attributable to the minor systems may be observed. Generally, fluoroquinolones, tetracycline, and chloramphenicol are among the most common agents capable of being exported by these systems (refer to Table 1 for the substrate profile of each system). Both antibiotics and
Table 1. Substrates exported by RND-type efflux pumps in *P. aeruginosa.*

<table>
<thead>
<tr>
<th>Efflux Pump</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MexAB-OprM</td>
<td>β-lactams, fluoroquinolones, tetracycline, chloramphenicol, macrolides, SDS, ethidium bromide</td>
</tr>
<tr>
<td>MexCD-OprJ</td>
<td>β-lactams, fluoroquinolones, chloramphenicol, tetracycline, macrolides, chlorhexidine, crystal violet, SDS, ethidium bromide</td>
</tr>
<tr>
<td>MexEF-OprN</td>
<td>Fluoroquinolones, chloramphenicol,</td>
</tr>
<tr>
<td>MexXY</td>
<td>Fluoroquinolones, tetracycline, erythromycin, aminoglycosides</td>
</tr>
<tr>
<td>MexGHI-OpmD</td>
<td>Vanadium</td>
</tr>
<tr>
<td>MexJK</td>
<td>Tetracycline, erythromycin</td>
</tr>
<tr>
<td>MexVW</td>
<td>Norfloxacin, chloramphenicol, tetracycline, ethidium bromide</td>
</tr>
<tr>
<td>MexPQ-OpmE</td>
<td>Erythromycin, chloramphenicol, tetracycline</td>
</tr>
<tr>
<td>MexMN</td>
<td>Chloramphenicol</td>
</tr>
</tbody>
</table>

Adapted from (Schweizer, 2003).
other antimicrobial compounds may be exported by a single efflux system; for example, the MexCD-OprJ system is capable of exporting many antibiotic compounds including β-lactams, fluoroquinolones, chloramphenicol, and tetracycline, and non-antibiotic compounds such as crystal violet, ethidium bromide, and sodium dodecyl sulfate (SDS) (Poole, 2001).

1.5.2 Genetic control and expression of RND-type efflux systems in *P. aeruginosa*

With the exception of the constitutively expressed MexAB-OprM system, expression of each of the RND efflux systems in *P. aeruginosa* typically occurs following exposure to an inducing agent or mutational activation. The genetic control and expression of the 4 major RND-type efflux systems (MexAB-OprM, MexCD-OprJ, MexXY and MexEF-OprN) will be examined. Activating mutations have been identified for each of the 4 systems, while only the MexXY and MexCD-OprJ systems have been demonstrated to be inducible in the presence of certain agents. Although the MexAB-OprM system is constitutively expressed under normal laboratory conditions, *nalB* mutants have been identified that hyperexpress this system (Saito *et al.*, 1999). These strains possess mutations in the *mexR* gene, which encodes a negative regulator located upstream of *mexAB-oprM*. The MexR protein is able to bind twice in the *mexA-mexR* intergenic region and represses transcription of both the *mexAB-oprM* operon and itself (Evans *et al.*, 2001). The regulation of MexAB-OprM, however, is quite complex and other mutations that hyperexpress this system have been identified in *nalC* and *nalD* mutants (Srikumar *et al.*, 2000).
The MexEF-OprN system is not normally expressed in wild-type cells but is present in so-called *nfxC* mutants (Fukuda *et al.*, 1990). Unlike MexAB-OprM, a positive regulatory protein, MexT, is responsible for the regulation of this system; like the other genes encoding a regulatory protein, *mexT* is located upstream of *mexEF-oprN* (Kohler *et al.*, 1997). The *mexT* gene, however, is usually found inactivated in most so-called wild-type *P. aeruginosa* strains (Maseda *et al.*, 2000). In *nfxC* mutants, *mexEF-oprN* expression and multi-drug resistance, results from the mutational reversion of these pre-existing *mexT* mutations. In MexT+ strains, which contain an active *mexT* gene, mutations in a second gene, *mexS*, are also able to increase *mexEF-oprN* expression (Sobel *et al.*, 2005).

Similar to the MexEF-OprN system, MexXY is not normally found expressed in wild-type cells but expression may occur following the accumulation of activating mutations (Masuda *et al.*, 2000a). Normally, MexXY is repressed due to the presence of a negative regulatory protein, MexZ, which is found linked to the *mexXY* operon; MexZ represses expression of both *mexXY* and itself (Aires *et al.*, 1999; Matsuo *et al.*, 2004). Mutations in the *mexZ* gene, therefore, result in the increased expression of this system. In contrast to both MexAB-OprM and MexEF-OprN, the MexXY system may also be induced when specific agents are present. MexXY may be induced in MexZ+ strains by the presence of ribosome-targeting drugs, such as aminoglycosides and tetracyclines, where these compounds are also MexXY substrates (Masuda *et al.*, 2000b; Morita *et al.*, 2006). Interestingly, it is not the presence of these inducing substrates that interacts with MexZ but rather another by-product of their influence on the cell, since the presence of
these substrates fail to inhibit MexZ binding to the *mexXY* promoter region in gel retardation assays (Matsuo *et al.*, 2004).

Finally, expression of *mexCD-oprJ* may occur following the occurrence of mutations in the gene encoding its regulatory protein or the presence of inducing agents (Poole *et al.*, 1996; Morita *et al.*, 2003). This system is regulated by the NfxB repressor protein, where the *nfxB* gene is found divergently transcribed from the *mexCD-oprJ* operon. Like the negative regulators MexR and MexZ, NfxB represses expression of both its associated efflux system (*mexCD-oprJ*) and itself (Shiba *et al.*, 1995; Poole *et al.*, 1996). Since this system is regulated by a repressor protein, *P. aeruginosa* strains with inactivating mutations in *nfxB* will express *mexCD-oprJ*. MexCD-OprJ may also be induced following exposure to certain agents such as the biocides benzalkonium chloride and chlorhexidine, as well as cytotoxic agents such as tetrphenylphosphonium chloride and ethidium bromide (Morita *et al.*, 2003). In these instances, this expression occurs in the presence of a functional *nfxB* gene. While some of these compounds are also exported by MexCD-OprJ, not all inducers are substrates; for example, benzalkonium chloride is not exported by this system yet, as stated, is an inducing compound. Interestingly, expression of this system results in an increased susceptibility to certain β-lactams (e.g. imipenem) and aminoglycosides (Masuda *et al.*, 1996). Although a reduced expression of *ampC* has been suggested to be the cause of the increased β-lactam susceptibility in MexCD-OprJ producing strains, studies have demonstrated that this is not the case and work is currently ongoing to determine a link (Wolter *et al.*, 2005; Wolter *et al.*, 2007).
1.6 **Bacterial envelope structure and function**

The gram-negative bacterial envelope consists of two membranes, namely the inner and outer membrane, and the periplasmic space found between these membranes, which contains the peptidoglycan layer (Figure 3). The inner membrane, or cytoplasmic membrane, is found in contact with the cytoplasm. It consists of almost equal amounts of phospholipid and protein (Schumann, 2006). Phospholipids are composed of a hydrophobic tail region containing two fatty acids and a hydrophilic head region that consists of glycerol and a phosphate group. The phospholipids are arranged in a bilayer with the hydrophobic tails in contact with one another. This formation results in a hydrophobic barrier that prevents movement of polar molecules. Additionally, these hydrophobic interactions assist in maintaining inner membrane structure. Further stabilization occurs through the cross-linking of phospholipids by Ca\(^{2+}\) and Mg\(^{2+}\) cations; these cations form ionic bonds with neighbouring negatively charged phosphates in the phospholipid headgroups (Kadner, 1996; Schumann, 2006).

Proteins that are associated with the inner membrane are often amphipathic in nature; they possess hydrophobic regions that interact with the interior of the membrane bilayer, while other regions are hydrophilic and interact with the cytoplasmic or periplasmic compartments (Kadner, 1996). These proteins are involved in many functions within the cell including the transport of compounds and proteins across the inner membrane and the generation of energy (Kadner, 1996; Schumann, 2006). The transfer of electrons between various electron acceptors in the inner membrane is used to generate energy and to maintain the PMF. The PMF, in turn, may also be used in the generation of ATP by ATP synthetases. Since the inner membrane is involved in energy
Figure 3. Structure of the gram-negative cell envelope.

Two membranes are present in the gram-negative cell wall, an outer membrane and an inner membrane; these membranes are composed of phospholipids with hydrophobic headgroups and hydrophilic tail regions. A peptidoglycan layer is located within the periplasmic space between these two membranes. Various proteins are located within the two membranes, while lipoproteins within the outer membrane are anchored to the peptidoglycan layer. Outer membrane integrity requires the presence of lipopolysaccharide (LPS) and the association of divalent cations (Ca\(^{2+}\) and Mg\(^{2+}\)).
production, inner membrane damage is lethal to the bacterial cell (Kadner, 1996; Schumann, 2006).

The periplasmic compartment is the next section of the gram-negative cell envelope that is encountered moving from the inside of the organism outwards. It is located between the inner and outer membrane and contains the peptidoglycan layer. The periplasmic space also contains many proteins including hydrolytic enzymes, detoxifying enzymes, transport proteins, and proteins involved in biogenesis of peptidoglycan and outer membrane components (Park, 1996; Schumann, 2006). Hydrolytic enzymes function to degrade complex molecules, thus allowing their uptake across the inner membrane, while detoxifying enzymes are able to degrade substances harmful to the bacterium, and include enzymes such as β-lactamases. Transport proteins work to either move substances, often hydrophobic in nature, from the outer membrane to the inner membrane or to chaperone proteins from the inner membrane whose destination is the outer membrane or beyond. This compartment is also the location of the peptidoglycan layer, which gives cells shape and prevents lysis (Park, 1996). It is essential for cell survival, and antibiotics that inhibit cross-linking of this layer, such as β-lactams, are, thus, bacteriocidal. The peptidoglycan layer is attached to the outer membrane through the association of lipoproteins, which are embedded in the outer membrane and form covalent links with the peptidoglycan layer (Nikaido, 1996; Park, 1996).

The final layer of the bacterial envelope, which is in contact with the cell’s surrounding, is the outer membrane. The outer membrane is an asymmetric lipid bilayer, where the inner leaflet is composed of phospholipids and the outer leaflet of lipopolysaccharide (LPS). This layer functions as another barrier to hydrophobic
molecules, inhibits movement of hydrophilic molecules, and encloses the periplasm (Nikaido, 1996; Schumann, 2006). Porins are found within the outer membrane to facilitate the movement of hydrophilic compounds through this barrier (Nikaido, 1996). The low permeability of the outer membrane is principally due to the presence of LPS. LPS is composed of three components: a hydrophobic lipid A region, a hydrophilic O-antigen polysaccharide region, and a core oligosaccharide region that links these two regions (Figure 4) (Park, 1996; Schumann, 2006). The lipid A region, found within the outer membrane bilayer, contains two D-glucosamine (GlcN) residues that have six or seven saturated fatty acids attached. Sugars, such as 2-keto-3-deoxy-octonic acid (KDO), heptose, galactose, and glucosamine, are attached to this region and make up the core polysaccharide. Finally, repeating sugar subunits make up the distal O-antigen. Of all regions of this molecule, only the presence of lipid A and KDO is essential (Park, 1996). Since LPS functions in decreasing the outer membrane permeability, so-called “deep rough” mutants, which only express lipid A and KDO, demonstrate greatly increased outer membrane permeability (Ruiz et al., 2006). The lipid A region, is also the endotoxic component of the outer membrane that is toxic to many mammals (Nikaido, 1996).

The structure of the outer membrane is maintained, again by hydrophobic interactions among lipid components on the interior of the bilayer, and through cross-linking by the divalent cations, \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \). Cross-linking occurs through the interaction between these cations and the negatively charged phosphate groups of the lipid A region or the negatively charged KDO sugars of the core polysaccharide (Nikaido, 1996; Ruiz et al., 2006). These divalent cations are required to minimize
Figure 4. Structure of lipopolysaccharide.

Diagram of the outer membrane component lipopolysaccharide. It is composed of three regions: the lipid A, the core polysaccharide, and the O-antigen. Taken from (Miller et al., 2005).
repulsive forces between adjacent LPS molecules, due to the large number of negatively charged groups found on LPS (Nikaido, 1996). Since divalent cations are crucial in the maintenance of outer membrane integrity, ion chelators, such as ethylenediamine tetraacetic acid (EDTA) have been used to study the LPS leaflet of the outer membrane. The presence of EDTA removes the Ca\(^{2+}\) and Mg\(^{2+}\) ions, destabilizes the LPS containing monolayer of the membrane and results in the liberation of LPS from this layer (Nikaido, 1996); phospholipids may flip across the leaflet and occupy the regions where LPS was once located, resulting in regions that contain only a phospholipid bilayer, which are more permeable to hydrophilic agents.

1.7 Membrane-damaging antimicrobials

Since the envelope is so crucial to the survival of the bacterial cell, many agents that interact and disrupt this region often have antimicrobial activity. The physical characteristics of the membrane previously described, often dictate what properties membrane-active antimicrobial compounds must possess. Molecules that interact with membranes, therefore, often contain cationic regions that interact with the numerous negatively charged portions on the surface of either the inner or outer membrane, or amphipathic/hydrophobic sections, which allow movement of the molecules into the membrane bilayer.
1.7.1 Cationic membrane-damaging agents

Many biocides, antibiotics, and immune components are amphipathic cationic agents that are able to damage both the inner and outer membrane of gram-negative cells. Representative examples of compounds that fall into these categories include chlorhexidine, polymyxin B, and cationic antimicrobial peptides (CAPs), respectively. This damage results in bacterial death, due to the loss of essential membrane function, which is principally associated with damage to the inner membrane and the resulting loss of energy production (Gilbert and Moore, 2005).

The biocide chlorhexidine is a commonly used cationic agent found in surgical hand-washes, in topical antiseptics, and in oral hygiene products where it kills many gram-positive and gram-negative bacteria (Hope and Wilson, 2004; Gilbert and Moore, 2005). The chlorhexidine molecule possesses two cationic sites that are separated by six saturated carbons, which create a small hydrophobic region (Davies, 1973). Disruption of the outer membrane occurs through the binding of the cationic regions of chlorhexidine to available anionic sites on LPS, displacing Ca\(^{2+}\) and Mg\(^{2+}\) cations in the process. This displacement results in the release of LPS molecules, which increases membrane permeability since, as described previously, the release of LPS results in areas composed of only a phospholipid bilayer, which is more permeable to hydrophilic substances (Nikaido, 1996; Yasuda et al., 2003). The hydrophobic region of chlorhexidine is too small to integrate into the outer membrane and instead remains above the outer leaflet. Normally the tight association of LPS molecules with the small divalent cations stabilizes the outer membrane but the larger chlorhexidine molecules do not allow these stabilizing interactions to form, thereby membrane permeability is
increased. These changes are believed to allow chlorhexidine molecules to move across
the outer membrane (Gilbert et al., 1990). On the inner membrane, chlorhexidine is able
to interact with the negatively charged phospholipid headgroups found in this layer.
Once again, a chlorhexidine molecule replaces the small divalent cations bridging these
headgroups, resulting in increased membrane permeability that, in turn, leads to the
leakage of ions and protons, the disruption of the PMF, and the loss of energy production
(Gilbert and Moore, 2005).

The antibiotic polymyxin B is typically used topically, as nephrotoxicity is a
common result of internal administration (Spann et al., 2004). Despite its toxic effects on
the kidney, however, it may be used internally to combat antibiotic resistant gram-
negative infections, particularly those associated with P. aeruginosa, with appropriate
monitoring of kidney function (Ouderkirk et al., 2003). Polymyxin B is a lipoprotein that
contains five cationic regions and a long hydrophobic tail (Storm et al., 1977; Nikaido,
1996). At low concentrations, it is able to selectively permeabilize the outer membrane
(Vaara, 1992). An interaction with the anionic regions of LPS is believed to be the first
step in membrane disruption, while integration into the membrane, due to the presence of
the hydrophobic tail, likely promotes its uptake through this membrane (Nikaido, 1996).
This interaction with LPS is believed crucial since alterations in LPS structure,
specifically the replacement of a negatively charged phosphate group in the lipid A
region with 4-aminoarabinose, confers resistance to polymyxin B (Lee et al., 2004).
Interestingly, in contrast with chlorhexidine, polymyxin B does not lead to the release of
LPS from the outer membrane (Nikaido, 1996). The lethal action of polymyxin B, which
occurs at higher concentrations, is believed to result from the leakage of cellular
components, and a generalized disorganization of the inner membrane. This occurs following binding to negatively charged phospholipid headgroups in the inner membrane and the subsequent incorporation of polymyxin B into this layer (Vaara, 1992).

Finally, CAPs are proteins commonly found in nature and include proteins such as defensins, found in immune cells, and melittin, found in honey bee venom (Nikaido, 1996; Mookherjee and Hancock, 2007; de Leeuw and Lu, 2007). These proteins are believed to create channels in the bacterial inner membrane, which in turn, cause cell death (Nikaido, 1996). Melittin, in particular, is highly toxic to both bacterial and blood cells (Watala and Gwozdzinski, 1992). Although very few studies have examined the interaction of CAPs with the outer membrane, it is believed that interactions with the anionic regions of LPS are critical to promote self-uptake (Nikaido, 1996). This is partially based on an examination of defensins, which have been demonstrated to bind with high affinity to LPS (Sawyer et al., 1988). Although the exact mechanism of peptide-membrane interaction, as well as their specific lethal effects, is still under investigation, it has been demonstrated that melittin is able to permeabilize the outer membrane (Vaara, 1992; Piers et al., 1994). The partial disruption of the gram-negative outer membrane is believed to be a sub-lethal effect, which allows the peptide to penetrate this barrier and subsequently disrupt the inner membrane that, in turn, results in cell death.
1.7.2 Other membrane-damaging agents

In addition to cationic compounds that exhibit membrane-damaging activity, other compounds are also capable of damaging membranes, including detergents (e.g. SDS), solvents, ethanol, and ion chelators (e.g. EDTA). With the exception of EDTA, all of these agents possess hydrophobic properties, which are likely important features for membrane disruption. SDS is an ionic detergent that is commonly found in household items and is often used in the laboratory. Little is known about its interaction with bacterial cells but studies using model membranes have been performed. Since SDS is an amphipathic molecule, it interacts non-specifically with phospholipids moving between the inner and outer leaflets of a membrane bilayer; this integration and “flip-flop” between the inner and outer leaflets is believed to be the first stage in membrane disruption (LeMaire et al., 2000). With the eventual saturation of the membrane by SDS molecules, phospholipid-detergent micelles readily form resulting in the sequestration of these phospholipids away from the membrane. Although the mechanism of action in bacterial cells in not defined, SDS does effectively permeabilize the inner and outer membrane (Filip et al., 1973)

A similar mechanism exists for organic solvents, including alkanes such as \( n \)-hexane, and cyclic hydrocarbons, such as \( p \)-xylene. Since these compounds are both hydrophobic, they are able to enter membrane bilayers (Sikkema et al., 1995). Toxicity is related to this ability and it is believed that the accumulation of these compounds in a membrane causes the loss of membrane integrity, which, in the case of the inner membrane, alters function (through the loss of the proton gradient) (Sikkema et al., 1995; Ramos et al., 2002); this would likely be the main lethal effect. The importance of
membrane disruption by these agents as a means to kill bacterial cells comes from studies in resistant bacterial species. Modifications that increase membrane rigidity (e.g. an increased number of trans fatty acids and a more ordered phospholipid configuration) or that increase the biosynthesis of membrane phospholipids, thus, are able to confer resistance to organic solvents, since their target site, the membrane, is altered (Sikkema et al., 1995; Pinkart and White, 1997; Sardessai and Bhosle, 2002).

It has always been suggested that the adverse effects of ethanol on bacterial membranes are due to the hydrophobic nature of the compound (Jones, 2007). There is, however, debate surrounding this concept since ethanol is a polar compound that readily hydrogen bonds and is soluble in water; partitioning of ethanol into the hydrophobic core of the membrane bilayer, therefore, is likely minimal. Instead, this agent likely competes with water for hydrophilic regions of LPS on the outer membrane surface, or proteins in the case of the inner membrane (Tsuchido et al., 1985; Jones, 2007). Regardless of a specific site of interaction, it is important to note that ethanol does alter membrane structure (e.g. increases proportion of unsaturated fatty acids) and results in the loss of membrane integrity (Jones, 2007).

Unlike the previous compounds that possessed hydrophobic regions, EDTA, a common laboratory ion chelator, mediates outer membrane-damage through the sequestration of Ca$^{2+}$ and Mg$^{2+}$ ions associated with LPS (Watt and Clarke, 1994). This, as stated previously, results in the loss of LPS from the bacterial surface and, thus, increases permeability; although this disruption normally causes cell lysis, cells kept in an osmotically stable environment may survive (Asbell and Eagon, 1966). Lysis does occur, however, with concomitant damage to the peptidoglycan layer caused by
autolysins, which become activated following EDTA exposure and degrade small areas of the peptidoglycan, weakening its structure (Watt and Clarke, 1994). Regardless of the mechanism of cell death by EDTA exposure, the presence of EDTA results in outer membrane perturbations by destabilizing and releasing LPS from the outer leaflet of this membrane.

1.8 The bacterial envelope stress response

Since the structural integrity of the bacterial envelope is critical to the survival of the organism, envelope integrity is continuously monitored by a bacterial cell (Ades, 2004). Envelope stress, particularly damage that results in the accumulation of misfolded outer membrane proteins in the periplasm, is monitored during both normal cellular growth and during various stressing situations; this information, in turn, is communicated to the interior of the cell (Raivio and Silhavy, 2001). Two-component pathways and sigma factors are key mechanisms that are used to sense and convey this information. In *E. coli*, three signal transduction pathways have been identified that monitor envelope status, namely CpxAR, BaeSR, and σ^E (Raivio, 2005).

The primary stresses that the CpxAR pathway senses are the presence of misfolded proteins in the periplasm (e.g. misfolded PapE or PapG pilin subunits that do not get incorporated into the pilin structure but rather remain in the periplasm) and changes to inner membrane structure (e.g. changes to lipid composition) (Dorel *et al.*, 2006). Expression of genes involved in the folding of proteins (e.g. *dsbA*, *ppiA*, *ppiD*), the degradation of misfolded proteins (e.g. *degP*), the chaperone of proteins (e.g. *dnaK*)
and the production of membrane proteins (e.g. *ompF* and *ompC*) are activated by the Cpx system (or possess potential CpxR binding domains); together the activation of these proteins allows cells to repair or produce new membrane proteins, which may be required with envelope stress and damage (De Wulf *et al.*., 2002; Dorel *et al.*, 2006). The Cpx system also activates the heat-shock transcription factor $\sigma^{32}$ (RpoH) (De Wulf *et al.*, 2002; Batchelor *et al.*, 2005). The BaeSR pathway is not yet well understood, but is known to co-ordinate expression of *spy* with the Cpx system, where Spy senses envelope stresses produced during spheroplast formation (Raffa and Raivio, 2002). Both the CpxAR and BaeSR pathways are two-component systems; CpxA and BaeS are histidine kinase sensors located within the inner membrane of the cell, while CpxR and BaeR are response regulators localized to the cytoplasm and function as transcriptional activators. In contrast, $\sigma^E$ (RpoE, $\sigma^{22}$), an alternative sigma factor and member of the extracytoplasmic function (ECF) subfamily of sigma factors, is normally sequestered to the cytoplasmic side of the inner membrane by the anti-sigma factor RseA and released in the presence of an inducing stimulus (see below).

### 1.8.1 Induction of the $\sigma^E$ stress response in *E. coli*

In *E. coli*, $\sigma^E$ carries out many roles in the maintenance of envelope integrity, during both normal growth or when damage occurs, and is essential for viability (De Las Penas *et al.*, 1997b; Ades, 2004). Originally identified as both a heat- and ethanol-inducible sigma factor, which was thought to complement the $\sigma^{32}$ cytoplasmic stress response by monitoring the cell envelope, $\sigma^E$ is responsive to the presence of misfolded
outer membrane proteins; misfolded OmpC, OmpF, and OmpX proteins have been shown to increase $\sigma^E$ expression the greatest (Mecsas et al., 1993). Consistent with this model of $\sigma^E$ as an envelope stress response sigma factor, many genes involved in maintaining envelope integrity and LPS synthesis are part of the $\sigma^E$ regulon (Dartigalongue et al., 2001; Tam and Missiakas, 2005). Genes regulated by $\sigma^E$ that are involved in envelope integrity include periplasmic proteases to degrade misfolded proteins (e.g. $degP$) and proteins involved in folding envelope proteins (e.g. $fkpA$) (Dartigalongue et al., 2001). Interestingly, there is partial overlap between the Cpx and $\sigma^E$ pathways, for instance, expression of the protease DegP is regulated by both systems (Dartigalongue et al., 2001; De Wulf et al., 2002). Additionally, like the Cpx pathway, $\sigma^E$ may increase expression of $\sigma^{32}$. LPS biosynthetic genes regulated by $\sigma^E$ include $lptA$ and $lptB$, whose products have been proposed to be involved in the transport of LPS to the outer membrane, and $lptD$, which is involved in lipid A synthesis (Dartigalongue et al., 2001; Sperandeo et al., 2007).

Regulation of $\sigma^E$ occurs primarily through its interaction with RseA, an anti-sigma factor located in the inner membrane (Figure 5) (Campbell et al., 2003). In the absence of stress, $\sigma^E$ is bound to RseA, thereby sequestering $\sigma^E$ to the inner membrane. In the presence of stress, however, $\sigma^E$ is released through the controlled proteolysis of RseA by the proteases DegS and RseP (formerly known as YaeL) (Alba et al., 2002). Proteolysis occurs in a successive manner where DegS first cleaves the periplasmic domain of RseA, followed by the successive degradation of RseA by RseP; RseP activation, and subsequent degradation of RseA, requires the initial periplasmic cleavage of RseA by DegS. Activation of DegS occurs as a result of binding to misfolded outer
Figure 5. Induction of the $\sigma^E$ pathway in *E. coli* in response to envelope stress.

Misfolded outer membrane proteins (OMPs) bind and activate DegS, which cleaves the anti-sigma factor RseA. Following this cleavage, the protease RseP degrades RseA. Degradation of RseA releases $\sigma^E$ from sequestration. Alternatively other stresses appear to influence RseB, which regulates $\sigma^E$ release by stabilizing the RseA-$\sigma^E$ interaction.
membrane proteins, where it is a exposed motif in the C-terminus of these misfolded proteins that interacts with and activates DegS (Walsh et al., 2003).

Additional DegS-independent pathways also exist to modulate $\sigma^E$ activity and involve the periplasmic protein RseB (Collinet et al., 2000). Normally, RseB interacts with RseA, the anti-sigma factor; this interaction stabilizes RseA and, therefore, the $\sigma^E$-RseA complex (De Las Penas et al., 1997a). With the accumulation of an unknown signal in the periplasm, unrelated to the previous C-terminal protein motif recognized by DegS, RseB is titrated away from RseA, thus allowing for an increased rate of RseA degradation by RseP (Ruiz and Silhavy, 2005). In the end, this pathway increases the $\sigma^E$-mediated response, however, an inducing signal through DegS is believed required for full activation.

1.8.2 Regulation of AlgU in P. aeruginosa

In P. aeruginosa, AlgU (AlgT or $\sigma^{22}$) is homologous to $\sigma^E$ found in E. coli. The role of AlgU as an ECF sigma factor belonging to the same family as $\sigma^E$ can be best shown by the ability of $\sigma^E$ to complement algU deletions in P. aeruginosa (Yu et al., 1995). A further similarity with $\sigma^E$ is the ability of both genes to be induced following heat-shock (Schurr et al., 1995). One protein MucE, believed to be either a periplasmic or outer membrane protein based on a cleavable signal sequence, was found to increase algU expression (Qiu et al., 2007). The C-terminus of this protein is similar to other outer membrane proteins, such as OprP and OprO, and it is believed misfolded proteins that are exposing this region may act as a signal to increase algU expression. Although
most work regarding the AlgU regulon pertains to its role in alginate production, many genes controlled by AlgU are not directly associated with the production of alginate and encompass different aspects of the cell envelope, specifically the outer membrane, in a similar manner to $\sigma^E$. For example, genes that encode lipoproteins, porins, and a protein predicted to be involved in protein folding, specifically $lptA$, $oprF$, and $lptB$, respectively, are positively regulated by AlgU (Firoved et al., 2002). In addition, like both the Cpx and $\sigma^E$ pathways in *E. coli*, AlgU is able to mediate transcription of the heat-shock sigma factor $\sigma^{32}$ (Schurr and Deretic, 1997). Since $\sigma^{32}$ mediates most cytoplasmic stress responses, these systems (Cpx, $\sigma^E$, AlgU) likely increased $rpoH$ transcription as a way to prepare the cell for a generalized stress; the cell envelope would be the first area to sense an environmental stress and could prepare the bacterium for further cytoplasmic disturbances through the activation of $rpoH$ transcription.

In *P. aeruginosa*, AlgU in the cytoplasm is normally bound by the RseA homologue MucA, a protein localized to the inner membrane; this sequestration inhibits AlgU activity (Schurr et al., 1996; Mathee et al., 1997). Under inducing stress conditions, believed to be the presence of a misfolded outer membrane protein, another protein AlgW is activated, where AlgW is a protease homologous to DegS that cleaves MucA. This model is based on activation of AlgW following the over-expression of MucE (Qiu et al., 2007). MucE, as stated earlier, is a proposed periplasmic or outer membrane protein that possesses a specific C-terminal motif that is capable of interacting with and activating AlgW. The cleavage of MucA by AlgU is followed by degradation of MucA by MucP, a homologue of RseP. As with the regulation of $\sigma^E$, an accessory
protein, MucB is normally bound to MucA stabilizing MucA and the AlgU-MucA interaction; generally, MucB plays a similar role to that of RseB in *E. coli*.

### 1.9 Experimental focus

Previous studies have shown that the MexCD-OprJ efflux system is inducible by various agents (e.g. benzalkonium chloride and chlorhexidine), which are known to target and disrupt membranes. To assess the significance of membrane damage on the regulation of MexCD-OprJ induction, additional agents known to target membranes were tested for the ability to increase *mexCD-oprJ* expression. As well, given the known role of AlgU in the bacterial envelope stress response, and expecting that membrane damage would elicit this envelope stress response, the influence of AlgU on *mexCD-oprJ* expression by membrane-damaging agents was examined. We sought, therefore, to provide evidence for the involvement of MexCD-OprJ as part of the envelope stress response, a possible first step in determining the natural substrates for this efflux system. Based on our data we propose that two signals are involved in the induction of MexCD-OprJ, where these signals originate due to membrane damage; one of the signals is mediated by AlgU while the second signal is responsible for relieving NfxB repression (Figure 6).
The presence of membrane damage causes the release (or accumulation) of an outer membrane component (component X) that binds to, and activates, the protease AlgW. Activated AlgW cleaves the anti-sigma factor MucA allowing for degradation of MucA by MucP. MucA degradation releases the sigma factor AlgU; free AlgU is able to mediate transcription of a variety of genes including a protein believed to be involved in mediating *mexCD-oprJ* expression (protein Y). A signal is also required to relieve NfxB repression.
CHAPTER 2: Materials and methods

2.1 Bacterial strains, plasmids, and growth conditions

Bacterial strains used in this study are presented in Table 2, while the plasmids used in this work are in Table 3. All overnight liquid cultures of *E. coli* or *P. aeruginosa* were grown in Luria-Bertani (LB) Broth (Difco) that contained 2 g/L of NaCl. These cultures were incubated at 37°C, unless otherwise indicated, with shaking at approximately 90 rotations per minute (rpm). All solid media contained the same components as liquid media with the addition of 1.5% (w/v) Bacto Agar (L-agar); plates were also incubated for growth at 37°C. Antibiotics, at the concentrations indicated in Table 4, were used when required to maintain plasmids.

2.2 DNA isolation

Plasmid DNA was isolated from *E. coli* cells using the method described by Sambrook and colleagues (Sambrook *et al.*, 1989). Briefly, 3.0 mL of overnight culture was pelleted in a microfuge tube using a desktop centrifuge (Heraeus Instruments, Biofuge Pico) at 13000 rpm for 1 minute. The pellet was resuspended in 250 μL of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 μg/mL RNase A to which 250 μL of 200 mM NaOH, 1% (w/v) SDS was added. Finally, 350 μL of 3 M potassium acetate (pH 5.5) was added and the tube centrifuged (10 min, 13000 rpm). The supernatant was transferred to a clean microfuge tube and 500 μL of isopropanol was used to precipitate the plasmid DNA during the following centrifugation (10 min, 13000 rpm). With the supernatant discarded, 500 μL of 70% (v/v) ethanol was added to the pellet and again the
Table 2. Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td><em>thi, pro, hsdR (rm^+), recA,</em> carries plasmid RP4 derivative (AMP; TET::Mu; KAM::Tn7) integrated into the chromosome; TET^+, KAM^+; tra^+ used in the mobilization of plasmids carrying a <em>mob</em> region</td>
<td>(Simon et al., 1983)</td>
</tr>
<tr>
<td>DH5α</td>
<td>Φ80dlacZ ΔM15 Δ(lacZYA-argF) endA1 recA1</td>
<td>(Ausubel et al., 1992)</td>
</tr>
</tbody>
</table>

*Abbreviations: AMP, ampicillin; CAM, chloramphenicol; KAM, kanamycin; TET, tetracycline.*

| **P. aeruginosa strains** | | |
| K767   | PAO1 prototroph | (Masuda and Ohya, 1992) |
| K1521  | K767 ΔmexCD-oprJ | (Srikumar et al., 1997) |
| K1536  | K767 ΔnfxB | (Hirakata et al., 2002) |
| K1542  | K767 ΔmexB-ΔmexXY | (Dean et al., 2003) |
| K2443  | K767 ΔalgU | S. Fraud |
| K2841  | K1536 ΔalgU | This study |
| K2873  | K1542 ΔmexCD-oprJ | This study |
Table 2. Bacterial strains used in this study. (continued)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>K2861</td>
<td>K1542 ΔalgU</td>
<td>Z. Chen</td>
</tr>
<tr>
<td>K2858</td>
<td>K2873 ΔalgU</td>
<td>Z. Chen</td>
</tr>
<tr>
<td>K2874</td>
<td>K1542 carrying a Pₚₙᵢₓ₆₅₋ₖₑₓ₃⁻lacZ transcriptional fusion at the attB site</td>
<td>This study</td>
</tr>
<tr>
<td>K2875</td>
<td>K1542 carrying a promoter-less lacZ gene at the attB site</td>
<td>This study</td>
</tr>
<tr>
<td>K2876</td>
<td>K1542 ΔalgU carrying a Pₚₙᵢₓ₆₅₋ₖₑₓ₃⁻lacZ transcriptional fusion at the attB site</td>
<td>This study</td>
</tr>
<tr>
<td>K2877</td>
<td>K1542 ΔalgU carrying a promoter-less lacZ gene at the attB site</td>
<td>This study</td>
</tr>
<tr>
<td>K2878</td>
<td>K767 ΔPA4596</td>
<td>This study</td>
</tr>
<tr>
<td>K2879</td>
<td>K1536 ΔPA4596</td>
<td>This study</td>
</tr>
<tr>
<td>PA01-JL</td>
<td>Wild-type strain</td>
<td>J. Lam</td>
</tr>
<tr>
<td>PA01-JL::rmlC</td>
<td>PA01 ΔrmlC</td>
<td>J. Lam</td>
</tr>
<tr>
<td>PA01-JL::waaL</td>
<td>PA01 ΔwaaL</td>
<td>J. Lam</td>
</tr>
<tr>
<td>PA01-JL::wapR</td>
<td>PA01 ΔwapR</td>
<td>J. Lam</td>
</tr>
</tbody>
</table>

*a Abbreviations: AMP, ampicillin; CAM, chloramphenicol; KAM, kanamycin; TET, tetracycline.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKmobsacB</td>
<td>Gene replacement vector derived from plasmid pK18; Mob&lt;sup&gt;+&lt;/sup&gt; sacB KAM&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(Schäfer et al., 1994)</td>
</tr>
<tr>
<td>pEX18Tc</td>
<td>Gene replacement vector, oriT&lt;sup&gt;+&lt;/sup&gt; sacB, TET&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(Hoang et al., 1998)</td>
</tr>
<tr>
<td>miniCTX-lacZ</td>
<td>Integration vector with promoterless lacZ, oriT; TET&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(Becher and Schweizer, 2000)</td>
</tr>
<tr>
<td>pFLP2</td>
<td>Source of Flp recombinase; AMP&lt;sup&gt;r&lt;/sup&gt;/CAR&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(Hoang et al., 1998)</td>
</tr>
<tr>
<td>pMMB206</td>
<td>Broad host range low-copy number cloning vector; CAM&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(Morales et al., 1991)</td>
</tr>
<tr>
<td>pRSP05</td>
<td>pKmobsacB::ΔmexCD-oprJ</td>
<td>(Srikumar et al., 1997)</td>
</tr>
<tr>
<td>pSF01</td>
<td>pKmobsacB::ΔalgU</td>
<td>S. Fraud</td>
</tr>
<tr>
<td>pAJC01</td>
<td>pEX18TC::ΔPA4596</td>
<td>This study</td>
</tr>
<tr>
<td>pSF02</td>
<td>pMMB206::algU</td>
<td>S. Fraud</td>
</tr>
<tr>
<td>pAJC02</td>
<td>pMMB206::PA4596</td>
<td>This study</td>
</tr>
<tr>
<td>pAJC03</td>
<td>miniCTX-P&lt;sub&gt;nfxB-mexC-lacZ&lt;/sub&gt;</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup>Abbreviations: KAM, kanamycin; CAM, chloramphenicol; CAR, carbenicillin; AMP, ampicillin; TET, tetracycline.
Table 4. Antibiotic concentrations used to maintain plasmids.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration (µg/mL)</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>n/a</td>
<td>100 – 150</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>10</td>
<td>50 – 100</td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50</td>
<td>750 – 1500</td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10</td>
<td>50 – 100</td>
<td></td>
</tr>
</tbody>
</table>
tube was centrifuged (5 min, 13000 rpm). Once again the supernatant was discarded and the DNA pellet was resuspended in 30 μL of water. For isolation of plasmid DNA from *E. coli* on a larger scale, the Qiagen plasmid midi kit (Qiagen, Mississauga, ON) was used. Isolation of plasmid DNA from *P. aeruginosa* strains was done with a minor modification to the above procedure. Prior to the addition of isopropanol, the supernatant underwent two phenol-chloroform (250 μL of Tris-EDTA [TE; 10 mM Tris-HCl (pH 8.0), 1 mM EDTA]-buffered phenol and 250 μL of chloroform) and one chloroform (500 μL) extraction.

Chromosomal DNA was isolated from 1.5 mL of overnight culture that had been pelleted (1 min, 13000 rpm) and resuspended in 500 μL of 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 50 μg/mL of RNase A. Following the addition of 60 μL of 10% (w/v) SDS, the mixture was heated for 10 minutes at 55°C. Two phenol-chloroform extractions were performed with 250 μL TE-buffered phenol and 250 μL chloroform, followed by one chloroform (500 μL) extraction. The upper phase was removed and added to 1 mL of isopropanol; the DNA precipitate that formed was removed using a pipette tip, placed into 70% (v/v) ethanol and subsequently put into a clean microfuge tube to dry. The dry sample was resuspended in 50 μL of water.

To visualize DNA, a 0.8 – 1.0% (w/v) agarose gel was prepared. Agarose gels were prepared and run in Tris-acetate-EDTA [TAE; 40 mM Tris-HCl (pH 8.5), 20 mM acetic acid, 1 mM EDTA] buffer. Ethidium bromide was added to the gels prior to solidification at a concentration of 0.5 μg/mL to allow for visualization of any DNA fragments under UV illumination; fragment size was assessed by comparison to either the
GeneRuler DNA ladder, a 1 kb DNA ladder, or a 100 bp DNA ladder (all from Fermentas Canada Inc., Burlington, ON).

2.3 Restriction digests and ligation of DNA fragments

All restriction digests were carried out according to the directions provided by the manufacturer and in the buffers accompanying the restriction enzymes (New England Biolabs Ltd., Pickering, ON). Normally, digests were carried out in a total volume of 30 μL and contained 5 – 10 μL of DNA, 1 μL restriction enzyme (equivalent to 20 units of enzyme), and 3 μL of the appropriate 10X buffer, with dH₂O added to 30 μL; 0.3 μL of 10 μg/mL bovine serum albumin (BSA) was added when necessary. Digests were performed sequentially when two enzymes were to be used. Digested DNA was purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI). To ligate digested DNA fragments into a digested DNA vector, 3 – 5 μL of insert and 1 μL of vector were mixed with 1 μL of 10X ligase buffer, 1 μL of T4 DNA ligase (NEB Ltd.), and 3 – 5 μL of dH₂O; the total final volume was 10 μL. This mixture was incubated overnight at 16°C as described by Sambrook et al. (Sambrook et al., 1989).

The pMMB206 and pEX18Tc vectors are designed with multiple cloning sites (MCS) at the beginning of a gene that encodes the lacZ α-subunit, therefore, allowing blue-white screening. Without an insert, these vectors express the α-subunit required for complementation in E. coli DH5α; this results in the expression of a fully functional LacZ protein which is able to cleave the substrate 5-bromo-4-chloro-3-indolyl-β-D-
galactoside (X-gal) and produce a blue colour. Vectors with inserts would appear white in colour, since the inserted gene disrupts the \(\text{lacZ}\) \(\alpha\)-subunit.

### 2.4 PCR amplification and nucleotide sequencing

Polymerase chain reaction (PCR) was carried out using 2 \(\mu\)L of a 1/10 dilution of chromosomal DNA (isolated as previously described), 1 \(\mu\)L dNTPs, which contained 10 mM each of dGTP, dCTP, dTTP, dATP (NEB Ltd.), 1 \(\mu\)L of the forward and 1 \(\mu\)L of the reverse primers (each primer was diluted to 30 \(\rho\)M, with primer sequences described below), 2.5 \(\mu\)L of dimethyl sulfoxide (DMSO), 5 \(\mu\)L of Thermopol buffer (NEB Ltd.) and 1 \(\mu\)L of either DNA Taq polymerase (NED Ltd.) or DNA Vent polymerase (NEB Ltd.). Distilled water was added to this reaction mixture to a final total volume of 50 \(\mu\)L and the mixtures were placed in a T-gradient thermocycler (Biometra, Goettingen, Germany). Colony PCR was performed by resuspending a single colony in 30 \(\mu\)L dH\(_2\)O that was subsequently boiled at 95°C for 5 minutes and then placed on ice for an additional 5 minutes. After a brief centrifugation (3 – 5 seconds), 10 \(\mu\)L of lysate was used per 50 \(\mu\)L reaction; all other components are identical to the PCR procedure described above. The typical PCR program used was as follows: an initial 94°C denaturation step for 3 minutes, followed by 23 – 35 cycles of a 1 minute denaturation step at 94°C, a 1 minute annealing step at 55°C – 72°C (actual annealing temperatures provided for specific reactions below), and a 1 minute per kb extension step at 72°C. This was followed by a final 10 minute extension step at 72°C. All DNA sequencing was
performed by ACGT Corporation (Toronto, ON) and aligned with previously published sequences when necessary (www.pseudomonas.com) (Stover et al., 2000).

2.5 DNA transformation

2.5.1 Calcium chloride competent *E. coli*

*E. coli* calcium chloride competent cells were made by growing 100 mL of cells in LB to an O.D._600_ of approximately 0.5; these cells were first pelleted by centrifugation (10 min, 8000 xg). Two washes (40 mL and 10 mL) with ice-cold 100 mM CaCl₂ were performed with cells centrifuged (10 min, 8000 xg) between washes. Finally, 4 mL of 100 mM CaCl₂ containing 15% (v/v) glycerol was used to resuspend the cells. Aliquots of 200 μL were frozen at -80°C until required. To transform CaCl₂ competent cells, 25 – 50 ng of DNA was added to one 200 μL aliquot and left on ice for 20 minutes. The cells were then heat shocked for 90 seconds at 42°C and then left on ice for 2 minutes. Eight-hundred microlitres of LB was then added and the cells were allowed to recover for 1 hour at 37°C. The cells were plated on L-agar plates containing the appropriate antibiotic (Table 4).

2.5.2 Electrocompetent *P. aeruginosa*

*P. aeruginosa* were made electrocompetent by the method described by Choi and colleagues (Choi et al., 2006). Six millilitres of an overnight LB culture was equally separated into 4 microfuge tubes and centrifuged (1 min, 13000 rpm). Cells in each tube
were resuspended in 250 μL of 300 mM sucrose and pelleted (1 min, 13000 rpm); this procedure was repeated an additional two times. Cells from each of the 4 microfuge tubes were resuspended in a total volume of 100 μL of 300 mM sucrose and 50 – 100 ng of plasmid DNA was added to the cells. The mixture was added to a 2 mm electroporation cuvette (Bio-Rad Laboratories, Hercules, CA) and pulsed at 2500 V, 25 μF, and 200 Ohm in a BTX ECM399 electroporation system (Inoviv Biomedical Corporation, San Diego, CA). Nine-hundred millilitres of LB was added to the cells, which were allowed to recover for 2 hours at 37°C. Once again the cells were plated on L-agar with appropriate antibiotics (Table 4).

2.6 Construction of gene deletions

2.6.1 Construction of a *P. aeruginosa* K1536 Δ*algU* mutant

Deletion of the *algU* gene was carried out by standard allelic exchange techniques that are described by Schäfer *et al.* (Schäfer *et al.*, 1994). The previously constructed plasmid pSF01 contained the Δ*algU* construct; this construct consists of an approximately 1 kb fragment of DNA upstream and downstream of *algU* in the gene replacement vector, pK18mobsacB. *E. coli* strain S17-1 was transformed with the plasmid pSF01 (pK18mobsacB::ΔalgU), which allowed pSF01 to be mobilized into *P. aeruginosa* strain K1536 via conjugal transfer. To accomplish this, 600 μL of *E. coli* S17-1 (pSF01) grown overnight at 37°C in LB containing 50 μg/mL kanamycin and 300 μL of *P. aeruginosa* K1536 grown overnight at 42°C in LB were added to a microfuge tube and centrifuged (3 min, 8000 rpm). The pellet was resuspened in 100 μL of LB and
spotted in the centre of an L-agar plate for incubation at 37°C for approximately 4 hours. Bacterial growth was suspended in 1 mL of LB and plated on L-agar plates containing 1500 μg/mL kanamycin (to select for pSF01 integration into the chromosome) and 0.5 μg/mL imipenem (to counterselect *E.coli* S17-1). To select for a second recombination event, which excises the plasmid from the chromosome (leaving colonies either a wild-type or Δ*algU* genotype), colonies were streaked onto plates containing 10% (w/v) sucrose. Single colonies from the sucrose plates were patched onto both kanamycin (1500 μg/mL) and L-agar plates. Colonies that were now sensitive to kanamycin, indicating that they have lost the plasmid backbone, were next screened by PCR amplification for a shortened *algU* region (e.g. Δ*algU*) by colony PCR using the primers Scr algU For (5’-GAGCCCGATGCAATCCATTTTC-3’) and Scr algU Rev (5’-GCAGGGTAGGCTCGCGGTGCAT-3’); an annealing temperature of 63.5°C was utilized.

### 2.6.2 Construction of a *P. aeruginosa* K1542 Δ*mexCD-oprJ* mutant

A Δ*mexCD-oprJ* strain of *P. aeruginosa* K1542 was constructed by a similar method as above. The previously constructed plasmid pRSP05 (pKmobsacB::Δ*mexCD-oprJ*) was transformed into the *E. coli* S17-1, where pRSP05 contained a 1 kb fragment of DNA upstream and downstream of the *mexCD-oprJ* operon. The plasmid pRSP05 was transferred to *P. aeruginosa* K1542 by conjugation with *E.coli* S17-1 (pRSP05). As the plasmid pK18mobsacB backbone was once again used, the product of this mating was plated on kanamycin (1000 μg/mL) and imipenem (0.5 μg/mL). Colonies were streaked
onto 10% (w/v) sucrose plates and sucrose resistant colonies were selected and patched onto both kanamycin (1000 μg/mL) and L-agar plates. Sucrose resistant and kanamycin sensitive colonies were screened for the ΔmexCD-oprJ genotype using colony PCR with the Reverse mexC (5’-ATGGCTGATTTGCGTGCAAT-3’) and oprJ Forward (5’-TCAACTCCTGCGCCTGTGAT-3’) primers; the annealing temperature for these primers was 56°C.

2.6.3 Construction of P. aeruginosa ΔPA4596 mutants

Deletions of PA4596 from strains of P. aeruginosa were carried out using standard allelic exchange protocols. A 1 kb DNA fragment upstream of the PA4596 gene was PCR amplified using the primers PA4596 del (UP FOR) (5’-GATCGAATTCAGGTGCTCGACCTTGGCGATC-3’; EcoRI site underlined) and PA4596 del (UP REV) (5’-GATCGGATCCGCCAGTTCCGCCAGTTCTC-3’; BamHI site underlined) and an annealing temperature of 72°C, while a 1 kb DNA fragment downstream of PA4596 was amplified using the primers PA4596 del (DOWN FOR) (5’-GATCGGATCCTTGAGCGGGCGGGCGAGTTG-3’; BamHI site underlined) and PA4596 del (DOWN REV) (5’-GATCAAGCTTCTGGTCGCGAGGTAGCC-3’; HindIII site underlined), which also had an annealing temperature of 72°C. The upstream and downstream fragments were digested with EcoRI/BamHI and BamHI/HindIII, respectively, and cloned individually into the vector pEX18Tc, which had been digested with the same pairs of restriction enzymes. After sequencing to ensure that no mutations were present, the fragments were jointly cloned into pEX18Tc in an EcoRI-BamHI-HindIII orientation resulting in plasmid pAJC01. Calcium competent E. coli S17-1 were
transformed with pAJC01 and subsequently used to transfer the plasmid into *P. aeruginosa* strains K767 and K1536 via conjugation as above. The products of these conjugal matings were plated on tetracycline (25 μg/mL) and imipenem (0.5 μg/mL). Colonies were streaked onto 10% (w/v) sucrose and subsequently patched onto tetracycline (25 μg/mL) or L-agar plates. Finally, sucrose sensitive and tetracycline resistant colonies were screened by colony PCR using the primers PA4596 del (UP FOR) and PA4596 del (DOWN REV) and an annealing temperature of 72°C. Colonies that had a shorter version of the PA4596 region were considered to be ΔPA4596 mutants.

2.7 Complementation of gene deletions

2.7.1 Complementation of ΔalgU mutants

To complement the *algU* deletions, a previously constructed plasmid expressing *algU* in the vector pMMB206 (pSF02) was isolated from *E. coli* DH5α (pSF02) by the method described earlier. The plasmid pSF02 was transformed into *P. aeruginosa* strains K2861 (K1542 ΔalgU) and K2841 (K1536 ΔalgU), which were made electrocompetent by the method described above, and plated onto L-agar plates containing 50 μg/mL and 125 μg/mL chloramphenicol, respectively. Colonies were screened for restored chlorhexidine resistance as an indicator of complementation.
2.7.2 Complementation of ΔPA4596 mutants

The PA4596 gene was PCR amplified using the primers PA4596 For (5’-GAATTCGAAATTCCCAACCCCGTCGTCCTCAGG-3’; EcoRI site underlined) and PA4596 Rev (5’-AAGCTTAAGCTTCGTGGGAAACGGTGCTA-3’; HindIII site underlined), using an annealing temperature of 62°C. The resulting product was digested using EcoRI and HindIII and ligated into the pMMB206 plasmid, also digested with EcoRI and HindIII, creating plasmid pAJC02. This plasmid was transformed into calcium competent E. coli DH5α, as described above. The transformed cells were plated onto L-agar containing 10 µg/mL of chloramphenicol; one plasmid containing the PA4596 gene insert was sent for sequencing to ensure that no mutations in the PA4596 gene were present. The sequenced plasmid was transformed into electrocompetent P. aeruginosa K2878 (K767 ΔPA4596) and K2879 (K1536 ΔPA4596) and selected on L-agar plates containing 125 µg/mL of chloramphenicol. Colonies were screened for restored resistance/susceptibility to chlorhexidine.

2.8 Antimicrobial susceptibility testing

Susceptibility of P. aeruginosa to various antibiotic and antimicrobial compounds was assessed in standard 96-well microtitre plates using a two-fold serial dilution method (Jo et al., 2003). Overnight bacterial cultures were diluted 1/2000 in LB and 50 µL was added to an equal volume of LB containing serial two-fold dilutions of antibiotic or membrane-damaging agent. Following incubation for 16 – 18 hours at 37°C, plates were examined for the presence or absence of microbial growth. The lowest concentration of
agent that prevented visible microbial growth was recorded as that agent’s minimal inhibitory concentration (MIC). To determine antibiotic resistance in the presence of membrane-damaging agents, overnight bacterial cultures, as well as the media used to make the 1/2000 dilution, contained a sub-inhibitory concentration of a membrane-damaging agent as per Table 5.

2.9 Construction of lacZ fusion reporter strains

To have a quantitative measure of mexCD-oprJ expression in P. aeruginosa, a lacZ-transcriptional fusion was created based on the methods described by Hoang and colleagues (Hoang et al., 1998). A 700 bp region containing the mexCD-oprJ promoter region and the upstream repressor gene nfxB (P_{nfxB-mexC}) was PCR amplified from the chromosome of P. aeruginosa K767 using the primers PmexC For (5’-GGATCCGGATCCGACACACCGGTTGATTGTTTACAGGG-3’; BamHI site underlined) and nfxB Rev (5’-GAATTCGAATTCATCCGGTCCTCCGTGCATGCCTGCG- 3’; EcoRI site underlined). The amplified region was digested with BamHI and EcoRI, and cloned upstream of the promoter-less lacZ gene in the plasmid mini-CTX-lacZ; this resulted in the creation of the plasmid pAJC03. After sequencing to ensure no mutations were present, pAJC03 was mobilized from E. coli S17-1 into P. aeruginosa K1542 and K2861 (K1542 ΔalgU) by conjugal mating as above. Colonies were selected on 25 μg/mL of tetracycline and 0.5 μg/mL of imipenem (the latter to counterselect for E. coli S17-1). The plasmid backbone was cured from the chromosome using the flp recombinase, pFLP2, to leave only the mexC-lacZ fusion in the chromosome. P. aeruginosa K1542 and K2861 with pFLP2 were initially selected on 100 μg/mL of carbenicillin, and
Table 5. Sub-inhibitory concentrations of membrane-damaging agents used to determine antibiotic MICs in *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Fractional MIC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorhexidine</td>
<td>1/16</td>
<td>0.04 – 0.625 μg/mL</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>1/16</td>
<td>0.016 – 0.031 μg/mL</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1/8</td>
<td>0.5%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SDS</td>
<td>1/128</td>
<td>0.3875 μg/mL</td>
</tr>
<tr>
<td>Melittin</td>
<td>1/16</td>
<td>1.875 – 3.75 μg/mL</td>
</tr>
</tbody>
</table>

<sup>a</sup>MIC, minimal inhibitory concentration.

<sup>b</sup>v/v.
subsequently streaked on 10% (w/v) sucrose to select for the loss of plasmid pFLP2 (following excision of the miniCTX backbone from the chromosome). Finally, colonies were patched on L-agar plates containing tetracycline (25 μg/mL) or carbenicillin (100 μg/mL) to confirm the loss of the miniCTX backbone (tetracycline sensitive) and the pFLP2 plasmid (carbenicillin sensitive). The presence of the \( P_{\text{nfxB-mexC}}-\text{lacZ} \) fusion was confirmed in the newly created strains K2874 and K2876, using a \( \beta \)-galactosidase assay with growth in the presence of chlorhexidine, a known inducer of \( \text{mexCD-oprJ} \) expression. A promoterless \( \text{lacZ} \) gene was also integrated into strains K1542 and K2861, thereby creating the strains K2875 and K2877, respectively, by identical procedures for use as a control.

### 2.10 \( \beta \)-galactosidase assay

Overnight cultures of LB-grown \( P. \text{aeruginosa} \) containing the \( P_{\text{nfxB-mexC}}-\text{lacZ} \) fusion (K2874 and K2876) or carrying the promoterless \( \text{lacZ} \) gene (K2875 and K2877) were sub-cultured 1/50 and grown at 37°C. After 2 hours, a membrane-damaging agent was added to the growing culture at the concentration and partial MIC indicated in Table 6. After an additional 2 hours, samples were placed on ice for 15 minutes. One-millilitre from each sample was removed, washed once in LB, and resuspended in 1 mL of LB. One-hundred microlitres of cell culture was added to 900 μL of Z buffer (60 mM \( \text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}, 40 \text{mM Na}_2\text{PO}_4 \cdot \text{H}_2\text{O}, 10 \text{mM KCl}, 1 \text{mM MgSO}_4 \cdot 7\text{H}_2\text{O}, \) and 50 mM \( \beta \)-mercaptoethanol), 50 μL of 0.02% (w/v) SDS, and 50 μL of chloroform. After vortexing for 10 seconds, tubes were incubated for 5 minutes at room temperature. Next,
Table 6. Sub-inhibitory concentrations of membrane-damaging agents used to determine *mexCD-oprJ* promoter activity in *P. aeruginosa* K1542 and K2861.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Fractional MIC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorhexidine</td>
<td>1/8</td>
<td>0.16 – 1.25 μg/mL</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>1/4</td>
<td>0.125 μg/mL</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1/8</td>
<td>0.5%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SDS</td>
<td>1/16</td>
<td>3.1 μg/mL</td>
</tr>
<tr>
<td>Melittin</td>
<td>1/4</td>
<td>15 μg/mL</td>
</tr>
<tr>
<td>EDTA</td>
<td>1/32</td>
<td>11.6 μg/mL</td>
</tr>
<tr>
<td><em>n</em>-Hexane</td>
<td>1/4</td>
<td>1.25%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>p</em>-Xylene</td>
<td>18</td>
<td>0.0156%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>V8</td>
<td>1/8</td>
<td>8 μg/mL</td>
</tr>
<tr>
<td>V681</td>
<td>1/8</td>
<td>16 μg/mL</td>
</tr>
</tbody>
</table>

<sup>a</sup>MIC, minimal inhibitory concentration.

<sup>b</sup>v/v.
20 μL of the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG; 4 mg/mL in Z buffer) was added to the solution and the reaction allowed to proceed for 3 – 10 minutes or until a yellow colour developed. The reaction was stopped by the addition of 0.5 mL of 1 M Na₂CO₃. The solution was transferred to a disposable cuvette and the O.D₅₅₀ and O.D₄₂₀ was determined; optical density of the original cultures was also determined at 600 nm. β-galactosidase activity was calculated in Miller units using the equation (Miller, 1972):

\[
1000 \times \frac{O.D_{420} - (O.D_{550} \times 1.75)}{v \times t \times O.D_{600}}
\]

(ν is volume of culture added in mL, t is time of incubation with ONPG in minutes)

2.11 Quantification of \textit{mexCD-oprJ} expression using RT-PCR

2.11.1 RNA isolation

During RNA isolation, all water was first treated with diethylpyrocarbonate (DEPC) (Sigma-Aldrich Ltd., Oakville, ON) in order to eliminate RNases that could degrade RNA. DEPC-treated water was made by the addition of 500 μL of DEPC to 500 mL of sterile water. After incubation overnight at 37°C, the water was autoclaved on liquid cycle for 20 minutes to eliminate any residual traces of DEPC. Total bacterial RNA was isolated from 1.5 mL of early-log phase cultures of \textit{P. aeruginosa} grown overnight in LB in the presence or absence of a sub-inhibitory concentration of a membrane-damaging agent according to Table 7. RNA was isolated with the Qiagen
Table 7. Sub-inhibitory concentrations of membrane-damaging agents used in the RNA isolation from *P. aeruginosa*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Agent</th>
<th>Fractional MIC&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1542/K2861</td>
<td>Chlorhexidine</td>
<td>1/8</td>
<td>0.16 – 1.25 μg/mL</td>
</tr>
<tr>
<td></td>
<td>Polymyxin B</td>
<td>1/4</td>
<td>0.125 μg/mL</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>1/8</td>
<td>0.5%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>SDS</td>
<td>1/32</td>
<td>1.56 μg/mL</td>
</tr>
<tr>
<td></td>
<td>Melittin</td>
<td>1/4</td>
<td>15 μg/mL</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>1/64</td>
<td>5.8 μg/mL</td>
</tr>
<tr>
<td></td>
<td><em>n</em>-Hexane</td>
<td>1/4</td>
<td>1.25%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td><em>p</em>-Xylene</td>
<td>1/4</td>
<td>0.125%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Norfloxacin</td>
<td>1/4</td>
<td>0.019 μg/mL</td>
</tr>
<tr>
<td></td>
<td>Fosfomycin</td>
<td>1/4</td>
<td>16 μg/mL</td>
</tr>
<tr>
<td>K767/K2443</td>
<td>Chlorhexidine</td>
<td>1/4</td>
<td>0.625 – 3.1 μg/mL</td>
</tr>
<tr>
<td></td>
<td>Polymyxin B</td>
<td>1/4</td>
<td>0.25 μg/mL</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>1/2</td>
<td>2%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Melittin</td>
<td>1/2</td>
<td>30 μg/mL</td>
</tr>
<tr>
<td></td>
<td><em>n</em>-Hexane</td>
<td>1/4</td>
<td>1.25%&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>K2879</td>
<td>Chlorhexidine</td>
<td>1/4</td>
<td>5 μg/mL</td>
</tr>
</tbody>
</table>

<sup>a</sup>MIC, minimal inhibitory concentration.

<sup>b</sup>v/v.
RNeasy PLUS Mini Kit (Qiagen Inc.) according to the manufacturer’s directions. Samples were assessed for DNA contamination by performing a PCR reaction using the rpsL Forward (5’-GCAACTATCAACCAGCTG- 3’) and rpsL Reverse (5’-GCTGTGCTCTTGCAGGTTGTG- 3’) primers and an annealing temperature of 55°C; the reverse transcription step was omitted when determining if contaminating DNA was present.

2.11.2 RT-PCR

Reverse transcriptase-PCR (RT-PCR) was carried out using the QIAGEN OneStep RT-PCR Kit (Qiagen Inc.) according to manufacturer’s directions. For equilibration of RNA samples, rpsL Forward (5’-GCAACTATCAACCAGCTG- 3’) and rpsL Reverse (5’-GCTGTGCTCTTGCAGGTTGTG-3’) primers were used, while mexD Forward (5’-TCTTCATCAAGCGGCCGAAC-3’) and mexD Reverse (5’-AGGGTAGCGGTCTGGATCGC-3’) primers were used to assess mexCD-oprJ expression. Reaction mixtures were incubated at 50°C for an initial reverse transcription step for 30 minutes, and a 15 minute HotStart step at 95°C, followed by the normal PCR program previously described; 23 and 25 PCR cycles were used for rpsL amplification and 30 to 35 cycles were used in the amplification of mexD. The annealing temperature used for rpsL and mexD amplification was 55°C.
CHAPTER 3: Results

3.1 Influence of membrane-damaging agents on MexCD-OprJ-mediated antibiotic resistance in *P. aeruginosa*

Previous studies have shown that MexCD-OprJ is inducible in the presence of benzalkonium chloride and chlorhexidine, both membrane-active agents (Morita *et al.*, 2003). MexCD-OprJ induction, therefore, in the presence of additional membrane-active agents was assessed in order to demonstrate a link between membrane damage and *mexCD-oprJ* expression. Susceptibility of *P. aeruginosa* strains K1542 and K2873 (K1542 Δ*mexCD-oprJ*) was assessed in the presence of chlorhexidine, polymyxin B, ethanol, SDS, and melittin, all of which cause membrane damage. With the exception of chlorhexidine, there was no difference between the determined MIC values for each of the strains (Table 8); strain K1542 was 4-fold more resistant to chlorhexidine than K2873. Resistance to erythromycin, norfloxacin, chloramphenicol, and carbenicillin was next determined in the presence and absence of a sub-inhibitory concentration of each membrane-damaging agent. With the exception of carbenicillin, there was an increase in the antibiotic resistance of *P. aeruginosa* K1542 in the presence of each membrane-damaging agent as compared to the absence of an agent (Table 9; K1542). Resistance to erythromycin increased the greatest, while the increase in chloramphenicol resistance was only modest or, in the case of SDS- and melittin-treated cells, absent. The largest increases in antibiotic resistance were seen in the presence of chlorhexidine and polymyxin B, while the antibiotic resistance increases observed with exposure to ethanol, SDS and melittin were more modest. This contrasts with the antibiotic susceptibility
Table 8. Susceptibility of *P. aeruginosa* K1542-derived strains to membrane-damaging agents.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Minimal inhibitory concentration&lt;sup&gt;cd&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHX</td>
<td>PxB</td>
</tr>
<tr>
<td>K1542</td>
<td>CDJ&lt;sup&gt;b&lt;/sup&gt; AlgU&lt;sup&gt;+&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>K2873</td>
<td>CDJ&lt;sup&gt;-&lt;/sup&gt; AlgU&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.5</td>
</tr>
<tr>
<td>K2861</td>
<td>CDJ&lt;sup&gt;b&lt;/sup&gt; AlgU&lt;sup&gt;-&lt;/sup&gt;</td>
<td>1.25</td>
</tr>
<tr>
<td>K2858</td>
<td>CDJ&lt;sup&gt;-&lt;/sup&gt; AlgU&lt;sup&gt;-&lt;/sup&gt;</td>
<td>0.625</td>
</tr>
</tbody>
</table>

<sup>a</sup>CDJ<sup>+</sup>, *mexCD-oprJ* present; CDJ<sup>-</sup>, Δ*mexCD-oprJ*; AlgU<sup>+</sup>, *algU* present; AlgU<sup>-</sup>, Δ*algU*.

<sup>b</sup>MexCD-OprJ inducible.

<sup>c</sup>Concentrations are in μg/mL unless otherwise indicated.

<sup>d</sup>Abbreviations: CHX, chlorhexidine; PxB, polymyxin B; EtOH, ethanol; SDS, sodium dodecyl sulfate; MEL, melittin; EDTA, ethylenediaminetetraacetic acid; HEX, *n*-hexane; XYL, *p*-xylene; NOR, norfloxacin; FOS, fosfomycin.

<sup>e</sup>% (v/v).
Table 9. Membrane-damaging agents induce *mexCD-oprJ* in an AlgU-dependent manner.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Agent</th>
<th>MIC (μg/mL)&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ERY</td>
<td>NOR</td>
</tr>
<tr>
<td>K1542</td>
<td>CDJ&lt;sup&gt;b&lt;/sup&gt;/AlgU&lt;sup&gt;+&lt;/sup&gt;</td>
<td>None</td>
<td>32-64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chlorhexidine (1/16)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>512</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polymyxin B (1/16)</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol (1/8)</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SDS (1/32)</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Melittin (1/16)</td>
<td>128</td>
</tr>
<tr>
<td>K2873</td>
<td>CDJ/AlgU&lt;sup&gt;+&lt;/sup&gt;</td>
<td>None</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chlorhexidine (1/16)</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polymyxin B (1/16)</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol (1/8)</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SDS (1/32)</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Melittin (1/16)</td>
<td>64</td>
</tr>
<tr>
<td>K2861</td>
<td>CDJ&lt;sup&gt;b&lt;/sup&gt;/AlgU&lt;sup&gt;−&lt;/sup&gt;</td>
<td>None</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chlorhexidine (1/16)</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polymyxin B (1/16)</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol (1/8)</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SDS (1/32)</td>
<td>64</td>
</tr>
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<td></td>
<td></td>
<td>Melittin (1/16)</td>
<td>64</td>
</tr>
<tr>
<td>K2858</td>
<td>CDJ/AlgU&lt;sup&gt;−&lt;/sup&gt;</td>
<td>None</td>
<td>32</td>
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<td></td>
<td></td>
<td>Chlorhexidine (1/16)</td>
<td>32</td>
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<td></td>
<td></td>
<td>Polymyxin B (1/16)</td>
<td>32</td>
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<td></td>
<td>Ethanol (1/8)</td>
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<td></td>
<td>SDS (1/32)</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Melittin (1/16)</td>
<td>64</td>
</tr>
</tbody>
</table>

<sup>a</sup>CDJ<sup>+</sup>, *mexCD-oprJ* present; CDJ<sup>−</sup>, Δ*mexCD-oprJ*; AlgU<sup>+</sup>, *algU* present; AlgU<sup>−</sup>, Δ*algU*.

<sup>b</sup>MexCD-OprJ inducible.

<sup>c</sup>Fraction of membrane-damaging agent MIC used.

<sup>d</sup>Abbreviations: ERY, erythromycin; NOR, norfloxacin; CAM, chloramphenicol; CAR, carbenicillin.
profiles for the ΔmexCD-oprJ strain, K2873. In this instance there were no observable
MIC changes for any antibiotic in the presence of the membrane-damaging agents (Table
9; K2873), suggesting that the increased antibiotic resistance observed was due to the
expression of mexCD-oprJ.

3.2 Influence of membrane-damaging agents on mexCD-oprJ expression in P. aeruginosa

To better assess the influence of membrane-damaging agents on mexCD-oprJ
expression in P. aeruginosa, RT-PCR was utilized to analyze gene expression in K1542
in the presence and absence of a sub-inhibitory concentration of each membrane-
damaging agent. Pump gene expression in the presence of three additional membrane-
active agents (EDTA, n-hexane and p-xylene), as well as the non-inducing antibiotic
substrate norfloxacin, was also examined. In order to determine the concentration of
each of these additional agents to use in RT-PCR experiments, an MIC for each agent
was first determined; these values are presented in Table 8. The RT-PCR experiments
demonstrate that an increased level of mexCD-oprJ expression occurs in the presence of
each membrane-damaging agent (Figure 7A and Figure 8; lanes 1 and 3). The
fluoroquinolone antibiotic norfloxacin, however, did not alter mexCD-oprJ expression
under these conditions (Figure 8; norfloxacin).
Figure 7. Chlorhexidine induces mexCD-oprJ expression in an AlgU-dependent manner in ΔmexB-ΔmexXY strains of P. aeruginosa.

The expression of mexD and rpsL was assessed in P. aeruginosa strains K1542 (ΔmexB-ΔmexXY; A, lanes 1 and 3) and K2861 (ΔmexB-ΔmexXY-ΔalgU; A, lanes 2 and 4) in the absence (A, lanes 1 and 2) or after continuous overnight exposure to 1/4 MIC chlorhexidine (A, lanes 3 and 4) by semiquantitative RT-PCR. Expression of mexD and rpsL in K2861 carrying pMMB206 (B, lanes 1 and 2) or pMMB206::algU (B, lanes 3 and 4) in the absence (B, lanes 1 and 3) or presence of 1/4 MIC chlorhexidine (B, lanes 2 and 4) using semiquantitative RT-PCR is also shown. The rpsL reaction served as an internal control to ensure equal amounts of RNA loading. The PCR portion was carried out at 33 (mexD) and 23 (rpsL) cycles (top) and 35 (mexD) and 25 (rpsL) cycles (bottom).
Figure 8. Influence of membrane-damaging agents and AlgU on mexCD-oprJ expression in ΔmexB-ΔmexXY strains of P. aeruginosa.

The expression of mexD and rpsL was assessed in P. aeruginosa strains K1542 (ΔmexB-ΔmexXY; lanes 1 and 3) and K2861 (ΔmexB-ΔmexXY-ΔalgU; lanes 2 and 4) in the absence (lanes 1 and 2) or after continuous overnight exposure to membrane-damaging agents at the indicated fractional MIC (lanes 3 and 4) by semiquantitative RT-PCR. The rpsL reaction served as an internal control to ensure equal amounts of RNA loading. The PCR portion was carried out at 33 (mexD) and 23 (rpsL) cycles (top) and 35 (mexD) and 25 (rpsL) cycles (bottom).
<table>
<thead>
<tr>
<th>Condition</th>
<th>mexD</th>
<th>rpsL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymyxin B (1/4 MIC)</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>EDTA (1/64 MIC)</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>Ethanol (1/4 MIC)</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>n-Hexane (1/4 MIC)</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td>SDS (1/32 MIC)</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
<tr>
<td>p-Xylene (1/4 MIC)</td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
<tr>
<td>Melittin (1/4 MIC)</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
</tr>
<tr>
<td>Norfloxacin (1/4 MIC)</td>
<td><img src="image15" alt="Image" /></td>
<td><img src="image16" alt="Image" /></td>
</tr>
</tbody>
</table>
3.3 Involvement of AlgU in MexCD-OprJ inducibility by membrane-damaging agents

Given that the alternative sigma factor AlgU is responsive to envelope stress, it was of interest to determine if AlgU is involved in the induction of MexCD-OprJ mediated by membrane damage. Expression of \textit{mexCD-oprJ}, therefore, was analyzed by RT-PCR in \textit{P. aeruginosa} strain K2861 (K1542 \textit{ΔalgU}), in the presence and absence of each membrane-damaging agent. Since this strain lacked the \textit{algU} gene, these experiments analyzed the requirement for AlgU for induction of \textit{mexCD-oprJ} expression in the presence of each membrane-damaging agent. Prior to the isolation of RNA, the susceptibility of K2861 to the membrane-damaging agents was determined (Table 8, K2861). Although absolute MIC values between K1542 and K2861 differed, K2861 was exposed to the same fractional MIC of each agent as used in the K1542 studies. In the absence of any membrane-damaging agent, little difference in \textit{mexCD-oprJ} expression was observed between K1542 and K2861 (Figure 7A and Figure 8; lanes 1 and 2). However, in contrast to K1542 (AlgU$^+$), where substantial increases in \textit{mexCD-oprJ} expression was observed upon exposure to membrane-damaging agents (Figure 7A and Figure 8; lanes 1 and 3), K2861 (AlgU$^-$) showed only a modest increase in \textit{mexCD-oprJ} expression following exposure to these same agents (Figure 7A and Figure 8; lanes 2 and 4). When the \textit{algU} deletion was complemented with the plasmid pSF02 (pMMB206::\textit{algU}), the increased level of \textit{mexCD-oprJ} expression was restored following exposure to chlorhexidine (Figure 7B; lanes 3 and 4). In this instance, chlorhexidine was used as a representative membrane-damaging agent.
Consistent with the AlgU-dependent increased expression of \textit{mexCD-oprJ} following exposure to membrane-damaging agents, increased MexCD-OprJ-mediated antibiotic resistance in the presence of these agents was also AlgU-dependent. First, the susceptibility of the $\Delta algU$ strains, K2861 (K1542 $\Delta algU$) and K2858 (K1542 $\Delta mexCD-oprJ-\Delta algU$), to the membrane-damaging agents chlorhexidine, polymyxin B, ethanol, SDS, and melittin was determined; no significant changes in resistance were observed between these two strains (Table 8; K2861 and K2858). Furthermore, compared to K1542, only resistance to chlorhexidine was altered; an 8 and 16-fold decrease in chlorhexidine MIC was observed in strains K2861 and K2858, respectively. Next, the antibiotic resistance of K2861 and K2858 was determined after overnight exposure to sub-inhibitory concentrations of each membrane-damaging agent. Following this sub-inhibitory exposure, neither strain K2861 nor K2858 showed any difference in antibiotic resistance when compared to the absence of these agents (Table 9; K2861 and K2858).

3.4 The influence of a 2-hour exposure to membrane-damaging agents and the loss of AlgU on \textit{mexCD-oprJ} promoter activity and expression

A \textit{mexC-lacZ} transcriptional fusion was subsequently used to examine the promoter activity of the \textit{mexCD-oprJ} operon in \textit{P. aeruginosa} strain K1542 in response to the presence of these membrane active agents, while this same transcriptional fusion in K2861 (K1542 $\Delta algU$) allowed for examination of the impact of AlgU loss. In both strains, this fusion construct enabled a quantitative measurement of \textit{mexCD-oprJ} promoter activity in response to the various membrane-damaging agents. In these assays, two additional membrane active agents were tested, V8 and V681; again, prior to their
use as potential inducing agents, the MIC of V8 and V681 MIC was assessed for the strains K1542 and K2861 (Table 8). Similar to the MIC and RT-PCR data, promoter activity in K1542 increased following exposure to each agent, including V8 and V681, as compared to the background promoter activity (Figure 9A). Although there was an increased background mexC-lacZ promoter activity in K2861, there was no difference in promoter activity between cells grown in the presence or absence of a membrane-damaging agent (Figure 9B).

The time periods used to examine mexCD-oprJ expression by RT-PCR and mexC-lacZ promoter activity differed; RT-PCR was performed after continuous overnight exposure to the membrane-damaging agents, while promoter activity was assessed following a 2-hour incubation with the agents. It was, therefore, of interest to determine if mexCD-oprJ expression, as determined by RT-PCR, also increased during a 2-hour time period. Consequently, RT-PCR was performed following the identical procedure used to assess mexC-lacZ promoter activity in order to correlate mexCD-oprJ expression and promoter activity. Although to a lesser degree than observed with overnight exposure to chlorhexidine, a 2-hour exposure to chlorhexidine was sufficient to increase mexCD-oprJ expression (Figure 10; lanes 1 and 3); this increased expression was greatly compromised in the ΔalgU strain, K2861, again highlighting the importance of this sigma factor in mexCD-oprJ expression (Figure 10; lanes 2 and 4).
Figure 9. Promoter activity of mexCD-oprJ after a 2-hour exposure to membrane-damaging agents in *P. aeruginosa* strains K1542 (A) and K2861 (B).

Lane 1, no agent; Lane 2, 1/8 MIC chlorhexidine; Lane 3, 1/4 MIC polymyxin B; Lane 4, 1/8 MIC ethanol; Lane 5, 1/16 MIC SDS; Lane 6, 1/4 MIC melittin; Lane 7, 1/32 EDTA; Lane 8, 1/4 MIC n-hexane; Lane 9, 1/8 MIC p-xylene; Lane 10, 1/8 MIC V8; Lane 11, 1/8 MIC V681. Data representative of three independent trials with the mean ± standard deviation shown.
Figure 10. Induction of mexCD-oprJ expression following a 2-hour exposure to chlorhexidine is AlgU-dependent in a ΔmexB-ΔmexXY strain of P. aeruginosa.

The expression of mexD and rpsL was assessed in P. aeruginosa strains K1542 (ΔmexB-ΔmexXY; A, lanes 1 and 3) and K2861 (ΔmexB-ΔmexXY-ΔalgU; A, lanes 2 and 4) in the absence (A, lanes 1 and 2) or after a 2-hour exposure to 1/4 MIC of chlorhexidine (A, lanes 3 and 4) by semiquantitative RT-PCR. The rpsL reaction served as an internal control to ensure equal amounts of RNA loading. The PCR portion was carried out at 33 (mexD) and 23 (rpsL) cycles (top) and 35 (mexD) and 25 (rpsL) cycles (bottom).
3.5 The effect of membrane-damaging agents and AlgU on \textit{mexCD-oprJ} expression in wild-type \textit{P. aeruginosa}

Until now, \textit{mexCD-oprJ} expression has been examined in a strain lacking both the MexAB-OprM and MexXY efflux systems, allowing experiments to focus principally on the MexCD-OprJ response. The ability of membrane-damaging agents to induce \textit{mexCD-oprJ} expression in a wild-type \textit{P. aeruginosa} strain (K767) was examined next; this would demonstrate that the presence of these agents were capable of modulating \textit{mexCD-oprJ} expression in a more natural and, therefore, more clinically relevant background. Antibiotic resistance was no longer used as a method to demonstrate increased \textit{mexCD-oprJ} expression, due to the ability of the other efflux systems (MexAB-OprM and MexXY) to export the same antibiotic compounds and, so, mask MexCD-OprJ-mediated resistance (Masuda \textit{et al.}, 2000b). Thus, the ability of select membrane-damaging agents to induce expression of \textit{mexCD-oprJ} was evaluated in \textit{P. aeruginosa} K767 through RT-PCR. First, the MIC for the membrane-damaging agents was determined (Table 10). The agents were selected so as to cover a range of different compound types: chlorhexidine, polymyxin B, ethanol, melittin and \textit{n}-hexane. All agents tested were found to increase expression of \textit{mexCD-oprJ} in this wild-type background, although to, what appeared to be, a lesser extent than in K1542 (Figure 11A and Figure 12; lanes 1 and 3).

The impact of \textit{algU} deletion on \textit{mexCD-oprJ} expression in \textit{P. aeruginosa} K767 was examined next using strain K2443 (K767 \textit{ΔalgU}). Susceptibility of this strain to the membrane-damaging agents was tested and, except in the case of chlorhexidine where \textit{algU} loss resulted in a decreased resistance to chlorhexidine, found to be identical to
Table 10. Susceptibility of *P. aeruginosa* K767 derived strains to membrane-damaging agents.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype(^a)</th>
<th>Minimal inhibitory concentration(^c,d)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CHX</td>
</tr>
<tr>
<td>K767</td>
<td>CDJ(^b) AlgU(^+)</td>
<td>12.5</td>
</tr>
<tr>
<td>K2443</td>
<td>CDJ(^b) AlgU(^-)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

\(^a\)CDJ\(^+\), *mexCD-oprJ* present; CDJ\(^-\), Δ*mexCD-oprJ*; AlgU\(^+\), *algU* present; AlgU\(^-\), Δ*algU*.

\(^b\)MexCD-OprJ inducible.

\(^c\)Concentrations are in μg/mL unless otherwise indicated

\(^d\)Abbreviations: CHX, chlorhexidine; PxB, polymyxin B; EtOH, ethanol; MEL, melittin; HEX, n-hexane.

\(^e\)% (v/v).
Figure 11. Chlorhexidine induces mexCD-oprJ expression in an AlgU-dependent manner in wild-type P. aeruginosa.

The expression of mexD and rpsL was assessed in P. aeruginosa strains K767 (A, lanes 1 and 3) and K2443 (ΔalgU; A, lanes 2 and 4) in the absence (A, lanes 1 and 2) or after continuous overnight exposure to 1/4 MIC of chlorhexidine (A, lanes 3 and 4) by semiquantitative RT-PCR. Expression of mexD and rpsL in K2443 carrying pMMB206 (B, lanes 1 and 2) or pMMB206::algU (B, lanes 3 and 4) in the absence (B, lanes 1 and 3) or presence of chlorhexidine (B, lanes 2 and 4) using semiquantitative RT-PCR is also shown. The rpsL reaction served as an internal control to ensure equal amounts of RNA loading. The PCR portion was carried out at 33 (mexD) and 23 (rpsL) cycles (top) and 35 (mexD) and 25 (rpsL) cycles (bottom).
Figure 12. Influence of membrane-damaging agents and AlgU on \textit{mexCD-oprJ} expression in wild-type \textit{P. aeruginosa}.

The expression of \textit{mexD} and \textit{rpsL} was assessed in \textit{P. aeruginosa} strains K767 (lanes 1 and 3) and K2443 (\textit{ΔalgU}; lanes 2 and 4) in the absence (lanes 1 and 2) or after continuous overnight exposure to membrane-damaging agents at the indicated fractional MIC (lanes 3 and 4) by semiquantitative RT-PCR. The \textit{rpsL} reaction served as an internal control to ensure equal amounts of RNA loading. The PCR portion was carried out at 33 (\textit{mexD}) and 23 (\textit{rpsL}) cycles (top) and 35 (\textit{mexD}) and 25 (\textit{rpsL}) cycles (bottom).
K767 (Table 10). Similar to the results presented for K1542 and K2861 (K1542 ΔalgU), the deletion of algU in K767 compromised MexCD-OprJ induction by membrane-damaging agents, indicating that it is required for mexCD-oprJ expression in wild-type cells (Figure 11A and Figure 12; lanes 2 and 4). Complementation of the algU deletion with plasmid pSF02 restored MexCD-OprJ inducibility by membrane-damaging agents, when challenged with the representative membrane-damaging agent chlorhexidine (Figure 11B; lanes 3 and 4).

3.6 Importance of AlgU for mexCD-oprJ hyperexpression in nfxB mutants

As nfxB encodes a negative regulator of mexCD-oprJ expression, mutations in this gene greatly increase pump gene expression. To determine if mexCD-oprJ hyperexpression in such mutants requires AlgU, mexCD-oprJ expression was studied in P. aeruginosa K1536 (K767 ΔnfxB). When compared to the wild-type strain K767, expression of mexCD-oprJ is constitutively elevated in K1536, even in the absence of any inducing agent (Figure 13; lanes 1 and 3). The loss of algU in K1536 resulted in a decreased mexCD-oprJ expression; the constitutive hyperexpression of this system was altered (Figure 13; lanes 3 and 4). Expression of mexCD-oprJ in K2841 (K1536 ΔalgU), however, was still elevated compared to the K767 or K2443 (K767 ΔalgU) (Figure 13; lanes 2 and 4). In agreement with the decrease in mexCD-oprJ expression in K2841, were the MIC values determined for the substrate chlorhexidine. Strain K2841 was 16-fold more susceptible to chlorhexidine than K1536 with the MIC decreasing from 20 μg/mL (K1536) to 1.25 μg/mL (K2841).
Figure 13. Influence of AlgU on mexCD-oprJ expression in wild-type and nfxB-mutant strains of *P. aeruginosa*.

The expression of *mexD* and *rpsL* was assessed in *P. aeruginosa* strains K767 (lanes 1), K2443 (ΔalgU; lane 2), K1536 (ΔnfxB; lane 3), and K2841 (ΔnfxB-ΔalgU; lane 4) by semiquantitative RT-PCR. The *rpsL* reaction served as an internal control to ensure equal amounts of RNA loading. The PCR portion was carried out at 31 (*mexD*) and 23 (*rpsL*) cycles (top) and 33 (*mexD*) and 25 (*rpsL*) cycles (bottom).
3.7 Effect of cell wall-damaging agents and LPS mutations on *mexCD-oprJ* expression

It was previously shown that fosfomycin, a cell wall-damaging agent, increased *algD* promoter activity, which was subsequently lost in an *algU* deletion strain, thus indicating that AlgU is required for *algD* expression (Wood *et al.*, 2006). In this same study, *algD* was also shown to be highly expressed by microarray analysis following exposure to another cell wall-damaging agent, D-cycloserine. Together, this indicates that cell wall-damaging agents activate the alginate biosynthetic operon, as *algD* is the first gene of the alginate biosynthetic operon, and that this activation requires AlgU. Microarray data from this study also indicated that D-cycloserine exposure resulted in a 2.5-fold increase in *mexC* expression (Wood *et al.*, 2006). It was, therefore, of interest to ascertain if we would observe this same increased *mexCD-oprJ* expression following exposure to a cell wall-damaging agent, if a more sensitive method was used to detect gene expression. Therefore, the susceptibility of strain K1542 to fosfomycin was determined (Table 8, FOS) and a fractional MIC was used to investigate the potential of this agent to induce *mexCD-oprJ* expression by RT-PCR. Under our conditions, growth in the presence of fosfomycin did not appear to alter *mexCD-oprJ* expression (Figure 14).

Next, *mexCD-oprJ* expression was examined using *P. aeruginosa* strains that possess mutations in LPS biosynthetic genes. These studies were performed based on a model in *E. coli* where intermediates in the LPS biosynthetic pathway function to activate $\sigma^E$ (Tam and Missiakas, 2005). Specifically, it was shown that when the lipid A component of LPS is altered by chemical means or the lipid A-inner core is not fully
Figure 14. Influence of fosfomycin on mexCD-oprJ expression in a ΔmexB-ΔmexXY strain of P. aeruginosa.

The expression of mexD and rpsL was assessed in P. aeruginosa strain K1542 (ΔmexB-ΔmexXY) grown without antibiotics (lane 1) or with continuous overnight exposure to 1/4 MIC fosfomycin (lane 2) by semiquantitative RT-PCR. The rpsL reaction served as an internal control to ensure equal amounts of RNA loading. The PCR portion was carried out at 33 (mexD) and 23 (rpsL) cycles (top) and 35 (mexD) and 25 (rpsL) cycles (bottom).
acetylated due to the presence of genetic mutations, \textit{rpoE} expression is increased. We, therefore, acquired strains possessing mutations in LPS biosynthetic genes and examined \textit{mexCD-oprJ} expression in these mutants. The strains tested contained deletions in \textit{waaL}, which is responsible for attaching the O-antigenic side chain to the core polysaccharide, and \textit{rmlC} and \textit{wapR}, which are involved in assembly of the outer region of core polysaccharide (Rahim \textit{et al.}, 2000; Lam \textit{et al.}, 2004; Abeyrathne \textit{et al.}, 2005). The deletion of any one of these three genes (\textit{waaL}, \textit{rmlC} or \textit{wapR}), had no effect on \textit{mexCD-oprJ} expression (Figure 15).

3.8 Influence of PA4596 on \textit{mexCD-oprJ} expression in \textit{P. aeruginosa}

Based on the observation that membrane stress mediates \textit{mexCD-oprJ} expression, through the alternative sigma factor AlgU, it was of interest to determine possible AlgU-dependent mediators of this response. Microarray data (provided by a colleague) reports the identification of a gene, PA4596, which, like \textit{mexCD-oprJ}, is both AlgU-dependent and chlorhexidine inducible and is located directly downstream of the \textit{mexCD-oprJ} operon, an organization also found in \textit{Pseudomonas putida} (Figure 16) (Stover \textit{et al.}, 2000). It was of interest to determine if, and how, PA4596 contributes to \textit{mexCD-oprJ} expression. The ability of chlorhexidine to induce \textit{mexCD-oprJ} expression in K767 had been previously demonstrated and, being both an inducing agent and a substrate for MexCD-OprJ, its presence alone would be sufficient to determine MexCD-OprJ activity based on MICs. The impact of PA4596 loss on \textit{mexCD-oprJ} expression, therefore, was assessed first by MIC and secondly by RT-PCR. Compared to K767, the \textit{\Delta PA4596}}
Figure 15. Influence of LPS mutations on mexCD-oprJ expression in *P. aeruginosa*.

The expression of *mexD* and *rpsL* was assessed in a wild-type *P. aeruginosa* PA01 strain (lane 1) and compared to *P. aeruginosa* strains that contained mutations in the LPS biosynthetic genes *rmlC* (lane 2), *waaL* (lane 3), or *wapR* (lane 4) by semiquantitative RT-PCR. The *rpsL* reaction served as an internal control to ensure equal amounts of RNA loading. The PCR portion was carried out at 33 (*mexD*) and 23 (*rpsL*) cycles (top) and 35 (*mexD*) and 25 (*rpsL*) cycles (bottom).
Figure 16. Genetic organization of PA4596 and the *mexCD-oprJ* operon.

Schematic diagram of the *P. aeruginosa* chromosomal region including the *mexCD-oprJ* operon and the upstream PA4596 gene, where PA4596 and NfxB share extensive homology.
PA4596 \quad oprJ \quad mexD \quad mexC \quad nfxB
strain, K2878, was two-fold more resistant to chlorhexidine; the chlorhexidine MIC increased from 10 μg/mL (K767) to 20 μg/mL (K2878). RT-PCR confirmed that the increase in \textit{mexCD-oprJ} expression was greater in K2878 (Figure 17A; lanes 3 and 4) than K767 (Figure 17A; lanes 1 and 2). Complementation of the PA4596 deletion with plasmid pAJC02 resulted in a restored sensitivity to chlorhexidine, as the MIC decreased to 7.5 μg/mL. RT-PCR also demonstrated that \textit{mexCD-oprJ} expression was restored to the wild-type levels in the complemented strain (Figure 17B).

3.9 Contribution of PA4596 to \textit{mexCD-oprJ} hyperexpression in an \textit{nfxB}-mutant strain of \textit{P. aeruginosa}

To determine if \textit{mexCD-oprJ} hyperexpression in the \textit{nfxB}-mutant strain requires PA4596, the chlorhexidine MIC was determined and RT-PCR was performed to compare strains K1536 (K767 ΔalgU) and K2879 (K1536 ΔnfxB-ΔPA4596). As shown previously, an \textit{nfxB} mutation results in a constitutively high level of \textit{mexCD-oprJ} expression and the impact of PA4596 loss was assessed in this background using the strain K2879. In contrast with above, K2879 was more susceptible to chlorhexidine (7.5 μg/mL) when compared to K1536 (20 μg/mL). The decreased expression of \textit{mexCD-oprJ} in strain K2879 compared to K1536 was confirmed by RT-PCR (Figure 18A). Complementation of the PA4596 deletion restored chlorhexidine resistance to 15 μg/mL and increased \textit{mexCD-oprJ} expression, as determined through RT-PCR (Figure 18B).
Figure 17. Influence of PA4596 on mexCD-oprJ expression in response to chlorhexidine treatment in wild-type P. aeruginosa.

The expression of mexD and rpsL was assessed in P. aeruginosa strains K767 (A, lanes 1 and 2) and K2878 (ΔPA4596; A, lanes 3 and 4) in the absence (A, lanes 1 and 3) or after continuous overnight exposure to 1/4 MIC chlorhexidine (A, lanes 2 and 4) by semiquantitative RT-PCR. Expression of mexD and rpsL in K2878 carrying pMMB206 (B, lanes 1 and 2) or pMMB206::PA4596 (B, lanes 3 and 4) in the absence (B, lanes 1 and 3) or presence of 1/4 MIC chlorhexidine (B, lanes 2 and 4) using semiquantitative RT-PCR is also shown. The rpsL reaction served as an internal control to ensure equal amounts of RNA loading. The PCR portion was carried out at 31 (mexD) and 23 (rpsL) cycles (top) and 33 (mexD) and 25 (rpsL) cycles (bottom).
Figure 18. Influence of PA4596 on mexCD-oprJ expression in an nfxB-mutant strain of P. aeruginosa.

The expression of mexD and rpsL was assessed in P. aeruginosa strains K1536 (ΔnfxB; A, lane 1) and K2879 (ΔnfxB-ΔPA4596; A, lane 2) by semiquantitative RT-PCR. Expression of mexD and rpsL in K2879 carrying pMMB206 (B, lane 1) or pMMB206::PA4596 (B, lane 2) by semiquantitative RT-PCR is also shown. The rpsL reaction served as an internal control to ensure equal amounts of RNA loading. The PCR portion was carried out at 31 (mexD) and 23 (rpsL) cycles (top) and 33 (mexD) and 25 (rpsL) cycles (bottom).
CHAPTER 4: Discussion

Multidrug efflux pumps in *P. aeruginosa* confer resistance to a wide range of antibiotics principally mediated by the MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY systems (Pumbwe and Piddock, 2000; Aeschlimann, 2003; Llanes *et al.*, 2004). Given that only the MexAB-OprM system is constitutively expressed under normal laboratory growth conditions, an understanding of the inducing compounds and conditions that select for expression of the other systems is important in the clinical management of *P. aeruginosa* infection. In determining these conditions, it is necessary to consider that the efflux of antibiotics, in many instances, may only be secondary to a natural function and agents that induce these systems likely alter conditions within the bacterium to a state that mimics the circumstances that naturally require efflux. Based on the data presented in this report regarding the MexCD-OprJ system, it appears that this system’s intended function may be to alleviate and/or compensate for membrane-associated stress. Previous data supports this model, since the inducing agents of MexCD-OprJ have all been membrane-active compounds, including benzalkonium chloride and chlorhexidine (Morita *et al.*, 2003).

To further demonstrate that *mexCD-oprJ* expression occurs in response to membrane damage, other membrane-active agents were chosen and the ability of these agents to induce pump expression monitored. The initial experiments, using increases in antibiotic resistance as a means to demonstrate that MexCD-OprJ was active, both allowed for a quick screening method and allowed us to demonstrate that the presence of these agents is able to mediate changes in antibiotic susceptibility (Table 9). Although some of the agents selected have additional activities not related to membrane disruption,
membrane damage is the key feature associated with all agents (chlorhexidine, polymyxin B, melittin, and ethanol). When determining antibiotic resistance, three of the membrane-damaging agents were cationic compounds (chlorhexidine, polymyxin B, melittin), while the fourth (ethanol) was not. The use of ethanol demonstrated that induction may occur with a more non-specific interaction with the membrane; the cationic agents cause outer membrane disruption, first, through the displacement of Ca\(^{2+}\) and Mg\(^{2+}\) ions that interact with LPS, followed by disruption of the inner membrane by the displacement of Ca\(^{2+}\) and Mg\(^{2+}\) ions associated with phospholipids, while ethanol is believed to partition into membranes and cause disruption (Gilbert and Moore, 2005; Jones, 2007).

Since the agents used in the current study are all membrane active, a likely MexCD-OprJ-inducing cellular compound would be found within the membrane, where membrane disturbances release, or cause the accumulation of, this product. Further supporting this model, where membrane stress produces an inducing “signal”, is the inability of norfloxacin or fosfomycin to induce expression. Norfloxacin, a fluoroquinolone antibiotic, binds and inhibits the DNA gyrase enzyme, although, inhibition of DNA topoisomerase IV may also occur (Pohlhaus and Kreuzer, 2005). This binding to DNA gyrase blocks DNA replication and causes double-stranded DNA breaks, creating a generalized cytosolic stress. Exposure of \textit{P. aeruginosa} K1542 to norfloxacin did not result in \textit{mexCD-oprJ} expression, indicating that cytosolic stress is unable to induce this efflux system. This is consistent with earlier studies that showed, while being a substrate for the MexCD-OprJ system, norfloxacin does not mediate induction (Morita \textit{et al.}, 2003). The cellular signal that induces pump gene expression also does not likely
originate in the peptidoglycan biosynthetic pathway, since disruptions in the
peptidoglycan layer, as demonstrated with the use of fosfomycin, do not increase mexCD-
oprJ expression. Fosfomycin is a phosphoenolpyruvate analogue, an essential compound
in the production of N-acetylgalactosamine (NAG), which is one of the two sugars required
in the synthesis of the peptidoglycan layer (Nilsson et al., 2003); the presence of
fosfomycin, therefore, inhibits the synthesis of peptidoglycan. The inability of
fosfomycin to induce MexCD-OprJ is consistent with previous studies where agents that
disrupt peptidoglycan structure, such as carbenicillin, were unable to induce pump
expression (Morita et al., 2003). Again, this suggests that the inducing signal of MexCD-
OprJ originates in the bacterial membrane.

Differences in resistance to each antibiotic (promoted by membrane-damaging
agents) may be due to differences in antibiotic recognition by MexCD-OprJ, where
MexCD-OprJ is able to better recognize and export agents whose MIC increased the
greatest (e.g. erythromycin); this is consistent with previous reports where the presence of
MexCD-OprJ was shown to greatly increase resistance to erythromycin (Masuda et al.,
2000b). Additionally, some of the differences in antibiotic resistance in the presence of
the membrane-damaging agents may be due to differences in the synergistic activity
between the agent and the antibiotic; a greater antibiotic activity may be potentiated in
the presence of certain membrane-damaging agents and, so, result in differences in
measured MICs. Every effort was made to determine a concentration of membrane-
damaging agent where the addition of an antibiotic did not kill the bacteria, however, the
same concentration of membrane-damaging agent was used for all antibiotics. This may
not have resulted in the use of an ideal concentration of membrane-damaging agent and
antibiotic in each instance. The RT-PCR experiments did not use MICs as a surrogate method to determine \textit{mexCD-oprJ} expression but rather directly measured efflux system gene expression; synergistic activity, therefore, was no longer a concern and this allowed for an expanded list of membrane-damaging agents to be used (EDTA, \textit{n}-hexane, and \textit{p}-xylene). The RT-PCR data was consistent with the MIC data and demonstrated that \textit{mexCD-oprJ} expression did increase upon exposure to each of the membrane-damaging agents, while the absence of induction following norfloxacin exposure, as stated previously, demonstrated that a general cytosolic stress did not induce \textit{mexCD-oprJ} expression.

The use of antibiotic resistance as a measure of \textit{mexCD-oprJ} expression did not eliminate the possibility of pump expression occurring as a result of mutations in the repressor gene \textit{nfxB} where, under these circumstances, exposure to an inducing agent is not necessary (Poole \textit{et al.}, 1996; Higgins \textit{et al.}, 2003). In these instances, it is the presence of MexCD-OprJ substrates that selects for \textit{nfxB} mutants and, thus, pump expression. The examination of \textit{mexCD-oprJ} expression by RT-PCR, however, did reduce the possibility of selecting for \textit{nfxB} mutants since the bacterial cells were exposed to only one agent, which was not usually a MexCD-OprJ substrate. With the exception of chlorhexidine, none of the membrane-damaging agents used were substrates of the MexCD-OprJ system, as demonstrated by identical MICs between K1542 and the \textit{ΔmexCD-oprJ} strain, K2873 (Table 9). This suggests that there is no selective pressure to express \textit{mexCD-oprJ} in order to reduce the accumulation of these membrane-damaging agents within the cell and, therefore, likely no selective pressure for \textit{nfxB} mutations. As stated earlier, in this scenario, expression likely occurs due to the
The β-galactosidase assays attempted to quantify the level of induction by each of the different membrane-active compounds. These experiments demonstrated that all agents increased *mexCD-oprJ* promoter activity, indicative of all agents being capable of inducing MexCD-OprJ. These experiments also introduced a new induction time period. The 2-hour exposure to the membrane-damaging agents differed from the continuous overnight exposure to the agents, used for the MIC and RT-PCR experiments. RT-PCR using a 2-hour exposure to chlorhexidine demonstrated that an increase in *mexCD-oprJ* expression occurs over this time period; this induction, however, was likely not as great compared with overnight exposure to chlorhexidine. This may have occurred since there is a constant inducing pressure in the overnight cultures. The β-galactosidase assay, also,
introduced two new peptide agents that targeted and disrupted the membrane, V8 and V681. Due to specific amino acid substitutions (and the presence or absence of proline residues), it has been demonstrated that, at the concentrations used in this study, V8 only disrupts outer membrane integrity while V681 is able to disrupt both the outer and inner membrane (Zhang et al., 1999; Zhang et al., 2001). In the current study, both agents increased mexCD-oprJ promoter activity, thereby indicating disruption of the outer membrane alone is able to increase pump gene expression. Furthermore, this suggests that the inducing signal is likely an outer membrane component.

The increased expression of mexCD-oprJ occurs in the presence of each membrane-damaging agent not only in the ΔmexB-ΔmexXY strain (K1542) (Figure 7A and Figure 8) but in wild-type cells (K767) (Figure 11A and Figure 12), as shown through RT-PCR. Expression of mexCD-oprJ in K767 demonstrates that these agents are able to induce this system in a wild-type background, which is a more clinically relevant genotype than K1542. No direct comparisons, however, can be made regarding the inducing ability of the different agents in these two backgrounds, since the amount of RNA is not normalized between these different strains; equal amounts of RNA are loaded only to compare the levels of mexCD-oprJ expression in the absence or presence of a particular membrane-damaging agent for a specific strain and not between agents or strains. Also, it is unclear the level of membrane damage that occurs as a result of exposure to each membrane-active agent; the fractional MICs used for each agent do not necessarily correspond to equivalent levels of membrane damage and, therefore, possible MexCD-OprJ-inducing potential. The RT-PCR data only allows us to determine if a membrane-damaging agent was capable of inducing mexCD-oprJ expression or not.
With this in mind, there still appears to be an overall difference in induction between K1542 and K767, where there is a greater difference in *mexCD-oprJ* expression (between the absence and presence of a membrane-damaging agent) in K1542 than is K767. The absence of MexAB-OprM and MexXY in K1542 may already elevate stress, making the cells more prone to express *mexCD-oprJ* when challenged; the absence of these two systems may result in a greater accumulation of a cellular product that is capable of affecting induction of MexCD-OprJ. In the case of the organic solvent, *n*-hexane, the reduced increase in *mexCD-oprJ* expression, upon exposure in K767 as compared to K1542, may be due to the efflux of the compound by systems other then MexCD-OprJ; MexAB-OprM has been shown to be capable of exporting this compound and, in turn, the necessity for *mexCD-oprJ* expression could be reduced (Li *et al.*, 1998).

Finally, supporting the notion that membrane-damage induces MexCD-OprJ, is the correlation between *mexCD-oprJ* expression and a functional *algU* gene. Antibiotic resistance mediated by MexCD-OprJ requires an intact *algU* gene (Table 9) and the RT-PCR results also supported the necessity of *algU* for optimal *mexCD-oprJ* expression in both K1542 and K767 (Figure 7A and Figure 8; Figure 11A and Figure 12). Additionally, the β-galactosidase assays demonstrated that there was no increased *mexCD-oprJ* promoter activity, when compared to background levels, in the presence of the membrane-damaging agents in the *algU* deletion strain K2861 (Figure 9B). In the β-galactosidase assays, however, there was an increased overall promoter activity in K2861 (K1542 Δ*algU*) compared to K1542. This may be due to changes in membrane integrity that occur with *algU* loss, resulting in an increased disruption of K2861 cells in the Miller
assay and, thus, an increased release of protein (LacZ) from the cells during permeabilization; this, in turn, would result in increased β-galactosidase activity.

Interestingly, expression is dependent on AlgU both in the presence of a membrane-active agent and in the nfxB mutant. The requirement for algU in the nfxB mutant, suggests that activation of mexCD-oprJ expression by AlgU does not occur by repressing NfxB binding, which is in contrast with a model where AlgU mediates the derepression of mexCD-oprJ through an interaction with NfxB. Expression of mexCD-oprJ likely occurs through the concomitant activation of AlgU and repression of NfxB (refer to Figure 6); activation of mexCD-oprJ expression by AlgU is still dependent on NfxB removal from the mexCD-oprJ promoter. Since mexCD-oprJ expression is still greater in K2841 (K1536 ΔalgU) compared to K2443 (K767 ΔalgU), it may be worthwhile to determine if the level of mexCD-oprJ expression following chlorhexidine treatment of K2443 is comparable to K2841. This would further demonstrate that a two-step process leads to the increased expression of mexCD-oprJ; there is first the release of the negative repressor NfxB followed by an AlgU-mediated activation of expression. This activation by AlgU does not appear to occur by the direct binding of AlgU to the mexCD-oprJ promoter region, since no consensus AlgU binding site [GAACCTT (-35 bp) and TCtgA (-10 bp)] is present in the area immediately upstream of mexCD-oprJ (Firoved et al., 2002).

If the modulation of mexCD-oprJ expression by AlgU is indirect, an ideal intermediary candidate is the product of the PA4596 gene, since this gene is linked to the mexCD-oprJ operon, a genetic organization also found in P. putida, and the PA4596 protein shares approximately 54% identity and 74% similarity to the mexCD-oprJ
regulatory protein NfxB (Figure 16) (Stover et al., 2000). As described earlier, microarray data indicates that the PA4596 gene has a similar induction profile as mexCD-oprJ, being both induced in response to chlorhexidine and requiring a functional algU gene for expression. In this study, we show that the absence of this gene has an effect on mexCD-oprJ expression, thereby demonstrating that it is involved in the regulation of MexCD-OprJ.

In wild-type cells (K767), the loss PA4596 results in the increased expression of mexCD-oprJ, indicative of PA4596 being a repressor protein. This differs, however, from the loss of AlgU, where there is a decreased mexCD-oprJ expression in K2443 (K767 ΔalgU), which would indicate that AlgU is mediating an activator of expression. Since each mediates a different effect, this data would suggest that the principal role of AlgU on mexCD-oprJ expression is not mediated through PA4596. In contrast, in K1536 (K767 ΔnfxB), the loss of PA4596 decreases mexCD-oprJ expression, which is identical to the loss of algU in this strain. This would indicate that PA4596 is functioning as an activator and is positively influencing mexCD-oprJ expression, which would be consistent with a potential mediator of the previously identified AlgU response.

Consistent with the previously proposed model, where an AlgU-activating and NfxB-repressing signal is required for mexCD-oprJ expression, the deletion of PA4596 in K1536 resulted in a decreased ability to induce MexCD-OprJ. This suggests that PA4596 induces mexCD-oprJ expression, like AlgU, in a manner that does not depend on an interaction with NfxB. This is a surprising revelation for the PA4596 protein, since it is similar to NfxB and, therefore, may have influenced mexCD-oprJ expression through an interaction with NfxB. Since PA4596 does not appear to influence mexCD-oprJ
expression through an interaction with NfxB, it is even more surprising that PA4596 appears to increase pump gene expression given its homology to a repressor protein. As PA4596 appears to mediate different affects in the wild-type and ΔnfxB strains, however, it may still be worthwhile to investigate if such an interaction (between PA4596 and NfxB) could occur. Since most LacI-type repressor proteins function as dimers to repress transcription, one function of PA4596, which would be dependent on the presence of NfxB, may be to alter the binding pattern of NfxB (Lewis, 2005). Both proteins are very similar, with the exception of a truncated N-terminal region in PA4596, and PA4596-NfxB dimers may have an altered binding affinity for the nfxB-mexCD-oprJ promoter region. Based on the mexCD-oprJ expression pattern in K767, PA4596 appeared to be a repressor, and, in turn, indicate that possible PA4596-NfxB dimers would have a greater affinity for this promoter region. A gel shift assay could be used to determine if PA4596 does have an effect on NfxB binding to this promoter region. The PA4596 promoter region could also be subjected to further experiments; for instance, the ability of AlgU to bind this region could be assessed, possibly through the use of in vitro transcription assay, to determine if it is capable of directing transcription of this region.

Further evidence suggesting that concurrent activation of AlgU and the removal of NfxB from the mexCD-oprJ promoter region is required for mexCD-oprJ expression, is supported by studies demonstrating that the activation AlgU alone does not mediate increased expression of mexCD-oprJ. Cell wall-damaging agents, including fosfomycin, have been shown to result in increased algD promoter activity, which is subsequently lost in an algU deletion strain (Wood et al., 2006). This suggests that AlgU activity is required for algD expression, where algD is the first gene in the 12 gene alginate
biosynthetic operon, which is in agreement with expression from the \textit{algD} promoter being AlgU-dependent (Govan and Deretic, 1996). While activation of AlgU is believed to be the principle inducing signal for \textit{algD} expression, the use of fosfomycin did not have an effect on \textit{mexCD-oprJ} expression in the current study. This suggests that activation of AlgU alone is not able to cause the up-regulation of \textit{mexCD-oprJ} expression; a signal that modulates NfxB is also required. Interestingly, too, the presence of cell membrane disrupting agents, including polymyxin B, had no effect on \textit{algD} expression in the study by Wood \textit{et al.} (2006), but polymyxin B was found to increase \textit{mexCD-oprJ} expression in our study. It is, therefore, likely that expression of the \textit{algD} operon (and alginate synthesis) also requires two signals, one being the activation of AlgU.

In \textit{E. coli}, the production of abnormal LPS is able to induce \( \sigma^E \) expression (Tam and Missiakas, 2005). For example, a decreased acylation, which results in a penta-acylated instead of a hexa-acylated lipid A structure, which occurs as a result of \textit{htrB} or \textit{msbB} loss, has been shown to increase \( \sigma^E \) expression using a \textit{rpoE-lacZ} reporter fusion. The deletion of \textit{tolA} or \textit{pal}, where TolA and Pal are periplasmic proteins that may be involved in the translocation of O-antigen across the periplasm, also activates \( \sigma^E \), as demonstrated by increased expression of genes that are \( \sigma^E \)-dependent (Vines \textit{et al.}, 2005). These mutations, which alter translocation of O-antigen, would likely not affect membrane integrity to the same degree as abnormal lipid A, since lipid A is embedded within the outer leaflet of the outer membrane and the O-antigen is not. This indicates that \( \sigma^E \), in these instances, is responsive to abnormal LPS structure. The chemical alteration of LPS by ammonium metavanadate (NH\textsubscript{4}VO\textsubscript{3}), which alters lipid A structure
through the addition of palmitate, phosphoethanolamine, and 4-amino-4-deoxy-L-arabinose (to the phosphate groups associated with lipid A), is also a strong inducer of $\sigma^E$ expression (Vines et al., 2005; Tam and Missiakas, 2005). These alterations may increase membrane permeability, since they alter the LPS bridging sites (the negatively charged phosphate groups of lipid A) where the divalent cations $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ localize on the outer membrane. In addition to a link between LPS changes and $\sigma^E$ induction, the activation of $\sigma^E$ may lead to alterations in LPS structure (Vines et al., 2005). The deletion of $rseA$, which encodes the anti-sigma factor that sequesters $\sigma^E$ and prevents its activity, results in the increased expression of $\sigma^E$-dependent promoters and altered LPS structure. Specifically, this activation leads to defects in O-antigen polymerization.

In the current study, therefore, gene mutations, which are involved in the attachment of O-antigen to core polysaccharide ($waaL$) or assembly of the outer region of the core polysaccharide ($rmlC$ or $wapR$) of LPS in $P.\ aeruginosa$, were assessed for the ability to increase $mexCD-oprJ$ expression (Rahim et al., 2000; Lam et al., 2004; Abeyrathne et al., 2005). These mutations alter LPS structure and, based on studies in $E.\ coli$, might alter AlgU activity and induce $mexCD-oprJ$ expression. Although no changes in $mexCD-oprJ$ expression were detected in the LPS mutant strains, this may be due to the specific mutations examined. In $E.\ coli$, the $htrB$ and $msbB$ deletions and the chemical alterations of LPS resulted in alterations of lipid A (Tam and Missiakas, 2005). The altered lipid A structure should result in a greater impact on membrane integrity since, as stated earlier, it is the portion embedded in the outer membrane. The ability of only lipid A modifications to alter membrane integrity is supported by the fact that “deep rough” LPS mutants, which possess only lipid A and KDO, have increased outer
membrane permeability (Nikaido, 1996). The LPS mutations in \textit{P. aeruginosa} that were tested did not affect the lipid A moiety and would likely have a lesser impact on membrane structure. In the future, \textit{mexCD-oprJ} expression could be assessed in \textit{P. aeruginosa} strains containing mutations in genes that alter the lipid A region, for example in PA0011 and PA3242, the \textit{P. aeruginosa} homologues of the \textit{E. coli htrB} gene, or \textit{pagL}, a deacylase present in \textit{P. aeruginosa}, whose loss would result in increased acylation of lipid A (Geurtsen \textit{et al.}, 2005; Winsor \textit{et al.}, 2005).

Together, this indicates that MexCD-OprJ is up-regulated as a result of membrane stress suggesting that a natural role may be in response to alterations or the release of components in these membranes. The natural role of most RND-type efflux systems is currently unclear, and unless it can be demonstrated that a specific compound interacts with a regulatory protein of an efflux system, it is difficult to determine the intended function of a system. In the case of MexCD-OprJ, although the identification of a specific of membrane component capable of inducing MexCD-OprJ may be difficult to find, the conditions that select for \textit{mexCD-oprJ} expression suggest that this system may have evolved to relieve membrane stress.
CHAPTER 5: Summary and conclusion

In the current report, MexCD-OprJ induction is linked to the presence of membrane stress. The ability of a variety of unrelated agents, many of which are not MexCD-OprJ substrates, to induce efflux pump gene expression was demonstrated. Subsequent analysis of \emph{mexCD-oprJ} promoter activity in the presence of these compounds also revealed that promoter activity increases in their presence. In addition, MexCD-OprJ appears to be partially controlled through the envelope stress response regulator, AlgU, as induction following membrane damage requires a functional \emph{algU} gene. Furthermore, AlgU does not appear to increase \emph{mexCD-oprJ} expression through an interaction with NfxB, since the deletion of \emph{algU} is still able to increase pump gene expression in the absence of a functional \emph{nfxB} gene. The effect of AlgU on \emph{mexCD-oprJ} expression may occur through the AlgU-dependent, chlorhexidine inducible product of the PA4596 gene. It is unclear the role of the PA4596 protein, since it appears to be able to function both as a repressor or activator, depending on the presence or absence of \emph{nfxB}, respectively. Together this data suggests that \emph{mexCD-oprJ} is part of the envelope stress response, which may be its natural function.
References


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