The *mexCD-oprJ* multidrug efflux operon in *Pseudomonas aeruginosa*: regulation by the NfxB-like novel regulator PA4596 and envelope stress

by

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Abstract

Expression of the *mexCD-oprJ* multidrug efflux operon is enhanced by the presence of membrane damaging agents [e.g., the biocide chlorhexidine (Chx)] or mutations in the *nfxB* gene encoding a repressor of efflux gene expression, both dependent on the AlgU envelope stress response sigma factor. Details of *mexCD-oprJ* regulation are, however, lacking. In examining the *mexCD-oprJ* locus, a gene, PA4596, encoding a homologue of NfxB (61% identity) was identified downstream of *oprJ*, a location conserved in all sequenced *Pseudomonas aeruginosa* isolates and in *Pseudomonas putida*. Opposite to *mexCD-oprJ*, PA4596 expression was reduced by Chx exposure, as assessed using RT-PCR; although like *mexCD-oprJ*, this was AlgU-dependent (i.e., lost in a Δ*algU* strain). Deletion of PA4596 had no impact on Chx resistance indicating that it is not required for Chx-inducible *mexCD-oprJ* expression/ MexCD-OprJ-dependent Chx resistance. In contrast, *mexCD-oprJ* expression and the attendant multidrug resistance of *nfxB* deletion mutants were compromised upon deletion of PA4596, indicating that PA4596 plays a positive role in *mexCD-oprJ* expression in such mutants. Consistent with this, PA4596 expression increased in *nfxB* deletion and missense mutants in parallel with *mexCD-oprJ*. Intriguingly, *mexCD-oprJ* expression and multidrug resistance were observed in a mutant lacking an *nfxB* mutation (demonstrating an NfxB-like phenotype) and in an *nfxB* missense mutant and these were not compromised upon deletion of PA4596. Thus, *mexCD-oprJ* hyperexpression can be both PA4596-dependent and -independent. A bacterial 2-hybrid assay revealed a PA4596-PA4596 interaction, consistent with the protein forming dimers as NfxB has been shown to do. Two-hybrid assays also demonstrated that NfxB and PA4596 interact. While the functional significance of this remains to be elucidated, it is consistent with their common role in regulating *mexCD-oprJ* expression and is suggestive of a complex and possibly novel regulatory mechanism. These data highlight the complexity of *mexCD-oprJ* regulation and the apparently
multiple pathways to efflux gene expression, suggestive of multiple roles for this efflux system in

*P. aeruginosa* independent of antimicrobial efflux.
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# Table of Contents

Abstract ................................................................................................................................. ii  
Acknowledgements ................................................................................................................ iv  
Table of Contents .................................................................................................................... v  
List of Figures ........................................................................................................................ viii  
List of Tables ........................................................................................................................ ix  
List of Abbreviations ............................................................................................................. x  

Chapter 1 : Introduction ........................................................................................................ 1  
  1.1 *Pseudomonas aeruginosa* .............................................................................................. 1  
  1.2 Antimicrobials and their mechanisms of action .............................................................. 3  
  1.3 Mechanisms of antimicrobial resistance ....................................................................... 5  
      1.3.1 Target Site Modification ....................................................................................... 5  
      1.3.2 Inactivation ........................................................................................................... 6  
      1.3.3 Efflux .................................................................................................................... 7  
  1.4 Non-RND type efflux systems ....................................................................................... 10  
  1.5 RND type efflux systems .............................................................................................. 13  
  1.6 Characterization of *P. aeruginosa* RND multidrug efflux pumps .............................. 15  
      1.6.1 MexAB-OprM ......................................................................................................... 15  
      1.6.2 MexEF-OprN ......................................................................................................... 18  
      1.6.3 MexXY ................................................................................................................ 19  
      1.6.4 MexCD-OprJ ......................................................................................................... 19  
  1.7 Paradigms in gene regulation ......................................................................................... 21  
      1.7.1 Sigma factors ....................................................................................................... 21  
      1.7.2 Activators ............................................................................................................. 23  
      1.7.3 Repressors ............................................................................................................ 24  
  1.8 Regulation of *P. aeruginosa* RND multidrug efflux pumps ....................................... 26  
      1.8.1 MexAB-OprM ......................................................................................................... 26  
      1.8.2 MexEF-OprN ......................................................................................................... 26  
      1.8.3 MexXY ................................................................................................................ 27  
      1.8.4 MexCD-OprJ ......................................................................................................... 28  
  1.9 Natural role of multidrug efflux pumps ....................................................................... 29  
      1.9.1 Natural roles of MexAB-OprM, MexEF-OprN, and MexXY ................................. 30  

v
1.9.2 Natural role of MexCD-OprJ ............................................................................ 31
1.10 Experimental aim .............................................................................................. 32

Chapter 2 : Materials and Methods ........................................................................ 33
2.1 Bacterial strains, plasmids, and growth conditions ........................................... 33
2.2 DNA isolation and visualization ......................................................................... 33
2.3 Restriction digests and ligation of DNA fragments ............................................. 37
2.4 PCR amplification and nucleotide sequencing .................................................... 38
2.5 DNA transformation .............................................................................................. 41
   2.5.1 Calcium chloride competent *E. coli* ............................................................ 41
   2.5.2 Electrocompetent *P. aeruginosa* ................................................................. 42
2.6 Conjugation ......................................................................................................... 42
2.7 Construction of gene deletions ............................................................................ 43
   2.7.1 Construction of *P. aeruginosa K767 ΔnfxB* ................................................ 43
   2.7.2 Construction of *P. aeruginosa K767 ΔPA4596* strains ................................ 44
2.8 Complementation of gene deletions .................................................................... 45
   2.8.1 Complementation of nfxB mutants ............................................................... 45
   2.8.2 Complementation of PA4596 deletion strains ............................................. 46
2.9 Antimicrobial susceptibility testing ...................................................................... 51
2.10 LexA 2-hybrid experiments ............................................................................... 51
2.11 Assessment of gene expression using RT-PCR .................................................. 53
   2.11.1 RNA isolation .............................................................................................. 53
   2.11.2 RT-PCR .................................................................................................... 53

Chapter 3 : Results .................................................................................................... 54
3.1 Influence of NfxB on mexCD-oprJ expression ..................................................... 54
3.2 Influence of chlorhexidine on PA4596 expression ............................................. 58
3.3 Influence of PA4596 on mexCD-oprJ expression in wild-type *P. aeruginosa* .... 61
3.4 Influence of PA4596 on mexCD-oprJ expression in *P. aeruginosa nfxB* mutants .......................................................... 68
3.5 Influence of NfxB on PA4596 expression ............................................................ 79
3.6 Homodimerization of NfxB and its importance for repressor activity ............... 79
3.7 Homodimerization of PA4596 and the interaction between PA4596 and NfxB .... 84

Chapter 4 : Discussion ................................................................................................ 92
4.1 Determination of the nfxB gene .......................................................................... 92

vi
4.2 A novel mutation causing \textit{mexCD-oprJ} hyperexpression........................................... 93
4.3 Instability of antimicrobial resistance in the K767 \textit{nfxB} deletion strain ...................... 94
4.4 Role of PA4596 in \textit{mexCD-oprJ} expression under wild-type conditions ....................... 95
4.5 Role of PA4596 in \textit{mexCD-oprJ} expression during the NfxB-phenotype ....................... 97
4.6 PA4596 and NfxB may functionally interact............................................................... 98
Chapter 5 : Conclusions ..................................................................................................... 101
References ......................................................................................................................... 102
List of Figures

Figure 1. Organization of antibiotic efflux pumps in Gram-negative bacteria. ......................... 8
Figure 2. Gene organization of clinically relevant RND multidrug efflux transporters in P. aeruginosa. ........................................................................................................................................ 16
Figure 3. 5’ region of nfxB-containing DNA fragments ......................................................... 47
Figure 4. 5’ region of PA4596-containing DNA fragment ..................................................... 49
Figure 5. Impact of nfxB on mexCD-oprJ expression in P. aeruginosa. ............................... 56
Figure 6. Impact of chlorhexidine and AlgU on PA4596 expression in P. aeruginosa ........... 59
Figure 7. Impact of chlorhexidine and PA4596 on mexCD-oprJ expression in P. aeruginosa .... 63
Figure 8. Impact of cloned PA4596 on mexCD-oprJ and PA4596 expression ....................... 69
Figure 9. Expression of PA4596 from vector pACP07. ....................................................... 71
Figure 10. Impact of nfxB and PA4596 on mexCD-oprJ expression in P. aeruginosa .......... 75
Figure 11. Impact of nfxB on PA4596 expression in K767-derivative P. aeruginosa ............ 80
Figure 12. Impact of PA4596 on nfxB expression in P. aeruginosa ........................................ 82
Figure 13. Self-association of NfxB and PA4596 ................................................................. 85
Figure 14. Self-association of NfxB_{H87R}. ............................................................................ 87
Figure 15. Interaction between NfxB and PA4596 ................................................................. 90
List of Tables

Table 1. Strains used in this study ........................................................................................................... 34
Table 2. Plasmids used in this study ........................................................................................................ 35
Table 3. Antibiotic concentrations used to maintain plasmids and strains ........................................... 36
Table 4. PCR primers ................................................................................................................................ 39
Table 5. Impact of cloned nfxB on susceptibilities to antimicrobials and biocides effluxed by MexCD-OprJ ........................................................................................................................................... 55
Table 6. Impact of cloned nfxB on susceptibilities to antimicrobials and biocides effluxed by MexCD-OprJ ........................................................................................................................................... 62
Table 7. Impact of cloned PA4596 on susceptibilities to antimicrobials and biocides effluxed by MexCD-OprJ ........................................................................................................................................... 66
Table 8. Antimicrobial susceptibility of stains isolated from pACP07 electroporation .................... 67
Table 9. Impact of cloned PA4596 on susceptibility to antimicrobials effluxed by MexCD-OprJ73 Table 10. Impact of PA4596 the nfxB strain K385 and the NfxB-like mexCD-oprJ hyperexpressor K1536........................................................................................................................................... 77
Table 11. Impact of cloned PA4596 on susceptibilities to antimicrobials and biocides effluxed by MexCD-OprJ ........................................................................................................................................... 78
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>Ap</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
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<td>Cb</td>
<td>carbenicillin</td>
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<td>chlorhexidine</td>
</tr>
<tr>
<td>Cm</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>CTP</td>
<td>cytosine 5’-triphosphate</td>
</tr>
<tr>
<td>DHPPP</td>
<td>7,8 dihydro-6-hydroxymethylpterin-pyrophosphate</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
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<td>gentamicin</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTTR</td>
<td>LyrR-type transcriptional regulators</td>
</tr>
<tr>
<td>MATE</td>
<td>multidrug and toxic compound extrusion</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug resistance</td>
</tr>
<tr>
<td>MF</td>
<td>major facilitator</td>
</tr>
<tr>
<td>MFP</td>
<td>membrane fusion protein</td>
</tr>
<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>Nal</td>
<td>nalidixic acid</td>
</tr>
<tr>
<td>Nor</td>
<td>norfloxacin</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OMP</td>
<td>outer membrane protein</td>
</tr>
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<td>ONPG</td>
<td>o-nitrophenyl-β-D-galactopyranoside</td>
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<td>PABA</td>
<td>para-aminobenzoic acid</td>
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<td>PBP</td>
<td>penicillin binding protein</td>
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<tr>
<td>PCP</td>
<td>pentachlorophenol</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PMF</td>
<td>proton motive force</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAP</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>RND</td>
<td>resistance-nodulation-division</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal RNA</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SMR</td>
<td>small multidrug resistance</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate-EDTA</td>
</tr>
<tr>
<td>Tc</td>
<td>tetracycline</td>
</tr>
<tr>
<td>TTP</td>
<td>thymidine 5'-triphosphate</td>
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Chapter 1: Introduction

1.1 Pseudomonas aeruginosa

*Pseudomonas aeruginosa* is a Gram-negative opportunistic human pathogen. It is known to cause a variety of community-acquired infections such as ulcerative keratitis, otitis externa, and skin infections (252). Additionally, *P. aeruginosa* is known to be an important cause of nosocomial infections especially in relation to serious burns, surgery and immunocompromised patients (210). In fact, *P. aeruginosa* was deemed the causative agent of 11 to 13.8% of nosocomial infections when a microbiological isolate is identifiable (49). It was further demonstrated to be the leading Gram-negative cause of hospital acquired pneumonia, second only to the Gram-positive organism *Staphylococcus aureus* (65, 106). In infections following serious burns, *P. aeruginosa* was found to be the causative agent in 57% of the cases (53). Additionally, *P. aeruginosa* nosocomial infections are associated with high morbidity and mortality when compared to other bacterial pathogens (176). *P. aeruginosa* also demonstrates a high incidence of infection in cystic fibrosis patients, where by the age of three, 97.5% of cystic fibrosis patients demonstrate evidence of being infected (25). This is complicated by the increasing incidence of epidemic multi-resistant strains of *P. aeruginosa* that infect cystic fibrosis patients (59).

The pathogenic potential of *P. aeruginosa* can be explained by the presence of several virulence factors. These include motility structures such flagella and pili, which have additional roles as adhesins and assist in tethering the microbe to epithelial cells during infection (58, 68, 102, 123, 147). Both pili dependent adhesion and twitching motility are considered critical to *P. aeruginosa* virulence (73, 76, 243). Lipopolysaccharide (LPS) is an essential component of the *P. aeruginosa* outer membrane and like flagella and pili, it can act as an adhesin (73).
Additionally, LPS is a bacterial endotoxin and an activator of the immune system (102). Following adherence, *P. aeruginosa* secretes several exotoxin effectors into host cells via a type III secretion system, which improves invasiveness during infection and helps to disseminate the microbe throughout the host (113). *P. aeruginosa* also secretes a wide variety of other virulence factors into the extracellular environment (8, 14, 74, 132, 134, 149, 155, 262). Additionally, this organism can secrete alginate and is able to form biofilms, which protect the bacteria from environmental stressors, inhibit phagocytosis, and prevent microbial clearance (199, 212). In fact, the impact of the *P. aeruginosa* virulence factors is so profound that new treatment approaches are being designed to counter their effects (253).

*P. aeruginosa* has often been observed to display considerable resistance to multiple antibiotics (48, 190). In a recent report surveying 314 isolates of *P. aeruginosa*, the majority were found to be resistant when treated with ampicillin alone (98.4%), ampicillin/sulbactam combinations (85.3%), or ampicillin supplemented with the β-lactamase inhibitor clavulanic acid (83.8%) (100). Additionally, widespread resistance was observed against the fluoroquinolone antibiotic ofloxacin (68.4%). These *P. aeruginosa* strains demonstrating multidrug resistance (MDR) statistically have four times the mortality rate, ten times the incidence of secondary bacteremia, and triple the duration of hospital stay, which substantially increases medical costs associated with this organism (31). A major contributor to this MDR is the Gram-negative envelope, which acts as a barrier that prevents many antimicrobials from entering the cell (11, 114). Furthermore, this barrier activity is supplemented by efflux pumps that export a variety of structurally unrelated antimicrobials from the bacterial cell, thereby preventing their accumulation to toxic levels (184).
1.2 Antimicrobials and their mechanisms of action

Antimicrobial agents are compounds that inhibit or disrupt essential cellular processes in order to prevent microbial growth (bacteriostatic) or to kill the microorganism (bactericidal). The targets of these compounds are often strongly conserved in bacteria but absent or unreachable in their mammalian hosts, thus allowing the specific destruction of pathogens. These targets include essential metabolic pathways, cell wall synthesis, DNA synthesis, RNA synthesis, and protein synthesis (169).

Drugs that target the cell wall typically do so by interfering with peptidoglycan synthesis. Peptidoglycan is essential to the structural integrity of the cell wall and its disruption allows the cell to succumb to osmotic lysis. The major class of drugs that impair peptidoglycan synthesis are β-lactams and include penicillins, monobactams, cephalosporins, and carbapenems (20, 56, 96, 156). β-lactams interfere with peptidoglycan binding proteins (PBP) and prevent them from cross-linking peptidoglycan via a transpeptidation reaction (28, 247, 271). Furthermore, β-lactams activate cell wall hydrolases and autolysins, which further degrade peptidoglycan. Glycopeptide antibiotics such as vancomycin also inhibit peptidoglycan synthesis by binding the terminal D-alanine residue and preventing further polymerization of the peptidoglycan strand (19, 248).

Protein synthesis is interrupted by several classes of antimicrobials including macrolides, aminoglycosides, tetracyclines, and chloramphenicol that act to abrogate bacterial ribosome activity (169). The 70S bacterial ribosome is composed of a 50S component and a 30S component and is structurally differentiable from the 80S eukaryotic ribosome. Macrolides and
chloramphenicol are known to bind to and interfere with the 50S subunit (60), whereas aminoglycosides and tetracyclines interfere with the 30S subunit (89, 218).

DNA replication is inhibited in microbes by a diverse class of antimicrobials called quinolones. Quinolones and their widely used derivatives, fluoroquinolones, inhibit DNA replication by binding to the enzymes DNA gyrase in Gram-negative organisms or DNA topoisomerase IV in Gram-positive organisms (12, 50, 86). These enzymes cleave and rejoin the DNA backbone to introduce negative supercoils into DNA, which disperses positive twists that accumulate owing to helicase-mediated separation of the DNA strands during replication (50). Quinolones interfere with this process by stabilizing the DNA-gyrase or the DNA-topoisomerase IV interaction post DNA cleavage and preventing the rejoicing of the DNA backbone, eventually leading to an accumulation of DNA breaks and the death of the microorganism (87).

Transcription is inhibited by inhibition of the DNA-dependent RNA polymerase (RNAP) found in prokaryotes by a structurally diverse class of drugs with examples such as rifampicin and streptolydigin (35, 255). It is, as of yet, unclear how rifampicin blocks transcription; however, it is theorized that by binding to RNAP these drugs occlude the channel by which the RNA transcript exits, thereby halting further transcription (29, 255).

Folate is an essential precursor of nucleic acid synthesis. Unlike humans who obtain folate through dietary intake, bacteria usually synthesize it from p-aminobenzoic acid (PABA) and 7,8 dihydro-6-hydroxymethylpterin-pyrophosphate (DHPPP) to form dihydropteroic acid. This reaction is catalyzed by the enzyme dihydropteroate synthetase; however, sulfonamide antimicrobials are able to competitively inhibit this enzyme because of their inherent structural similarity to PABA (228). Often sulfonamide drugs are administered with trimethoprim, a
structural analogue of dihydrofolic acid, which interferes with a later step in the folate synthesis pathway by inhibiting the enzyme dihydrofolate reductase (23, 203).

1.3 Mechanisms of antimicrobial resistance

While it was once suggested that the evolution of antibiotic resistance is solely a consequence of antibiotic usage, it is now believed that antibiotic resistance mechanisms originated naturally in the environment and that their prevalence have increased dramatically in the last century due to considerable exposure to and selection by prescribed antimicrobials (43, 54, 78, 135, 177). Compounded with the multiplicity of horizontal gene transfer mechanisms utilized by bacteria as well as random mutation accumulation, antimicrobial resistance has become a widespread problem in medicine (77, 115, 221). Resistance usually occurs by one of three general mechanisms: antimicrobial target site modification, enzymatic inactivation of the antimicrobial, or exclusion of the antimicrobial from the cell via efflux (187).

1.3.1 Target Site Modification

Antimicrobial drugs target unique bacterial components not found in their human hosts, which allows selective toxicity against pathogens. Additionally, these bacterial components are essential for proper cellular function, and thus evasion of antimicrobial toxicity cannot occur through dispensing with these targets entirely; however, mutations in or enzymatic modification of the targeted bacterial component can substantially decrease drug efficacy while retaining cellular function (111). For example, PBP2a, an alternative PBP, demonstrates very little affinity
for β-lactams in methicillin-resistant *S. aureus* (124, 271). Resistance to macrolides, ketolides, and chloramphenicol has been demonstrated in strains that methylate the peptidyl transferase centre of the 23S ribosomal RNA moiety by a variety of methyl transferases, which protect the ribosome by sterically hindering the binding of these antibiotics (60, 99, 133, 204). Both methylase-dependent modification and some mutations of the 16S subunit render aminoglycoside resistance (180, 200). Amino acid substitutions in topoisomerase IV and DNA gyrase have been found to considerably reduce binding affinity of quinolone antibiotics and confer resistance (46, 85, 86, 168, 268).

### 1.3.2 Inactivation

Antimicrobial inactivation is another widespread resistance mechanism, where enzymatic modification or destruction of the antimicrobial abrogates its activity (264). The most well known and earliest observed example of this mechanism concerns β-lactamases and their ability to cleave β-lactam antimicrobials (1, 26, 28, 209). These enzymes are believed to have evolved from PBPs through the addition of hydrolytic machinery to their active sites, and they inactivate β-lactams by opening the β-lactam ring (140, 264). Resistance to tetracycline and tigecycline can be achieved through expression of TetX, a monooxygenase capable of oxygen-dependent destruction of these antimicrobials (157, 231, 266). Most clinical aminoglycoside resistance occurs through enzymatic modification of the drug. This is carried out by a variety of enzymes, which modify the aminoglycosides through either O-phosphorylation, O-adenylylation, or N-acetylation so they are unable to bind to the ribosome (224, 254, 265). Likewise, acetylation of chloramphenicol by chloramphenicol acetyltransferases prevents their binding to the ribosome.
(225), whereas modification of quinolones by the enzyme acetyltransferase Aac(6)-Ib-cr prevents their binding to topoisomerases (137).

1.3.3 Efflux

Most bacterial organisms must protect themselves from a variety of naturally occurring noxious compounds such as the products of reactive oxygen species action (270). A largely impermeable double membrane prevents or slows the entry of many of these compounds (114). Additionally, this impermeability is supplemented in many cases by efflux pumps, which export a variety of compounds (196). In addition to providing protection from noxious compounds, efflux pumps have been proposed to have roles in detoxification of intracellular metabolites, host pathogenesis, and intercellular communication (54, 136, 170). Consequently, the role of efflux in clinical antimicrobial resistance may only be a fortuitous result of the intrinsic presence of efflux pumps and have little to do with their naturally evolved roles. Whatever their intended function, efflux pumps play an important role in antibiotic resistance. An efflux pump may be specific for a single antimicrobial or be able to recognize and export a wide range of often structurally unrelated antimicrobials (196, 206). Efflux pumps that are able to pump a variety of compounds are most often associated with MDR. There consists of five different families of efflux pumps found in bacteria including the major facilitator (MF) superfamily, the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (185) (Figure 1). Other than the ABC family, which is powered by ATP hydrolysis, these pumps are secondary efflux
Figure 1. Organization of antibiotic efflux pumps in Gram-negative bacteria.

Structure of the five families of multidrug efflux pumps found in Gram-negative bacteria including major facilitator (MF), ATP-binding cassette (ABC), resistance-nodulation-division (RND), small multidrug resistance (SMR), and multidrug and toxic compound extrusion (MATE). The tripartite multidrug efflux systems also include an outer membrane protein (OMP) and a membrane fusion protein (MFP). The single component multidrug drug efflux pumps are associated with a porin.
systems usually driven by the proton motive force (PMF) (196). Of all the efflux families mentioned, the RND family is thought to be the most important in conferring resistance to the large range of clinically-relevant antimicrobials (188).

1.4 Non-RND type efflux systems

The three efflux families MF, SMR, and MATE are usually single component and located in the inner membrane of Gram-negative bacteria. Alternatively, some pumps of the MF and ABC families are tripartite and also include an outer membrane component situated in the outer membrane connected to the inner membrane component by a periplasmic membrane fusion protein. This allows the export of compounds across the entire envelope in Gram-negative bacteria. Examples include the MF pump EmrAB-TolC and the ABC pump MacAB-TolC (103, 125). In Gram-negative bacteria, the MF, SMR, and MATE families’ single component pumps are typically associated with an outer membrane porin so that compounds that are extruded into the periplasm can be shuttled through the outer membrane (185), whereas in Gram-positive bacteria the absence of the outer membrane allows substrates to be pumped directly out of the cell (182).

The MF superfamily of transporters is the largest and most diverse family of secondary transporters and accounts for 25% of all transporters found in prokaryotes (213). As mentioned above, these pumps are dependent on the PMF. Individual members of the MF superfamily generally demonstrate tight substrate specificity; however, because of the overall multiplicity of this superfamily, MF efflux pumps are responsible for exporting an extensive variety of substrates (112). The Tet family is one of the most well known examples of the MF superfamily and is
responsible for providing resistance to tetracycline and tetracycline-related compounds via efflux (27). The Tet family demonstrates considerable horizontal transfer because most tet genes are found on mobile genetic elements (205). There are 23 different tet genes that are currently recognized to code for efflux pumps including: Tet A-E, G, H, I, J, Z, which are found in Gram-negative prokaryotes, and TetK and TetL, which are found mostly in Gram-positive prokaryotes (27, 185, 205). The Gram-negative Tet genes are typically regulated by a TetR repressor where tetracycline exposure results in derepression (185, 207). Gram-positive Tet genes, in contrast, are regulated by a translational attenuation mechanism where inducing conditions shift translation to occur at a second ribosome binding site and allow for translation of the Tet protein (207). Although rare, there exist MDR MF efflux pumps such as EmrAB-TolC (125, 127, 242).

The SMR family is a member of the much larger drug/metabolite transporter superfamily and is dependent on the PMF (178, 196). The SMR family has been shown to impart resistance to several classes of antibiotics including β-lactams, cephalosporins, dihydrofolate inhibitors, aminoglycosides, as well as a variety of quaternary ammonium compounds and lipophilic cations. Furthermore, this family is able to spread through horizontal transfer because genes encoding SMR members have been identified on plasmids and transposable elements (17). There are three SMR subfamilies; however, only two, small multidrug pumps (SMP) and paired SMR (PSMR) pumps, are associated with MDR (39). The SMP subclass of SMR has been identified in both Gram-positive and Gram-negative organisms. One of the earliest described examples of the SMP family in Gram-positive organisms is the S. aureus gene smr. It codes for an efflux pump that provides resistance to phosphonium derivatives and quaternary ammonium compounds such as the biocide benzalkonium chloride (72). An example of SMP in Gram-negative prokaryotes is EmrE, which is able to efflux several quaternary amines (267). The increasingly apparent, novel
subset of the SMR family known as PSMR requires two SMR gene homologues to be expressed in order to create the resistance phenotype such as EbrA and EbrB of *Bacillus subtilis* (90, 172). Surprisingly, these homologous copies have always been found to be separated by at least two other genes and found in separate loci from other SMR efflux genes (17, 172).

The MATE family is another class of transporters associated with antimicrobial resistance and has been reported in all domains of life (21). Efflux pumps associated with this family, including NorM from *Neisseria gonorrhoeae* and YdhE from *Escherichia coli*, have been shown to export fluoroquinolones, novobiocin, and several different quaternary ammonium antimicrobials such as acriflavine (128, 162). In contrast to the other PMF-dependent efflux families discussed, several pumps in the MATE family use a Na\(^+\) gradient instead of the PMF-associated proton gradient to export substrates (162).

The ABC superfamily of efflux pumps differs from the other families in that it utilizes ATP to drive substrate export (80, 196). Although ABC efflux pumps usually export a variety of substrates, ABC transporters associated with antimicrobial resistance are uncommon and typically export and provide resistance to only one antimicrobial (118, 185, 196). However, there do exist a few examples where ABC-type efflux pumps confer MDR such as LmrA in the Gram-positive organism *Lactococcus lactis* (57, 183) or the MacAB-TolC efflux system, capable of exporting macrolides, in the Gram-negative organism *E. coli* (103).

Multidrug efflux pumps play an especially important role in the development of clinical antimicrobial resistance. The wide spectra of antimicrobials, often structurally unrelated, effluxed by a single pump means that selection of these multidrug exporters can impart resistance to several classes of antibiotics, even non-selecting antibiotics (181). It is then crucial that
microbial exposure to antimicrobials is minimized to prevent selection of multidrug efflux pumps.

1.5 RND type efflux systems

Compared to the other classes of efflux pumps mentioned, the RND superfamily is most associated with antibiotic efflux and antimicrobial resistance in terms of distribution, breadth of clinically-related antimicrobial substrate profile, and impact on minimum inhibitory concentrations of antimicrobials (171). While their presence has been observed in eukaryotes and prokaryotes, their role as antibiotic resistance determinants has largely been confined to Gram-negative bacteria (189). Among this family of efflux pumps, AcrAB-TolC, broadly represented in Enterobacteriaceae, was the first secondary efflux system whose structure has been resolved, and which is, perhaps, one of the best understood multidrug efflux systems (7, 16, 166, 195). The structure of RND efflux pumps is typically tripartite including an inner membrane RND component, which is connected to an outer membrane protein (OMP) component by a membrane fusion protein (MFP). These are exemplified by AcrB, TolC, and AcrA, respectively, in E. coli (165, 195). Crystal structure determinations have demonstrated that AcrB exists as a homotrimer across the inner membrane (126). A three-step cycle is used for transporting substrates through this component where each transition requires a proton to be transferred across AcrB. In the access state, substrates may bind to AcrB. Upon conversion to the binding state a vestibule pocket is exposed and allows the substrate to bind deeper in the pump. In the third step, the substrate is extruded into the central pore to continue through the rest of the pump (165, 222). The periplasmic side of AcrB is proposed to open like a funnel where it can directly interact with
TolC (166, 241). TolC functions as the outer membrane component of the RND efflux system in \textit{E. coli} and, like AcrB, is assembled as a homotrimer inserted in the membrane. The periplasmic opening of the TolC trimer, which connects to AcrB, normally maintains a tightly closed configuration; however, upon substrate reception, helical coils around the opening relax and allow pore dilation and substrate entry (108). The AcrA MFP component completes the efflux pump by surrounding, stabilizing, and sealing the AcrB-TolC linkage (240, 246). The genes of RND efflux systems in Gram-negative bacteria are usually organized into operons with a predictable conserved organization consisting of the MFP coding gene preceding the RND component gene; often the OMP component gene is included as the last component in the efflux operon, although in some cases such as the described AcrAB-TolC example, it may be found elsewhere in the genome (24, 75, 130, 191, 220, 236).

The structure of the multidrug efflux pump of \textit{P. aeruginosa} MexAB-OprM has been rigorously characterized (4, 5, 223). Upon comparison to AcrAB-TolC, many strong similarities have been identified. All three components of MexAB-OprM have had their crystal structures resolved. The RND component, MexB, forms a homotrimer in the cellular membrane and adopts three conformational states synonymous with the transport process observed with AcrB (223). The crystal structure of the OMP component, OprM, also shows substantial similarities to TolC forming a homotrimeric outer membrane porin for unidirectional substrate transport (5). Structural studies of the MFP MexA have revealed that it acts as the MFP between MexB and OprM (4).
1.6 Characterization of \textit{P. aeruginosa} RND multidrug efflux pumps

Although the first four major RND efflux pumps were discovered as determinants of antimicrobial resistance, the remaining RND type efflux pumps in \textit{P. aeruginosa}, 12 putative, were discovered as a result of the whole genome sequencing of \textit{P. aeruginosa} PA01 (63, 81, 154, 201, 236). To date ten RND efflux pumps in \textit{P. aeruginosa} have been characterized including: MexAB-OprM (121, 191), MexCD-OprJ (193), MexEF-OprN (105), MexXY (154, 260), MexJK (36, 37), MexGHI-OpmD (2), MexVW (122), MexPQ-OpmE (153), MexMN(153), TriABC (152). Of the ten efflux systems characterized, efflux mediated antimicrobial resistance in \textit{P. aeruginosa} can be predominantly attributed to the presence of MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY (184), whose operons can be seen in Figure 2. All efflux pumps mentioned above that do not code for an OMP component gene in their operon have been found to utilize the constitutively expressed OprM with the exceptions of MexJK, which can also associate with OpmH, and TriABC, which to date has been shown only to associate with OpmH (37, 152).

1.6.1 MexAB-OprM

The first characterized multidrug efflux pump in \textit{P. aeruginosa}, MexAB-OprM, as described above consists of the RND component MexB, the MFP MexA, and the OMP OprM, all encoded by a single operon (236). MexAB-OprM hyperexpressing MDR strains have been observed amongst clinical isolates and laboratory isolates, and shown to carry a mutation in one of the following repressors: \textit{mexR} (previously \textit{nalB}), \textit{nalC}, or \textit{nalD} (30, 160, 230, 232).
Figure 2. Gene organization of clinically relevant RND multidrug efflux transporters in *P. aeruginosa*.

*P. aeruginosa* RND efflux pump genes are organized to encode the membrane fusion protein component (red), the RND component (blue), the outer membrane protein component (yellow), and the associated regulatory proteins (green).
MexAB-OprM, is considered to be one of the main determinants of intrinsic antimicrobial resistance, not only because it is the only constitutively expressed multidrug efflux pump in *P. aeruginosa*, but also because of its extensive substrate profile, which includes many structurally-unrelated and clinically-relevant antimicrobials (121, 145). MexB is known to determine substrate specificity; yet, it is unclear which residues are involved in determining selectivity (150, 167). The MexAB-OprM pump has been demonstrated to export a variety of antimicrobial compounds including but not limited to: β-lactams, chloramphenicol, fluoroquinolones, tetracycline, macrolides, sodium dodecyl sulfate, and ethidium bromide (121, 189, 234).

1.6.2 MexEF-OprN

In contrast to MexAB-OprM, MexEF-OprN is quiescent under normal laboratory conditions; however, it was found to be expressed in *mexT* (105, 142), *mexS* (229), or *mvaT* (261) mutant strains or if MexAB-OprM activity is impaired (119). *mexT* strains have also been derived as clinical isolates (63). In this efflux pump, MexE, MexF, and OprN function as the MFP, RND, and OMP components, respectively (105). The MexEF complex has been shown to determine substrate specificity (138). MexEF-OprN effluxes chloramphenicol, fluoroquinolones, trimethoprim, and aromatic hydrocarbons (138, 189).
1.6.3 MexXY

MexXY is not expressed under normal laboratory conditions but may be upregulated in mexZ or mexAB mutants (3, 119). As an operon, mexX codes for the MFP and mexY codes for the RND component. However, MexXY is the only major clinically-relevant RND multidrug efflux pump in *P. aeruginosa* that does not code for its own OMP component; instead, it utilizes the constitutively expressed OMP of MexAB, OprM (154). It has been suggested that MexXY may also be able to interact with other OMP components to form pumps, although it is unclear how much impact, if any, this has on MexXY functionality (93).

Loss of MexXY increases strain sensitivity to aminoglycosides, erythromycin, tetracyclines, the tetracycline-related glycylcyclines, and fluoroquinolones (3, 44, 84, 144); interestingly, all of these agents, with the exception of fluoroquinolones, induce mexXY expression (144). MexXY is essential in adaptive resistance to aminoglycosides where continuous exposure of a wild-type *P. aeruginosa* to sub-lethal concentrations of aminoglycosides results in cells that are more refractory to the antimicrobial. However, adaptive resistance is lost upon removal of the aminoglycoside (84).

1.6.4 MexCD-OprJ

MexCD-OprJ, while normally quiescent in wild-type *P. aeruginosa*, is inducible by a variety of non-antibiotic compounds such as tetraphenylphosphonium chloride, ethidium bromide, acriflavine, benzalkonium chloride and chlorhexidine, the latter two being biocides widely used in healthcare (61, 161, 163). Consequently, the healthcare associated resistance vis-à-vis biocide induction of MexCD-OprJ coupled with the frequency of *P. aeruginosa* nosocomial
infections elicits concerns regarding the treatment of this organism. Independent of induction circumstances, MexCD-OprJ hyperexpressors have been identified in the laboratory and clinically in nfxB strains (81, 92). This efflux system provides resistance to a variety of different antimicrobials including: β-lactams, fluoroquinolones, chloramphenicol, trimethoprim, sulfonamides, macrolides, tetracyclines, lincomycins, novobiocin as well as some biocides (e.g. chlorhexidine and triclosan), organic solvents, detergents, and dyes (38, 120, 145, 186, 233, 234). Earlier studies reported that exported β-lactam substrates were limited to 4th generation cephems; now refuted, the substrate profile of MexCD-OprJ also includes ordinary cephems (e.g. ceftazidime), many penicillins, carbapenems (although not imipenem), and penems (69, 143, 145, 173).

Expression of MexCD-OprJ in nfxB strains sensitized P. aeruginosa to some β-lactams, particularly carbenicillin via a concomitant reduction in MexAB-OprM expression (69); conversely, reduction in MexAB-OprM expression results in an increase, albeit smaller, in expression of MexCD-OprJ and MexEF-OprN (119). Additionally, nfxB strains are more susceptible to aminoglycosides (particularly streptomycin), because of a proposed reduction in MexXY expression that parallel the increase in mexCD-oprJ expression (145). Surprisingly, hyperexpression of MexCD-OprJ (or MexEF-OprN) has been associated with a reduction in growth rate and pathogenicity of P. aeruginosa strains likely as a result of interference with normal cellular processes (92). Considering mexCD-oprJ is not antibiotic inducible, it is possible that the intended functions of this pump is to temporarily respond to cellular stressors by effluxing cellular metabolites.
1.7 Paradigms in gene regulation

While *P. aeruginosa* is often known for its ability to cause infectious disease, the species also acts as an excellent model to examine numerous types of gene regulation systems. In fact, regulation is so critical to *P. aeruginosa* that 8.4% of its genes are involved in regulation (197). *E. coli*, in comparison, has a predicted 4.15% of its gene involved in regulation (18). *P. aeruginosa* has the incredible potential to survive and thrive in a vast array of environments in part owing to the versatility of its gene expression systems (252). While many systems of regulation were originally discovered in *E. coli*, most were later found to be present in *P. aeruginosa* (15).

Regulatory systems of gene expression are particularly diverse and complex. However, evolution has strongly favored specificity and tight control of gene expression. For example, the paucity of RNAP minimizes transcription of unnecessary genes (15). Gene regulation generally takes place at 3 different levels: transcriptional, translational, and post translational. A single gene may incorporate regulatory steps at some or all of these levels. Furthermore, a single regulator may affect several different genes allowing for a much more intricate control of gene expression (197). Some of the more common regulators of gene expression that will be discussed in this section are sigma factors (97), activators (71), and repressors (71).

1.7.1 Sigma factors

Sigma factors complex with the RNAP holoenzyme and direct the enzyme towards specific DNA recognition sequences and can assist in the separation of the DNA strands (97). The regulon for a single sigma factor can comprise of hundreds of genes, which highlights their
potential as global gene regulators (97). Most species of bacteria have a major sigma factor responsible for controlling the housekeeping functions of the cell, such as $\sigma^{70}$ in *P. aeruginosa* (88, 197). Also encoded are usually several other alternative sigma factors that upregulate additional sets of genes, are induced by specific environmental signals, and allow the cell to respond to environmental perturbations (88). They are usually responsible for creating a multifaceted, global cellular response such as responding to heat shock or directing flagella synthesis (97, 197, 258).

Anti-sigma factors act to specifically prevent their respective sigma factors from interacting with RNA polymerase and consequently downregulate genes that are targeted by the sigma factor (88). This definition excludes other competing sigma factors from being defined as anti-sigma factors because the competition is non-specific. Furthermore, an anti-sigma factor is sometimes inhibited by an anti-anti-sigma factor. Anti-anti-sigma factors have several mechanisms of inactivating the anti-sigma factor including enzymatic degradation, seen with AlgU ($\sigma^{22}$) activation in *P. aeruginosa*, or extrusion the anti-sigma factor from the cell (88, 263).

$\sigma^{E}$ is the envelope stress sigma factor in *E. coli* and is encoded by *rpoE*. It is known to have an extensive regulon, which consists of genes required for synthesis, maintenance, and repair of the cellular envelope. This includes genes encoding proteins that repair or degrade misfolded proteins such as foldases, proteases, peptidyl-prolyl-isomerases, chaperones, and thio-disulfide oxide-reductases and genes encoding enzymes that help rebuild the envelope, such as LPS biosynthesis genes (42, 202, 211).

AlgU in *P. aeruginosa* is thought to be the functional equivalent of the *E. coli* envelope stress response sigma factor $\sigma^{E}$. They have been found to share 66% amino acid sequence
identity (45, 236). The operons of AlgU, _algUmucABCD_, and _σ^E_, _rpoErseABC_, are known to be strikingly similar both in terms of the roles and the layout of the included genes (263). Perhaps most convincing of all, both cloned AlgU or _σ^E_ are functionally interchangeable (269).

The structure of the AlgU operon reveals another aspect of regulation. AlgU encoded by _algU_ (also called _algT_) can activate its own expression in the _algUmucABCD_ operon. However, the induction of the operon via AlgU also causes increased production of MucA, the AlgU anti-sigma factor, which creates negative feedback loop and helps prevent overactivation of AlgU (263).

### 1.7.2 Activators

Transcriptional activators increase gene expression by helping RNAP bind to the promoter via a mechanism known as recruitment (71, 258). In this mechanism, the activator binds to both a specific DNA sequence at the promoter and either the α or σ subunit of RNAP (71). As such, the RNAP complex is stabilized at the promoter and transcription is increased.

Activators can also increase transcription via allostery where the activator has no effect on the recruiting of RNAP and instead is required to aid the creation of an open complex in which the gene can be transcribed (71, 258). This mechanism can be further subdivided into activators that cause a conformational change in RNAP, like NtrC (71), and activators that cause a conformation change in the DNA, like MerR (22). In _P. aeruginosa_, NtrC forms an oligomeric complex with ATPase activity upstream from the promoter site. This ATPase causes a conformation change in RNAP, which allows it to melt the DNA strands at the transcription start site and initiate transcription (71, 258). The MerR family of transcriptional activators was
originally discovered in *P. aeruginosa* (235). MerR, when bound to both Hg$^{2+}$ and the mer*T* promoter, twists the DNA into a conformation in which RNAP can strongly initiate transcription (22, 235, 258).

### 1.7.3 Repressors

Repressors typically act to suppress gene transcription by binding to a specific operator sequence in the gene’s promoter and obstructing RNAP or a transcriptional activator from binding (71, 258). An additional, yet less common mechanism of repressors functions by locking the stable RNAP at the promoter start site and preventing promoter clearance as exemplified by p4 repression of the A2c promoter in *B. subtilis* (208). Repressors can be countered either by downregulating repressor expression or by sequestering the repressor with another protein. Repressors are often found to belong to one of several prototypical families.

The MarA family is known to regulate a diverse array of biological processes including antibiotic resistance, antimicrobial agents synthesis, aromatic compound sensing, and virulence (51, 64, 66, 245). Widely distributed in nature, MarA-like repressors are structurally similar, yet demonstrate considerable diversity at the amino acid level (6). This is thought to allow this family to interact with a variety of signal molecules and DNA targets (51).

As of 2004, the TetR family was represented by 2353 putative members. Only 85 have clearly defined functions, which include regulation of several antimicrobial resistance determinants, antimicrobial agent synthesis, catabolic pathway enzymes, osmotic stress responses, and virulence (32, 109, 110, 129, 198, 257). TetR-like regulators display a highly
conserved helix-turn-helix domain that is capable of binding to palindromic DNA recognition sequences in the operator during repressor homodimerization (198).

The regulator LysR is a transcriptional activator of lysA (encoding a diaminopimelate decarboxylase) in E. coli (237). Since then, LysR-type transcriptional regulators (LTTR) represent the most widespread transcriptional regulator class of prokaryotes with orthologues extending into eukaryotes and archaea and encompass both activators and repressors (131, 179, 239). LTTRs are often used during negative feedback in metabolic pathways where products of the pathway act as co-inducers necessary for transcriptional activation or repression by interacting with LTTRs (131, 216, 251). Furthermore, LTTRs are known to be important in a diverse set of cellular processes such as quorum sensing and oxidative stress responses (101, 249).

The Lac repressor system initially identified for its importance in efficiently regulating metabolic pathways especially with regard to diauxic growth, now acts as a template for a much larger family of LacI-like repressors (116, 259). Fully sequenced in 1978, the Lac repressor under non-inducing conditions binds to the target gene operator as a homotetramer (55, 117). However, interaction of an inducer with the Lac tetramer results in conformational change and greatly reduced affinity for the DNA operator (117). LacI-like regulators demonstrate considerable conservation of the helix-turn-helix domain involved in DNA binding, although otherwise vary enough that these regulators can accommodate a variety of inducer molecules (259).
1.8 Regulation of *P. aeruginosa* RND multidrug efflux pumps

1.8.1 MexAB-OprM

MexAB-OprM is the only constitutively expressed multidrug efflux pump in *P. aeruginosa* (121). Additionally, MexAB-OprM hyperexpressors have been observed in mexR, (formally *nalB*) (232), *nalC* (30), and *nalD* (160, 230) mutants or in strains that hyperexpress *armR* (30). *mexR* was found to be a MarA family repressor and is divergently expressed from *mexAB-oprM* (192). *mexAB-oprM* and *mexR* share an overlapping promoter region. MexR represses expression of both *mexR* and *mexAB-oprM* upon binding of this overlapping promoter region (141, 142, 201, 232). ArmR (PA3719) is a small protein of only 53 amino acids. ArmR overexpression caused an upregulation of *mexAB-oprM*; also upregulated was *mexR* suggesting that ArmR may function by modulating MexR repressor activity (30, 41, 232). NalC was found to be a TetR family repressor indirectly involved in regulating MexAB-OprM expression by repressing a downstream operon including *armR* (41). NalD, a TetR-like repressor, was found to repress *mexAB-oprM* by binding and blocking the *mexAB-oprM* promoter at a site distinct from the *mexR* binding site (160, 230).

1.8.2 MexEF-OprN

In contrast to MexAB-OprM, MexEF-OprN is quiescent under normal laboratory conditions; however, it was found to be expressed in NfxC strains (105, 142). The NfxC phenotype was attributed to mutations in the adjacent positive LysR-type transcriptional regulator *mexT*. *mexT* is normally encoded as an inactive form; however, certain mutations in *mexT* restore activity (104, 139). Additionally, *mexS* strains of *P. aeruginosa* were found to be
hyperexpressors of MexEF-OprN. Surprisingly, MexS is oxidoreductase/dehydrogenase homologue and in silico analysis suggests that it does not function as a regulator. Furthermore, mexS expression is activated by MexT. It has been suggested that MexS and MexEF-OprN are a dual response that is upregulated by an increase in toxic cellular metabolites and assists in their detoxification. Therefore, loss of MexS would enhance MexEF-OprN expression due to a further accumulation of toxic cellular metabolites (229). MvaT is a newly described indirect negative regulator of MexEF-OprN that acts independently of mexT and mexS (261); MvaT shows homology to H-NS regulatory proteins. Although H-NS proteins normally function to stabilize and compact DNA, they can also act as global regulators by compacting specific regions of DNA and silencing the enclosed genes (219). MexEF-OprN enhancement may occur via decompaction of the mexEF-oprN encoding DNA in the absence of MvaT (244, 261).

### 1.8.3 MexXY

MexXY, like MexEF-OprN, is not expressed under normal laboratory conditions but is derepressed in mexZ mutants or induced by ribosomal stress (3, 144, 146). Paralleled by regulation schemes seen with mexAB-oprM, mexZ is transcribed divergently from mexX and the MexZ protein product was shown to repress both mexZ and mexXY by binding to the overlapping intergenic promoter regions. MexZ was found to be a TetR-like repressor (146). Additionally, MexXY was found to be induced during impairment of ribosomal function vis-à-vis a direct interaction of ribosome-targeting antimicrobials with the ribosome (91). Induction of MexXY by ribosome-targeting antimicrobials was dependent on PA5471, a gene also upregulated in response to ribosomal-targeting antibiotics (159). However, PA5471 was not required for mexXY
derepression in the absence of MexZ indicating that PA5471 may directly or indirectly modulate MexZ activity. Interestingly, MexXY is one of few examples where agents that induce its expression (e.g. aminoglycosides, tetracyclines, and macrolides) are also substrates for efflux.

1.8.4 MexCD-OprJ

MexCD-OprJ is not expressed in wild-type *P. aeruginosa* but is induced by membrane-damaging agents (MDAs; e.g. chlorhexidine) (163) and is hyperexpressed in *nfxB* mutants. Such mutants (e.g. *P. aeruginosa* strain K385) carry a mutation in *nfxB* that encodes a repressor of *mexCD-oprJ* expression (174, 193, 226). NfxB was originally described as a LysR family regulator, although it is more likely that NfxB belongs to the LacI family of transcriptional regulators considering strong sequence homology (174, 226). Similar to regulation paradigms seen in *mexAB-oprM* and *mexXY*, *nfxB* is divergently transcribed from the MFP component, and its protein product binds to and represses overlapping promoter regions of *nfxB* and *mexCD-oprJ* (226). Electrophoretic mobility shifts assays (EMSAs) and DNase I protection assays suggest that not only does NfxB bind to the promoter regions of *nfxB* and *mexCD-oprJ*, it does so at two distinct sites possibly as a homodimer (226).

Agents that are known to cause membrane stress through disruption of cellular membranes (e.g. biocides) have been demonstrated to induce expression of *mexCD-oprJ* and result in the selection of mutants that stably hyperexpress this pump (38, 163). Moreover, MexCD-OprJ has been shown to provide resistance against biocides (e.g. triclosan, chlorhexidine) (38, 61). *mexCD-oprJ* induction in response to MDAs is mediated by the
envelope stress response sigma factor AlgU (61). However at this point, it is unclear mechanistically how membrane stress causes an induction of *mexCD-oprJ*.

PA4596 is encoded by a gene located adjacent to *oprJ* in both *P. aeruginosa* and *Pseudomonas putida*, and the PA4596 protein product has 61% identity with NfxB (236). Furthermore, RNA microarray data indicated that PA4596 may be upregulated by the membrane stressing biocide chlorhexidine in an AlgU dependent manner (D. Daigle, C. Dean; personal communication). Altogether, these observations suggest that PA4596 may have an important but as of yet undetermined role in the regulation of *mexCD-oprJ*.

1.9 Natural role of multidrug efflux pumps

*P. aeruginosa*, a fairly ubiquitous microbe, is exposed to noxious or antibiotic compounds synthesized by other organisms especially in highly competitive environments like soil. Mass production of penicillin, the first clinically used antimicrobial, began in 1944 (40). Therefore, only in the last 70 years have antibiotics been manufactured on a large enough scale to allow clinical selection for antimicrobial resistance. Moreover, *P. aeruginosa* isolates collected before the development of quinolone antibiotics are capable of extruding these drugs (9). Consequently, clinical antimicrobial exposure does not merit the diversity and complexity of multidrug efflux pumps produced by *P. aeruginosa*. More plausible is that these multidrug efflux pumps, while they do promote antimicrobial resistance, have additional natural functions. It is interesting, therefore, that microbes with the highest number of MDR efflux pumps are usually found in bacteria living in the soil or associated with plants where they have to resist a particularly wide variety of external stressors (107).
1.9.1 Natural roles of MexAB-OprM, MexEF-OprN, and MexXY

Pentachlorophenol (PCP), a well-known environmental contaminant, is known to be toxic to prokaryotes by acting as an uncoupler of oxidative phosphorylation and by disrupting the PMF (52). In response to PCP challenge, *P. aeruginosa* demonstrated a substantial increase in expression of the *mexAB-oprM* operon in parallel with a concomitant increase in *nalC* and *armR* expression, suggesting that secondary to PCP exposure some modulation of NalC activity is manifesting similarly to the *nalC* phenotype (164). Likewise *mexR* was upregulated, although this was likely a result ArmR modulation of MexR activity (41, 164). Still, MarA-like repressors such as MexR have been shown to respond to phenolic compounds, suggesting that MexR may be directly responding to PCP (238). Loss of MexAB-OprM increased sensitivity to PCP indicating that this efflux system contributes to tolerance to this energy stressor (164). MexAB-OprM has been demonstrated to provide organic solvent tolerance, likely directly through export of the solvent; however, MexAB-OprM may also have a role in exporting secondary toxic metabolites and damaged cellular components to neutralize the overall toxic effects of the organic solvent (120). Arguably, organic solvents do not occur naturally in the environment; however, many molecularly similar compounds including plant derivatives (e.g. vanillin) frequently do (256).

MexEF-OprN expression has also been associated with several non-antimicrobial signals. For example, interaction of *P. aeruginosa* for 12 hours with human airway epithelial cells (to simulate pulmonary infection circumstances) resulted in considerable increases in *mexEF-oprN* and *mexS* expression (62). MexEF-OprN is also known to be upregulated in response to oxidative stress (95). Altogether, MexEF-OprN may have a role in responding to host defenses response such as reactive oxygen species generation. Finally, it was found that MexEF-OprN hyperexpressors were selected for in the rat pneumonia model even in the absence of
antimicrobial exposure indicating that MexEF-OprN provides resistance against harmful agents generated in the rat lung environment (94).

MexXY, as discussed before, is induced by agents (e.g. antimicrobials) known to interrupt protein synthesis (91). Ribosome protection studies indicated that these agents had no direct modulating effect on the repressor MexZ binding to the mexX promoter (146). Additionally, artificial enhancement of ribosome protection mechanisms reduced the induction of MexXY in response to protein synthesis inhibitors suggesting that it is the direct inhibition of the ribosome that results in MexXY induction (91). Consequently, MexXY may have a natural function in a ribosome stress response by aiding in cells adaptive response to the adverse consequences of ribosome perturbations.

1.9.2 Natural role of MexCD-OprJ

Exposure to MDAs is known to cause upregulation of MexCD-OprJ. This is known to occur in an AlgU dependent fashion. The inactivation of algU sensitizes P. aeruginosa to high temperatures and superoxide-generating redox cycling compounds that are normally associated with envelope damage. AlgU was originally implicated in the control of alginate biosynthesis and export associated with the mucoid phenotype. However, the resistance provided by algU is not dependent on a mucoid phenotype being present (70). Furthermore, algU has been shown to be induced by membrane stress caused by heat shock and biocides (263). Altogether, it is likely that AlgU is involved in a larger role as an envelope stress response regulator.

MexCD-OprJ is likely part of the AlgU mediated envelope stress response. It may have some natural role in exporting toxic metabolites secondary to membrane stress or efflux of
host/competitor generated membrane disrupting factors. Furthermore, MexCD-OprJ may assist in adaptive membrane change by exporting old phospholipids so that new ones with different properties may be synthesized and mobilized into the membrane.

1.10 Experimental aim

Previous studies have yet to uncover what regulators are involved in allowing envelope stress to induce \textit{mexCD-oprJ} expression. The gene PA4596 has several characteristics which indicate that PA4596 may act as one of those regulators. For example, PA4596 expression was demonstrated to be influenced by envelope stress. Additionally, PA4596’s protein product has close amino acid identity (61\%) to the protein product of the known \textit{MexCD-oprJ} repressor, NfxB. Furthermore, like \textit{nfxB}, PA4596 is encoded close to the \textit{mexCD-oprJ} locus. Therefore, it is possible that PA4596 may play a role in the regulation of \textit{mexCD-oprJ} expression and may be required during \textit{mexCD-oprJ} induction during membrane stress. This study will attempt to identify what role, if any, that PA4596 plays in the regulation of \textit{mexCD-oprJ} expression.
Chapter 2: Materials and Methods

2.1 Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are summarized in Tables 1 and 2, respectively. All strains, *P. aeruginosa* and *E. coli*, were grown overnight in Luria-Bertani (LB) Broth (Difco) that contained 2 g/L NaCl. Incubation conditions consisted of 37°C and shaking at 90 rotations per minute (rpm), unless stated otherwise. All solid media consisted of LB Broth supplemented with 1.5% (w/v) Agar (Difco) and was solidified in 10 cm diameter Perti dishes (Fisherbrand). Once inoculated the LB-agar plates were incubated overnight at 37°C. Antibiotics, when required for the selection or maintenance of plasmids, were included in the growth media at concentrations listed in Table 3.

2.2 DNA isolation and visualization

Plasmid DNA was isolated from 3 ml *E. coli* overnight cultures using the alkaline lysis procedure as described by Sambrook (214) and was resuspended in 50 µl distilled water (dH₂O). For isolation of plasmid DNA to be used for sequencing, the GeneJET™ Plasmid Miniprep Kit (Fermentas) was used according to the protocol suggested by the manufacturer. Plasmid DNA was stored at -20°C.

*P. aeruginosa* chromosomal DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen) using a modified protocol. 1.5 ml of an overnight *P. aeruginosa* culture was pelleted in a microfuge tube (2 minutes, 13000 rpm). The pellet was resuspended in 180 µl of the kit buffer ATL and then combined with 20 µl of the kit Proteinase K solution, briefly vortexed,
<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or Reference</th>
</tr>
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<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td><em>thi pro hsdR recA</em> Tra&lt;sup&gt;+&lt;/sup&gt;</td>
<td>227</td>
</tr>
<tr>
<td>DH5α</td>
<td><em>Φ80d lacZΔM15 endA1 recA1 hsdR17 (rK&lt;sup&gt;-&lt;/sup&gt; mK&lt;sup&gt;+&lt;/sup&gt;) supE44 thi-1 gyrA96 relA1 F (lacZYA-argF)U196</em></td>
<td>13</td>
</tr>
<tr>
<td>SU101</td>
<td>Promoter&lt;sub&gt;sulA&lt;/sub&gt;(op+/op+)-lacZ</td>
<td>47</td>
</tr>
<tr>
<td>SU202</td>
<td>Promoter&lt;sub&gt;sulA&lt;/sub&gt;(op408/op+)-lacZ</td>
<td>47</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K767</td>
<td>PA01 prototroph</td>
<td>141</td>
</tr>
<tr>
<td>K1536</td>
<td>K767 NfxB-Phenotype, mexCD-oprI hyperexpressor</td>
<td>82</td>
</tr>
<tr>
<td>K372</td>
<td>met-90111 amiE200 rpsL pvd-9 pchR</td>
<td>79</td>
</tr>
<tr>
<td>K385</td>
<td>K372 NfxB&lt;sub&gt;H87R&lt;/sub&gt;</td>
<td>191</td>
</tr>
<tr>
<td>K2951</td>
<td>K767 ΔnfxB</td>
<td>This study</td>
</tr>
<tr>
<td>K2952</td>
<td>K767 ΔPA4596</td>
<td>This study</td>
</tr>
<tr>
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<td>K1536 ΔPA4596</td>
<td>This study</td>
</tr>
<tr>
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<td>K385 ΔPA4596</td>
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<td>K767 ΔPA4596 ΔnfxB</td>
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</tr>
<tr>
<td>K2878</td>
<td>K767 ΔPA4596</td>
<td>Aaron Campigotto</td>
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<tr>
<td>K2879</td>
<td>K1536 ΔPA4596</td>
<td>Aaron Campigotto</td>
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Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or Reference</th>
</tr>
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<tr>
<td>pEX18Tc</td>
<td>Gene Replacement Vector; oriT&lt;sup&gt;+&lt;/sup&gt; sacB Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>83</td>
</tr>
<tr>
<td>pPS856</td>
<td>Source of Gm&lt;sup&gt;+&lt;/sup&gt; cassette</td>
<td>83</td>
</tr>
<tr>
<td>pFLP2</td>
<td>Source of Flp recombinase; Ap&lt;sup&gt;+&lt;/sup&gt; Cb&lt;sup&gt;+&lt;/sup&gt;</td>
<td>83</td>
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<tr>
<td>pACP01</td>
<td>pEX18Tc::ΔnfxB</td>
<td>This study</td>
</tr>
<tr>
<td>pACP02</td>
<td>pEX18Tc::ΔPA4596</td>
<td>This study</td>
</tr>
<tr>
<td>pRK415</td>
<td>Low-copy-number, broad-host-range cloning vector; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>98</td>
</tr>
<tr>
<td>pACP03</td>
<td>pRK415::nfxB (including only downstream ATG start site)</td>
<td>This study</td>
</tr>
<tr>
<td>pACP04</td>
<td>pRK415::nfxB (including both possible ATG start sites)</td>
<td>This study</td>
</tr>
<tr>
<td>pACP05</td>
<td>pRK415::nfxB (including nfxB-mexC intergenic region)</td>
<td>This study</td>
</tr>
<tr>
<td>pNFX-1</td>
<td>pAK1900::nfxB (on a 2.2kb restriction fragment) Amp&lt;sup&gt;+&lt;/sup&gt; Cb&lt;sup&gt;+&lt;/sup&gt;</td>
<td>193</td>
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<td>pACP06</td>
<td>pAK415::PA4596 (including only downstream ATG start site)</td>
<td>This study</td>
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<tr>
<td>pACP07</td>
<td>pAK415::PA4596 (including both possible ATG start sites)</td>
<td>This study</td>
</tr>
<tr>
<td>pACP08</td>
<td>pAK415::PA4596 (including PA4596-PA4595 intergenic region)</td>
<td>This study</td>
</tr>
<tr>
<td>pMS604</td>
<td>LexA&lt;sub&gt;1-87&lt;/sub&gt;WT-Fos Zipper Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>194</td>
</tr>
<tr>
<td>pACP09</td>
<td>pMS604 LexA&lt;sub&gt;1-87&lt;/sub&gt;WT-NfxB Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
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<td>pACP10</td>
<td>pMS604 LexA&lt;sub&gt;1-87&lt;/sub&gt;WT-NfxB&lt;sub&gt;H87R&lt;/sub&gt; Tc&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pACP11</td>
<td>pMS604 LexA&lt;sub&gt;1-87&lt;/sub&gt;WT-PA4596 Tc&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
<td>pDP804</td>
<td>LexA&lt;sub&gt;1-87&lt;/sub&gt;408-Jun Zipper Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pACP12</td>
<td>pDP804 LexA&lt;sub&gt;1-87&lt;/sub&gt;408-NfxB Ap&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pMMB206</td>
<td>Broad host range low-copy number cloning vector; Cm&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pAJC02</td>
<td>pMMB206::PA4596</td>
<td>Aaron Campigotto</td>
</tr>
</tbody>
</table>

† Abbreviations: Tc<sup>+</sup>, tetracycline resistance; Gm<sup>+</sup>, Gentamicin resistance; Ap<sup>+</sup>, Ampicillin resistance; Cb<sup>+</sup>, Carbenicillin resistance; Cm<sup>+</sup>, Chloramphenicol resistance
Table 3. Antibiotic concentrations used to maintain plasmids and strains

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>10</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>10</td>
</tr>
</tbody>
</table>

*Not determined
and incubated (20 minutes, 55°C). Forty µl of 10 mg/ml RNase A was added and the solution was incubated (2 minutes, room temperature). The solution was vortexed for 15 s, subsequently supplemented with 200 µl of kit buffer AL, vigorously vortexed, and incubated (10 minutes, 70°C). Two-hundred µl of absolute ethanol was added to the solution. After mixing, the solution was transferred to a DNeasy® spin column and centrifuged (1 minute, 8000 rpm). Five-hundred µl of kit buffer AW1 was spun through the column (1 minute, 8000 rpm) followed by 500 µl of kit buffer AW2 (3 minutes, 13000 rpm). Finally, chromosomal DNA was eluted with 200 µl of kit buffer AE (1 minute, 8000 rpm) and stored at 4°C.

All DNA was visualized using agarose gel [0.8% (w/v)] electrophoresis. Gels were supplemented with 0.5 µg/mL of ethidium bromide and run in Tris-acetate-EDTA buffer (TAE; 40 mM Tris-HCl pH 8.5, 20 mM acetic acid, 1 mM EDTA) at 130 V. Either a one kb or 100 bp Generuler DNA ladder (Fermentas) was used to approximate fragment lengths.

2.3 Restriction digests and ligation of DNA fragments

Restriction endonuclease (New England Biolabs) DNA digests were carried out as specified in the manufacturer’s instructions using provided buffers. Briefly, digests were carried out in 50 µl volumes that included 30 µl of plasmid DNA prepared as described above, 1.5 µl of restriction enzyme (0.3 µl/ 10 µl reaction volume), 5 µl of the appropriate 10X enzyme buffer (10% v/v), 5 µl of 10X bovine serum albumin if required by the endonuclease, and 7 µl dH₂O. The reaction was incubated for two hours at the temperature designated by the manufacturer for that enzyme. When two enzymes needed to be used and had identical reaction conditions, both enzymes were added simultaneously to the reaction mixture. If the reaction temperatures of the
enzymes differed, the DNA was digested with the endonuclease that functioned at the lower temperature first. Subsequently, the reaction mixture was supplemented with the second endonuclease and incubated at the higher temperature. Digested DNA was purified either directly from the reaction mixture or following excision from an agarose gel electrophoresis using the Wizard SV Gel and PCR Clean-Up System (Promega).

Ligations were performed with T4 DNA ligase (New England Biolabs) prepared according to the manufacturer’s instructions. The ligation mixture contained 2 µl of the purified digested fragment, 6 µl of the purified digested vector, 2 µl of 10X T4 ligase buffer, 1 µl of T4 ligase, and 7 µl of dH₂O, and was incubated overnight at 16°C.

2.4 PCR amplification and nucleotide sequencing

Polymerase chain reaction (PCR) was used for amplification of DNA fragments. Where high fidelity was required Phusion™ DNA polymerase and associated buffers (New England Biolabs) was used in a 50 µl reaction mixture consisting of: 34.5 µl dH₂O, 10 µl of 5X Phusion Buffer HF, 1.5 µl of dimethyl sulfoxide (DMSO), 1 µl of 10mM dNTPs, 1 µl of each forward and reverse primer (Table 4), 0.5 µl of template chromosomal DNA, and 0.5 µl of Phusion™ DNA polymerase. The dNTP mixture consisted of 10 mM each of dGTP, dCTP, dTTP, and dATP (New England Biolabs). PCR was carried out in a T-gradient thermocycler (Biometra). PCR programs, unless otherwise specified, consisted of an initial denaturation step (3 minutes, 94°C) followed by 30 cycles of denaturation (1 minute, 94°C), annealing (1 minute, 55 – 72 °C depending on the primer pair), and extension (30 seconds per 1 kb of fragment length, 72°C) and concluded with a final extension step (10 minutes, 72°C). The annealing temperature for each
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2 nfxB U F BamHI</td>
<td>AACCGGATCCCTCGATCTGGAACAGCAGG</td>
<td>This study</td>
</tr>
<tr>
<td>A2 nfxB U R Gm</td>
<td>TCAGAGCGCTTTTGAAGCTAATTCGGGAATCAGGGTCATCG</td>
<td>This study</td>
</tr>
<tr>
<td>A2 nfxB D F Gm</td>
<td>AGGAACTTCAAGATCCCCCAATTCTGACCCCTGGAGCAGATGTTC</td>
<td>This study</td>
</tr>
<tr>
<td>A2 nfxB D R HindIII</td>
<td>AACAAAGCTTCAACAGGACCAGCAGAA</td>
<td>This study</td>
</tr>
<tr>
<td>Gm-F</td>
<td>CGAATTAGCTTCAAAAGCGCTCTGA</td>
<td>33</td>
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<tr>
<td>Gm-R</td>
<td>CGAATTGGGATCTTTGAAGTTCCT</td>
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</tr>
<tr>
<td>A2 PA4596 U F BamHI</td>
<td>AACCGGATCCAGGGAGATGTCCTTGAGG</td>
<td>This study</td>
</tr>
<tr>
<td>A2 PA4596 U R Gm</td>
<td>TCAGAGCGCTTTTGAAGCTAATTCGGGAATCAGGGTCATCG (This study is repeated)</td>
<td></td>
</tr>
<tr>
<td>A2 PA4596 D F Gm</td>
<td>AGGAACTTCAAGATCCCCCAATTCTGACCCCTGGAGCAGATGTTC (This study is repeated)</td>
<td></td>
</tr>
<tr>
<td>A2 PA4596 D R HindIII</td>
<td>AACAAAGCTTCAACAGGACCAGCAGA (This study is repeated)</td>
<td></td>
</tr>
<tr>
<td>Com nfxB F HindIII</td>
<td>ACTGAAGCTTACATCAGAAAACCAACCG</td>
<td>This study</td>
</tr>
<tr>
<td>Com nfxB R BamHI</td>
<td>ACTGGGATCC TCCGTGCCATGCGCGACGA</td>
<td>This study</td>
</tr>
<tr>
<td>nfxB long F HindIII</td>
<td>ACTGAAGCTTTAATTCCTTTGAGACGGA</td>
<td>This study</td>
</tr>
<tr>
<td>nfxB long R BamHI</td>
<td>GGTGGATCCCTGAATCCCAGGTGAGTGG</td>
<td>This study</td>
</tr>
<tr>
<td>nfxB igr F HindIII</td>
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<tr>
<td>Com PA4596 F HindIII</td>
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<td>This study</td>
</tr>
<tr>
<td>Com PA4596 R BamHI</td>
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<td>This study</td>
</tr>
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<td>PA4596 long F HindIII</td>
<td>ACTGAAGCTTATTGATCACAATCAGGAGTCCA</td>
<td>This study</td>
</tr>
<tr>
<td>Primer</td>
<td>Forward/Reverse</td>
<td>Sequence</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>PA4596 igr F HindIII</td>
<td></td>
<td>ACTGAAAGCTTAAACGAGCACCCTCATGAAAGAA</td>
</tr>
<tr>
<td>BstEII nfxB forward</td>
<td></td>
<td>GATCAGTGACCATGACCCTGTATTTCCCATGAC</td>
</tr>
<tr>
<td>PuuII nfxB reverse</td>
<td></td>
<td>GTCGACGCTGTCAAGGACGAGCCGGATT</td>
</tr>
<tr>
<td>BstEII PA4596 forward</td>
<td></td>
<td>GATCAGTGACCATGACCCTGTATTTCCCATGAC</td>
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<tr>
<td>XhoI PA4596 reverse</td>
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<td>GATCCTCGAGTCAAGGCTGGCAGTTG</td>
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<tr>
<td>BglII nfxB reverse</td>
<td></td>
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</tr>
<tr>
<td>rpsL F</td>
<td></td>
<td>GCAACTATCAACCAGCTG</td>
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<td></td>
<td>GCTGTCCTCTGAGGTTGTG</td>
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<tr>
<td>mexD F</td>
<td></td>
<td>TCTTCATCAAGCGGCGGAC</td>
</tr>
<tr>
<td>mexD R</td>
<td></td>
<td>AGGGTAGCGGTCTGGATCGC</td>
</tr>
<tr>
<td>RT-nfxB For</td>
<td></td>
<td>TTCCATGACGAGCGGACTC</td>
</tr>
<tr>
<td>RT-nfxB Rev</td>
<td></td>
<td>AACACGCTTTTCTGCTGTC</td>
</tr>
<tr>
<td>RT-PA4596 For</td>
<td></td>
<td>AACTGGCAGGAGTGAGCAGG</td>
</tr>
<tr>
<td>RT-PA4596 Rev</td>
<td></td>
<td>GCCGTAGATCAGCGGAGCTGAA</td>
</tr>
</tbody>
</table>
primer pair was determined using gradient PCR. DNA sequencing was performed by ACGT Corporation and aligned against \textit{P. aeruginosa} genome sequences to ensure no mutations had been introduced into the PCR product (236).

Colony PCR was performed using whole cell lysates as the source of chromosomal DNA. The cell lysates were prepared by resuspending a single colony in 30 µl of dH2O, which was boiled (5 minutes, 95°C) and then centrifuged (1 minute, 13000 rpm). The PCR mixture consisted of 6.8 µl dH2O, 1 µl of 10X Thermopolymerase Buffer (New England Biolabs), 0.5 ul DMSO, 0.2 µl of 10 mM dNTPs, 0.2 µl of each forward and reverse primer, 1 µl colony lysate (as prepared above), and 0.5 µl of Taq DNA polymerase (New England Biolabs). The PCR program was run as specified above except the extension time was doubled to one minute per kb of fragment.

2.5 DNA transformation

2.5.1 Calcium chloride competent \textit{E. coli}

An overnight culture of \textit{E. coli} was subcultured into 100 ml, grown to an optical density at 600 nm (OD\textsubscript{600}) of 0.4, and then cooled on ice. Cells were centrifuged in a JA-20 rotor (Beckman; 15 minutes, 8000 rpm, 4°C) and thoroughly resuspended in 40 ml of ice cold 100 mM CaCl\textsubscript{2}. The cells were again pelleted (15 minutes, 8000 rpm, 4°C), resuspended in 4 ml of 100 mM CaCl\textsubscript{2} + 15% (v/v) glycerol, and left overnight at 4°C. The next day cells were separated into 200 µl aliquots for future use. To transform, 2 µl of purified plasmid DNA was added to the competent cells and incubated at 4°C for 30 minutes. Cells were then heated (90 seconds, 42°C) and returned to ice to recover (2 minutes, 4°C). After recovery, 800 µl of LB broth was added to
the competent cells and they were incubated at 37°C for one hour. Transformed cells were plated on LB-agar plates supplemented with the appropriate antibiotic to select for the plasmid.

2.5.2 Electrocompetent \textit{P. aeruginosa}

Electrocompetent \textit{P. aeruginosa} were prepared by a protocol developed by Choi and colleagues (34). Briefly, overnight cultures of the \textit{P. aeruginosa} strain were grown, separated into 1.25 ml aliquots, and pelleted (1 minute, 13000 rpm). Pellets were resuspended in 250 µl of 300 mM sucrose. Cells were again pelleted (1 minute, 13000 rpm) and resuspended in 250 µl of 300 mM sucrose. Finally, cells were pelleted (1 minute, 13000 rpm) and resuspended in 80 µl of 300 mM sucrose. One µl of purified plasmid DNA was added to the cells. The mixture was transferred to electroporation cuvettes (Bio-Rad) and pulsed at 2500 V, 25 µF, and 200 Ω in a BTX ECM399 electroporation apparatus (Inovio Biomedical Corporation). After electroporation, 1 ml of LB broth was added and the cells were left to recover (2 hours, 37°C). Cells were plated on LB-agar plates containing the appropriate antibiotic to select for the plasmid.

2.6 Conjugation

Donor S17-1 \textit{E. coli} cells containing the plasmid to be transferred were grown overnight in LB containing the appropriate antibiotic and subcultured to an OD$_{600}$ of 0.8 in the absence of antibiotics. Recipient \textit{P. aeruginosa} cells were grown overnight in 10 ml non-shaking cultures at 42°C. 600 µl of donor cells were combined with 300 µl of recipient cells and pelleted together (2 minutes, 8000 rpm). Cells were carefully resuspended in 100 µl of LB broth, spotted onto an LB-
agar plate and incubated at 37°C for four hours to permit conjugation. Cells were resuspended in 1 ml of LB broth and plated on LB-agar containing 50 µg/ml gentamicin (Gm) selecting for pEX18Tc Gm' derivatives as well as 5 µg/ml chloramphenicol (Cm) counter-selecting against the *E. coli* donors.

2.7 Construction of gene deletions

2.7.1 Construction of *P. aeruginosa* K767 Δ*nfxB*

Gene deletions in this study were carried out using the standard allelic exchange techniques described by Schäfer and colleagues (215). This protocol was modified to include inserting a gentamicin resistance cassette between the two fragments bracketing the sequence to be deleted and allows for positive selection of the gene deletion as pioneered by Choi and Schweizer (33). PCR was used to amplify a fragment upstream of *nfxB* using the primers A2 nfxB U F BamHI and A2 nfxB U R Gm using a standard PCR protocol with an annealing temperature of 58°C to create a product of 460 bp. A fragment downstream of *nfxB* was amplified using the primers A2 nfxB D F Gm and A2 nfxB D R HindIII and a standard PCR protocol with an annealing temperature of 58°C to create a product of 403 bp. The gentamicin resistance cassette was amplified from the vector pPS856 using the primers Gm-F and Gm-R using a standard PCR protocol with an annealing temperature of 50°C to create a product of 1053 bp. The 5’ and 3’ ends of the gentamicin resistance cassette overlapped with the ends of the *nfxB* upstream and *nfxB* downstream fragments, respectively, and allowed joining of the three fragments through fusion PCR. Briefly, the fusion PCR mixture consisted of 33 µl dH$_2$O, 8 µl 5X Phusion Buffer HF, 1.5 µl DMSO, 2 µl MgCl$_2$, 1 µl of 10 mM dNTPs, 1 µl *nfxB* upstream (50
ng), 0.5 µl nfxB downstream (50 ng), 0.3 µl gentamicin resistance cassette (50 ng), 0.5 µl Phusion™ polymerase. The PCR program was run with an annealing temperature of 55°C for the first three cycles. After which, the primers A2 nfxB U F BamHI and A2 nfxB D R HindIII were added to the reaction. The reaction ran for 25 cycles using an annealing temperature of 63°C. The final product of 2 kb was visualized on an agarose gel and excised for purification. The Gm'-tagged deletion fragment digested with BamHI and HindIII was cloned into the BamHI-HindIII-restricted plasmid pEX18Tc to form the plasmid pACP01. This plasmid was transformed into E. coli S17-1 and conjugated into P. aeruginosa K767 selecting on Gm 50 µg/ml Cm 5 µg/ml LB-agar plates. Gm' transconjugants were then patched onto plates containing tetracycline (Tc) 100 µg/ml to ensure pEX18Tc integration into the chromosome. Gm' Tc' resultant colonies were streaked onto 10% sucrose (w/v) Gm 50 µg/ml plates to select for a second recombination leaving only the Gm' nfxB deletion construct. These colonies were electroporated with the flp recombinase encoding vector pFLP2, which is selected with Carbenicillin (Cb) 200 µg/ml. pFLP2 allows for the excision of the Gm' cassette. pFLP2-free cells carrying the untagged nfxB deletion were recovered on LB plates supplemented with 10% sucrose (w/v). The resultant K767 ΔnfxB strains were confirmed with colony PCR using the primers A2 nfxB U F BamHI and A2 nfxB D R HindIII and named K2951.

2.7.2 Construction of P. aeruginosa K767 ΔPA4596 strains

Construction of the K767 ΔPA4596 strain was carried out similar to deleting nfxB. PCR was used to amplify a fragment upstream of PA4596 using the primers A2 PA4596 U F BamHI and A2 PA4596 U R Gm using a standard PCR protocol with an annealing temperature of 58°C.
to create a product of 585 bp. The fragment downstream of PA4596 was amplified with the primers A2 PA4596 D F Gm and A2 PA4596 D R HindIII using a standard PCR protocol with an annealing temperature of 58°C to create a product of 369 bp. The PA4596 upstream and downstream fragments were fused with the gentamicin resistance cassette using the primers A2 PA4596 U F BamHI and A2 PA4596 D R HindIII and the PCR program described in the deletion of nfxB. The PA4596 Gm' deletion fusion was ligated into pEX18Tc using the same methods and parameters as was described in the nfxB deletion to generate pACP02. This construct was introduced into E. coli S17-1 and subsequently conjugated into P. aeruginosa K767. Selection of the PA4596 deletion was carried out as previously described for the deletion of nfxB. The final P. aeruginosa K767 ΔPA4596 was confirmed with colony PCR using the primers A2 PA4596 U F BamHI and A2 PA4596 D R HindIII and named K2952. pACP02 was additionally used to generate K1536 ΔPA4596 and K385 ΔPA4596, which were named K2953 and K2954, respectively. A P. aeruginosa K767 ΔPA4596 ΔnfxB was generated from the K767 ΔPA4596 strain using plasmid pACP01 and named K2955.

2.8 Complementation of gene deletions

2.8.1 Complementation of nfxB mutants

The gene nfxB includes two putative ATG start sites, 33 bp apart and both in the same reading frame, which would allow for translation of the NfxB protein product. An nfxB fragment including only the downstream ATG was amplified using the primers Com nfxB F HindIII and Com nfxB R BamHI and cloned into the vector pRK415 to create pACP03. A second fragment including the second upstream nfxB start site as well as the first was amplified with the primers
nfxB long F HindIII and nfxB long R BamHI and cloned into pRK415 to create pACP04. A third nfxB fragment additionally including the 124 bp upstream nfxB-mexC intergenic region and both start sites was amplified with the primers nfxB igr F HindIII and nfxB long R BamHI and cloned into pRK415 to create pACP05. The upstream region of these three nfxB-containing DNA fragments is shown in Figure 3. Each of these three vectors and a previously described nfxB vector pNFX-1 were electroporated into P. aeruginosa strains K1536, K385, and K2951. pRK415 derivative plasmids were selected by Tc 100 µg/ml, whereas vector pNFX-1 was selected by Cb 200 µg/ml.

2.8.2 Complementation of PA4596 deletion strains

Similar to nfxB, the PA4596 gene includes two putative ATG start sites, 117 bp apart and both in the same reading frame, which would allow for translation of the PA4596 protein product. The PA4596 gene including only the downstream ATG start site was amplified using the primers Com PA4596 F HindIII and Com PA4596 R BamHI and cloned into the vector pRK415 to create pACP06. A second PA4596 fragment including the second possible upstream PA4596 start site was amplified with the primers PA4596 long F HindIII and Com PA4596 R BamHI and cloned into pRK415 to create pACP07. A third PA4596 fragment additionally including the 375 bp upstream intergenic region between PA4596 and PA4595 was amplified with the primers PA4596 igr F HindIII and Com PA4596 R BamHI and cloned into pRK415 to create pACP08. The 5’ region of these three PA4596-containing DNA fragments is shown in Figure 4. Each of these three vectors was electroporated into P. aeruginosa strains K767 and K2955.
Figure 3. 5’ region of nfxB-containing DNA fragments

5’ regions of the nfxB-containing cloned fragments in pACP03 (purple), pACP04 (green), and pACP05 (orange) aligned against the nfxB gene. The two possible start sites of nfxB are bolded and their respective putative Shine-Dalgarno sequences are underlined. The mexC gene is shown in blue.
mexC

1

ATTCGACGCAAATCGCCATCGACACCCCGACCCTTTTGGATTGAAACGATCGAATGGTAG
TAACGTGCGTTTAGTGCAGTTACGTGTGCTGGCTCGCAACTAATCTTTGCTTAGTTACCATG

61

TTTTTCGAGCTAATTTGAGCATTATATTGTCTCAAAATGATCTTTTGGACAGCTAATTCTCTTTG
AAAAAGCTGCATTTAACTCATTTTACAGTTACAAGAGTTTACTCTGAAAACGTCGATTTAGGAAAC

121

GAGCNGGAGGGCGTTTCGCAAAATGCGCAACAATCTGAAACGACCAGCCGACATCG
CTGCCTCCGGTCGCAAAAGACGTGGTACCGTGTTAGCTTTTTGTTGCGCCCTGGCTAGC

181

ATGACCCCTGATT
TACTGGGACTAA

pACP03  pACP04  pACP05
Figure 4. 5’ region of PA4596-containing DNA fragment

5’ regions of the PA4596-containing cloned fragments in pACP06 (purple), pACP07 (green), and pACP08 (orange) aligned against the PA4596 gene. The two possible start sites of PA4596 are bolded. The PA4595 gene is shown in blue.
2.9 Antimicrobial susceptibility testing

The susceptibility to various antimicrobial compounds was assessed in microtitre plates with a two-fold dilution technique as suggested by Jo and colleagues (93). For assessing chlorhexidine susceptibility, five overlapping two-fold dilution series were used to increase precision. The lowest concentration of antimicrobial that did not permit growth was considered the minimum inhibitory concentration (MIC).

2.10 LexA 2-hybrid experiments

For assessing homodimerization, the standard LexA 2-hybrid approach pioneered by Schmidt-Dörr et al. (217) and expanded by Porte et al. (194) was used. An nfxB fragment was amplified with the primers BstEII nfxB forward and PvuII nfxB reverse. Restriction digest of pMS604 with endonucleases BstEII and PvuII allowed excision of the fos zipper and insertion of the nfxB BstEII-PvuII-restricted fragment to create pACP09. Additionally, the nfxB gene of P. aeruginosa K385 coding for the mutant protein NfxB_{H87R} was amplified and cloned into pMS604, using the same restriction sites as above, to make pACP10. Similarly, a PA4596 fragment was amplified with the primers BstEII PA4596 forward and XhoI PA4596 reverse and cloned into pMS604 via a BstEII XhoI restriction digest to make pACP11.

Each plasmid was transformed into the E. coli strain SU101, a strain harboring a lacZ gene modified to include the wild-type LexA operator. The natural LexA is able to dimerize and bind to this operator, thus repressing transcription of lacZ. The LexA protein encoded on the
plasmid pMS604 lacks the dimerization domain and can only function as a repressor if fused to a protein that is able to self-associate. Consequently, lacZ expression is downregulated in the event of fusion protein self-association, which is measurable by a β-galactosidase assay under conditions recommended by Dmitrova et al. (47). Briefly, E. coli SU101 strains transformed with a pMS604 derivative plasmid were grown overnight in LB broth containing Tc 10 µg/ml and then subcultured in 1 mM IPTG and allowed to grow to an OD\textsubscript{600} of 0.4 - 0.6. The β-galactosidase assay was carried out as described by Miller (151).

For assessment of an NfxB-PA4596 interaction, the modified LexA 2-hybrid approach developed by Dmitrova et al. was used (47). The E. coli strain SU202 includes a lacZ gene with a hybrid LexA operator engineered into the promoter. This hybrid operator can only be bound and repressed by the wild-type LexA DNA-binding domain dimerized with the mutant DNA-binding domain LexA\textsubscript{1-87}408. To facilitate dimerization and repression of lacZ, proteins fused to the two DNA-binding domains must be able to interact. As above, LacZ activity is quantified with the β-galactosidase assay as described by Miller (151). The gene nfxB was amplified with the primers BssHII nfxB forward and BglII nfxB reverse and cloned into pDP804 after digestion with BssHII and BglII to create pACP12 so that pACP12 expresses NfxB fused to the mutant Lex\textsubscript{1-87}408 DNA-binding domain. The previously constructed pMS604 PA4596-expressing derivative, pACP11, and pACP12 were transformed into SU202 and selected with Tc 10 µg/ml and Ampicillin (Ap) 100 µg/ml. The PA4596-NfxB interaction was assessed using a β-galactosidase assay under conditions recommended by Dmitrova et al. (47).
2.11 Assessment of gene expression using RT-PCR

2.11.1 RNA isolation

Overnight cultures were subcultured into 10 ml of LB broth grown to log phase. In cases where membrane stress was being simulated, one quarter the concentration of that strains chlorhexidine MIC was included in the LB broth during subculture. Extraction of RNA was carried out using the Ribopure™-Bacteria kit (Ambion) according to the manufacturer’s instructions including the DNase treatment typically recovering 50 µl of 200 ng/µl RNA. Purified RNA was stored at 4°C.

2.11.2 RT-PCR

RT-PCR was carried out on isolated RNA samples using the Qiagen OneStep RT-PCR Kit according to the manufacturer’s recommendations. RNA samples were tested for DNA contamination by performing PCR in the absence of reverse transcriptase with the primers rpsL F and rpsL R. To standardize RNA samples, RT-PCR was performed using rpsL F and rpsL R primers with an annealing temperature of 60°C for 20 and 22 cycles. RNA quantities were equilibrated accordingly. The primers mexD F and mexD R with an annealing temperature of 55°C were used to assess mexCD-oprJ expression. Alternatively, nfxB expression was assessed with the primers RT-nfxB For and RT-nfxB Rev and PA4596 expression was assessed with the primers RT-PA4596 For and RT-PA4596 Rev, both using an annealing temperature of 55°C.
Chapter 3: Results

3.1 Influence of NfxB on mexCD-oprJ expression

*nfxB* is annotated in the *Pseudomonas* genome database to be a gene of 564 nucleotides that codes for a protein of 187 amino acids with a molecular mass of ~21 kDa (236). However, two forms of NfxB have been observed, the predicted ~21 kDa form and a ~23 kDa form (226). This ~23kDa form correlates with a second upstream ATG start site that is in frame with the rest of the *nfxB* coding sequence (236).

K2951 is an *nfxB* deletion strain derived from the *P. aeruginosa* PA01 wild-type strain K767. This strain demonstrated quinolone resistance comparable to the MexCD-OprJ hyperproducing strain K385 (Table 5) and hyperexpressed mexCD-oprJ as shown with RT-PCR (Figure 5, lanes 1 and 2). It has been observed that the K2951 strain can spontaneously change to wild-type-like quinolone resistance if stored on LB-agar plates. It has been shown that this change must be through some non-*nfxB* based mechanism as a PCR check confirmed the quinolone-susceptible strain is still *nfxB* deleted. Interestingly, neither of the other two *nfxB* strains K385 nor K1536 demonstrated a tendency to revert when stored on LB-agar plates suggesting that there is stronger selection for a compensatory mutation with a complete deletion of *nfxB*.

It is unclear which of the two putative ATG start sites is used to initiate translation of NfxB. In an attempt to complement the loss of *nfxB* in strain K2951, two *nfxB*-containing DNA
Table 5. Impact of cloned *nfxB* on susceptibilities to antimicrobials and biocides effluxed by MexCD-OprJ

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Functional NfxB</th>
<th>Antibiotic / Biocide MICs (μg/ml)$^+$</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td>Nal</td>
</tr>
<tr>
<td>K372</td>
<td>+</td>
<td></td>
<td>128</td>
</tr>
<tr>
<td>K385</td>
<td>-</td>
<td></td>
<td>512</td>
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<td>K385</td>
<td>pRK415</td>
<td>-</td>
<td>512</td>
</tr>
<tr>
<td>K385</td>
<td>pACP03</td>
<td>+</td>
<td>256</td>
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<tr>
<td>K385</td>
<td>pACP04</td>
<td>+</td>
<td>256</td>
</tr>
<tr>
<td>K385</td>
<td>pACP05</td>
<td>+</td>
<td>- $^a$</td>
</tr>
<tr>
<td>K767</td>
<td>+</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>K2951</td>
<td>-</td>
<td></td>
<td>256</td>
</tr>
<tr>
<td>K2951</td>
<td>pRK415</td>
<td>-</td>
<td>256</td>
</tr>
<tr>
<td>K2951</td>
<td>pACP03</td>
<td>+</td>
<td>256</td>
</tr>
<tr>
<td>K2951</td>
<td>pACP04</td>
<td>+</td>
<td>256</td>
</tr>
<tr>
<td>K2951</td>
<td>pACP05</td>
<td>+</td>
<td>- $^a$</td>
</tr>
</tbody>
</table>

$^+$Abbreviations: Nal, Nalidixic Acid; Cp, Ciprofloxacin; Nor, Norfloxacin; Cm, Chloramphenicol; Chx, Chlorhexidine

$^a$Not determined
Figure 5. Impact of nfxB on mexCD-oprJ expression in P. aeruginosa.

RT-PCR was used to assess the expression of mexD as an indicator of mexCD-oprJ and rpsL in K767 (wild-type; lane 1), K2951 (K767 ΔnfxB; lane 2), K2951 (pAK1900) (lane 3), K2951 (pNFX-1) (lane 4). mexD was assessed at 30 and 32 cycles. rpsL was assessed at 20 and 22 cycles. All reactions were standardized using rpsL reactions as an internal control to ensure that equal amounts of RNA are added to all RT-PCR reactions. All RNA samples were checked for DNA contamination. Results presented are typical of at least two independent experiments.
fragments, the first including only the downstream annotated start site and the second including both start sites, were cloned into the vector pRK415 to make the plasmids pACP03 and pACP04, respectively. It was found that neither of these cloned \textit{nfxB}s restored susceptibility of K2951 (\textit{\textDelta nfxB}) or K385 (\textit{nfxB}) to antibiotics effluxed by MexCD-OprJ (Table 5); surprisingly, these strains did regain susceptibility to the biocide Chx, another substrate effluxed by MexCD-OprJ.

The plasmid pNFX-1 consists of the cloning vector pAK1900 with 2.2 kb fragment including all of \textit{nfxB}, most of \textit{mexC} (1.2 kb), and the intergenic region between these two genes. It has been shown to be able to complement the K385 NfxB\textsubscript{H87R} yielding missense mutant (193). RT-PCR showed \textit{mexCD-oprJ} hyperexpression in the K767 \textit{\textDelta nfxB} was also abrogated when pNFX-1 was expressed in this strain (Figure 5, lane 4). Another previously-described vector pNF253, which expresses \textit{nfxB} preceded by 230 bp of sequence upstream of the gene has been shown to complement an \textit{nfxB} strain (174). These results suggest that the intergenic region between \textit{nfxB} and \textit{mexC} may be important for \textit{nfxB} expression. Consequently a fragment including \textit{nfxB} and the \textit{nfxB-mexC} intergenic region was amplified and cloned into pRK415 to produce pACP05. However, this construct was also unable to restore susceptibility of K2951 (\textit{\textDelta nfxB}) or K385 (\textit{nfxB}) to antibiotics effluxed by MexCD-OprJ (Table 5).

### 3.2 Influence of chlorhexidine on PA4596 expression

Previous RNA microarray data showed that PA4596 was upregulated in response to Chx induced membrane stress in an AlgU-dependent fashion (i.e. PA4596 is not induced by Chx in a \textit{\textDelta algU} strain) (C. Dean and D. Daigle, unpublished). Contrary to this RNA microarray evidence, RT-PCR showed that PA4596 was downregulated in response to chlorhexidine induced membrane stress (Figure 6, lanes 1 and 2), albeit also in an AlgU-dependent fashion.
Figure 6. Impact of chlorhexidine and AlgU on PA4596 expression in *P. aeruginosa*.

RT-PCR was used to assess the expression of PA4596 and *rpsL* in K767 (wild-type; lanes 1 and 2), K2443 (*K767 ΔalgU*; lanes 3 and 4). Lanes 1 and 3 were grown in the absence of chlorhexidine, and lanes 2 and 4 were grown in ¼ MIC chlorhexidine for 2.5 hours. PA4596 was assessed at 28 and 30 cycles. *rpsL* was assessed at 20 and 22 cycles. All reactions were standardized using *rpsL* reactions as an internal control to ensure that equal amounts of RNA are added to all RT-PCR reactions. All RNA samples were checked for DNA contamination. Results presented are typical of at least two independent experiments.
(Figure 6, lanes 3 and 4). This result remains consistent with the hypothesis that PA4596 expression responds to envelope stress.

### 3.3 Influence of PA4596 on mexCD-oprJ expression in wild-type *P. aeruginosa*

The PA4596 deletion strain K2952 was used to investigate whether PA4596 had an effect on mexCD-oprJ expression. Since quinolones, chloramphenicol, and chlorhexidine are substrates of MexCD-OprJ, increased resistance to these compounds suggests mexCD-oprJ upregulation \(^{(193)}\). The K2952 strain showed similar susceptibility to these antimicrobials to that of wild-type *P. aeruginosa* K767 strain (Table 6). Moreover, RT-PCR evidence supported that mexCD-oprJ expression is not affected by the loss of PA4596 in the strain K2952 (Figure 7, lanes 1 and 2). As it was demonstrated that PA4596 expression responded to exposure to the biocide Chx, it was possible that PA4596 played a role in Chx-induced expression of mexCD-oprJ. However, PA4596 had no influence on mexCD-oprJ expression in the presence of the biocide chlorhexidine as assessed by RT-PCR (Figure 7, lanes 3 and 4). Moreover, an independently isolated *P. aeruginosa* K767 ΔPA4596 strain, K2878, also demonstrated similar susceptibility to antimicrobials to wild-type *P. aeruginosa* K767 and the K2952 PA4596 deletion strain (A.J. Campigotto, unpublished).

It was possible that expressing considerably higher amounts of PA4596 compared to wild-type levels could allow the creation of a more observable effect on mexCD-oprJ expression and quinolone resistance. Like nfxB, there exists a second potential upstream ATG start site, which could act as the translational start. Additionally, like nfxB, the adjacent upstream intergenic region occurring between PA4596 and PA4595, could be required for expression of
Table 6. Impact of cloned *nfxB* on susceptibilities to antimicrobials and biocides effluxed by MexCD-OprJ

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Antibiotic / Biocide MICs (μg/ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nal</td>
</tr>
<tr>
<td>K767 wild-type</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>K2952 K767 ∆PA4596</td>
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<td>64</td>
</tr>
<tr>
<td>K2951 K767 ∆nfxB</td>
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<td>256</td>
</tr>
<tr>
<td>K2955 K767 ∆PA4596 ∆nfxB</td>
<td></td>
<td>64</td>
</tr>
</tbody>
</table>

†Abbreviations: Nal, Nalidixic Acid; Cp, Ciprofloxacin; Nor, Norfloxacin; Cm, Chloramphenicol; Chx, Chlorhexidine
Figure 7. Impact of chlorhexidine and PA4596 on mexCD-oprJ expression in *P. aeruginosa*.

RT-PCR was used to assess the expression of *mexD* as an indicator of *mexCD-oprJ* and *rpsL* in K767 (wild-type; lanes 1 and 3), K2952 (K767 ΔPA4596; lanes 2 and 4). Lanes 1 and 2 were grown in the absence of chlorhexidine, and lanes 3 and 4 were grown in ¼ MIC chlorhexidine for 2.5 hours. *mexD* was assessed at 34 and 36 cycles. *rpsL* was assessed at 20 and 22 cycles. All reactions were standardized using *rpsL* reactions as an internal control to ensure that equal amounts of RNA are added to all RT-PCR reactions. All RNA samples were checked for DNA contamination. Results presented are typical of at least two independent experiments.
PA4596. Three DNA fragments of PA4596 were amplified in an attempt to express PA4596. These consist of PA4596 including the original annotated ATG start site, extending to the alternative upstream ATG start site, and including all of PA4596 and the upstream PA4596-PA4595 intergenic region. Each of which was cloned into the multicloning site of the cloning vector pRK415. This created the plasmids pACP06, pACP07, and pACP08, respectively.

No change in quinolone resistance was observed when pACP06 or pACP08 was transformed into the K767 wild-type. Interestingly, pACP07 transformants of the wild-type strain demonstrated an increase in quinolone and chloramphenicol resistance when compared to the wild-type strain and the pRK415 transformed wild-type strain suggesting that cloned PA4596 including both ATG start sites is able to enhance mexCD-oprJ expression. Surprisingly, however, K767 pACP07 transformants did not display increased resistance to the biocide chlorhexidine, which is associated with mexCD-oprJ hyperexpression (Table 7).

It was unusual that the pACP07 transformed strain K767, which is apparently upregulating mexCD-oprJ, did not demonstrate increased resistance to chlorhexidine (Table 7). However, the pACP07 transformed strain K767 did demonstrate increased resistance to chloramphenicol, thus excluding the possibility that resistance occurred from mutations in the fluoroquinolone’s cellular targets such as DNA gyrase (Table 7). Still possible was that a mutation occurred in the strain being transformed with pACP07 causing the upregulation of another P. aeruginosa multidrug efflux system, which is responsible for the observed antimicrobial resistance. Consequently MICs of antimicrobials that are effluxed by other multidrug efflux pumps were assessed including paromomycin (MexXY), erythromycin (MexCD-OprJ), carbenicillen (MexAB-OprM), and trimethoprim (MexEF-OprN) in addition to ciprofloxacin and norfloxacin (Table 8). It was found that strain K767 carrying pACP07 showed
Table 7. Impact of cloned PA4596 on susceptibilities to antimicrobials and biocides effluxed by MexCD-OprJ

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Antibiotic / Biocide (μg/ml)</th>
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<th>Cp</th>
<th>Nor</th>
<th>Cm</th>
<th>Chx</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
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<td>0.125</td>
<td>0.5</td>
<td>25</td>
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<td>0.125</td>
<td>0.5</td>
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</tr>
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<td>0.125</td>
<td>0.5</td>
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<tr>
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<td>pACP08</td>
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<td>0.125</td>
<td>0.5</td>
<td>-</td>
<td>7.5</td>
</tr>
</tbody>
</table>

†Abbreviations: Nal, Nalidixic Acid; Cp, Ciprofloxacin; Nor, Norfloxacin; Cm, Chloramphenicol; Chx, Chlorhexidine

*Not determined
Table 8. Antimicrobial susceptibility of strains isolated from pACP07 electroporation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Antibiotic / Biocide MICs (μg/ml)†</th>
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</thead>
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<td></td>
<td>Cp</td>
</tr>
<tr>
<td>K767</td>
<td>wild-type</td>
<td>pACP07</td>
<td>0.5</td>
</tr>
<tr>
<td>K2951</td>
<td>K767 ∆nfxB</td>
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<td>wild-type</td>
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<td>wild-type</td>
<td>pACP07</td>
<td>0.5</td>
</tr>
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</table>

†Abbreviations: Cp, Ciprofloxacin; Nor, Norfloxacin; Paro, Paromomycin; Ery, Erythromycin; Cb, Carbenicillin; Tmp, Trimethoprim
drug resistance more comparable to a *mexAB-oprM* hyperexpressor rather than a *mexCD-oprJ* hyperexpressor including increased resistance to carbenicillin, which is only effluxed by MexAB-OprM (145). Furthermore, increased susceptibility to paramomycin indicates MexXY downregulation, which occurs during MexAB-OprM hyperexpression (119,144,159). Additionally, *mexCD-oprJ* was not upregulated nor was there any increase in PA4596 expression in strains isolated following pACP07 electroporation as assessed by RT-PCR (Figure 8, lanes 1-3). Consequently, it is more likely that the colonies isolated after the pACP07 transformation were spontaneous mutants and not transformants.

Another *P. aeruginosa* strain K372, from which the *nfxB* mutant K385 was derived, was electroporated with pACP07 and showed high transformation efficiency similar to the transformation of pRK415. Strain K372 carrying pACP07 showed overexpression of PA4596 as assessed with RT-PCR (Figure 9) but did not demonstrate any increased resistance to quinolones indicating that cloned PA4596 is not able to enhance *mexCD-oprJ* expression (Table 9). Consequently, it seems that cloned PA4596 does not affect the expression of *mexCD-oprJ* in wild-type cells.

### 3.4 Influence of PA4596 on *mexCD-oprJ* expression in *P. aeruginosa nfxB* mutants

A PA4596 deletion mutant, K2955, was generated from the Δ*nfxB* strain, K2951, to assess the role of PA4596 in *mexCD-oprJ* hyperexpression in an *nfxB* deletion background. Strain K2955 demonstrated resistance to quinolones similar to the wild-type strain K767 (Table 6) supporting that PA4596 is essential to *mexCD-oprJ* hyperexpression seen in Δ*nfxB* strains.
Figure 8. Impact of cloned PA4596 on mexCD-oprJ and PA4596 expression.

RT-PCR was used to assess the expression of mexD as an indicator of mexCD-oprJ, PA4596, and rpsL in K767 (wild-type; lane 1), K767 (pRK415) (lane 2), K767 (pACP07) (lane 3), K2951 (K767 ΔnfxB; lane 4), K2955 (K767 ΔPA4596 ΔnfxB; lane 5), K2955 (pRK415) (lane 6), K2955 (pACP07) (lane 7). rpsL was assessed at 19 and 21 cycles. mexD was assessed at 30 and 32 cycles. PA4596 was assessed at 26 and 28 cycles. All reactions were standardized using rpsL reactions as an internal control to ensure that equal amounts of RNA are added to all RT-PCR reactions. All RNA samples were checked for DNA contamination. Results presented are typical of at least two independent experiments.
Figure 9. Expression of PA4596 from vector pACP07.

RT-PCR was used to assess the expression of PA4596, and *rpsL* in K372 (parent strain; lane 1), K372 (pRK415) (lane 2), K372 (pACP07) (lane 3). PA4596 was assessed at 24 and 26 cycles. *rpsL* was assessed at 19 and 21 cycles. All reactions were standardized using rpsL reactions as an internal control to ensure that equal amounts of RNA are added to all RT-PCR reactions. All RNA samples were checked for DNA contamination. Results presented are typical of at least two independent experiments.
Table 9. Impact of cloned PA4596 on susceptibility to antimicrobials effluxed by MexCD-OprJ

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Antibiotic / Biocide (μg/ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cp</td>
</tr>
<tr>
<td>K372</td>
<td>parent strain</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>K385</td>
<td>K372 nfxB</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>K372</td>
<td>parent strain</td>
<td>pRK415</td>
<td>0.25</td>
</tr>
<tr>
<td>K372</td>
<td>parent strain</td>
<td>pACP07</td>
<td>0.25</td>
</tr>
</tbody>
</table>

†Abbreviations: Cp, Ciprofloxacin; Nor, Norfloxacin
Consistent with this, loss of PA4596 in the K2951 nfxB deletion strain abolished mexCD-oprJ hyperexpression as assessed by RT-PCR (Figure 10, lanes 3 and 4). Alternatively, loss of PA4596 in the nfxB mutant K385 or the NfxB-like mexCD-oprJ hyperexpressor K1536 (Table 10) did not result in decreased resistance to quinolones. Nor did loss of PA4596 negate mexCD-oprJ hyperexpression in strain K1536 assessed by RT-PCR, indicating that PA4596 may not always be required for mexCD-oprJ hyperexpression (Figure 10, lanes 5 and 6).

To support that loss of PA4596 was in fact causing the abrogation of mexCD-oprJ hyperexpression in the nfxB deletion strain, each of the three PA4596-expressing plasmids were cloned into the ∆PA4596 ∆nfxB strain K2955. No change in quinolone resistance was observed when pACP06 was transformed into K2955 indicating that expression of PA4596 containing only the downstream putative ATG start site did not restore mexCD-oprJ hyperexpression (Table 11). pACP07 transformants of K2955 demonstrated an increase in quinolone and chloramphenicol resistance when compared to the parent strain and the pRK415 transformed parent strain; however, they did not demonstrate the increase in chlorhexidine resistance associated with mexCD-oprJ hyperexpressors (Table 11). Surprisingly, pACP08 transformants of K2955 remained susceptible to quinolones and chlorhexidine demonstrating that cloned PA4596 including the PA4596-PA4595 intergenic region was not able to restore mexCD-oprJ hyperexpression (Table 11). This is unexpected considering pACP07, also including both ATG start sites, was able to at least partially complement the loss of PA4596 in the strain K2955. However, plasmid pACP07, although not pACP06 nor pACP08, demonstrated very low electroporation efficiency when mobilized into K767 or any of its derivative strains. As with K767, pACP07 transformants of strain K2955 did not demonstrate increased chlorhexidine resistance. Moreover, these isolates did not show restored mexCD-oprJ hyperexpression
Figure 10. Impact of nfxB and PA4596 on mexCD-oprJ expression in P. aeruginosa.

RT-PCR was used to assess the expression of mexD as an indicator of mexCD-oprJ and rpsL in K767 (wild-type; lane 1), K1536 (NfxB phenotype, mutation unknown; lanes 2 and 5), K2951 (K767 ΔnfxB; lane 3), K2955 (K767 ΔPA4596 ΔnfxB; lane 4), K2953 (K1536 ΔPA4596; lane 6). mexD was assessed at 30 and 32 cycles. rpsL was assessed at 20 and 22 cycles. All reactions were standardized using rpsL reactions as an internal control to ensure that equal amounts of RNA are added to all RT-PCR reactions. All RNA samples were checked for DNA contamination. Results presented are typical of at least two independent experiments.
Table 10. Impact of PA4596 the \textit{nfxB} strain K385 and the \textit{NfxB}-like \textit{mexCD-oprJ} hyperexpressor K1536

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Antibiotic / Biocide MICs (μg/ml)(^\dagger)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nal</td>
</tr>
<tr>
<td>K372</td>
<td>parent strain</td>
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<tr>
<td>K385</td>
<td>K372 \textit{nfxB}</td>
<td>512</td>
</tr>
<tr>
<td>K2954</td>
<td>K372 \textit{nfxB} \textit{ΔPA4596}</td>
<td>512</td>
</tr>
<tr>
<td>K767</td>
<td>wild-type</td>
<td>(-)(^a)</td>
</tr>
<tr>
<td>K1536</td>
<td>\textit{nfxB}-like; mutation unknown</td>
<td>-</td>
</tr>
<tr>
<td>K2953</td>
<td>K1536 \textit{ΔPA4596}</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^\dagger\)Abbreviations: Nal, Nalidixic Acid; Cp, Ciprofloxacin; Nor, Norfloxacin; Cm, Chloramphenicol; Chx, Chlorhexidine

\(^a\) Not determined
Table 11. Impact of cloned PA4596 on susceptibilities to antimicrobials and biocides effluxed by MexCD-OprJ

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Antibiotic / Biocide MICs (μg/ml)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nal</td>
</tr>
<tr>
<td>K767</td>
<td>wild-type</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>K2951</td>
<td>K767 ΔnfxB</td>
<td></td>
<td>256</td>
</tr>
<tr>
<td>K2955</td>
<td>K767 ΔPA4596 ΔnfxB</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>K2955</td>
<td>K767 ΔPA4596 ΔnfxB pRK415</td>
<td></td>
<td>64</td>
</tr>
<tr>
<td>K2955</td>
<td>K767 ΔPA4596 ΔnfxB pACP06</td>
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<td>64</td>
</tr>
<tr>
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<td>wild-type</td>
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</tr>
<tr>
<td>K2951</td>
<td>K767 ΔnfxB</td>
<td></td>
<td>256</td>
</tr>
<tr>
<td>K2955</td>
<td>K767 ΔPA4596 ΔnfxB</td>
<td></td>
<td>64</td>
</tr>
<tr>
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<td>K2951</td>
<td>K767 ΔnfxB</td>
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</tr>
<tr>
<td>K2955</td>
<td>K767 ΔPA4596 ΔnfxB pACP08</td>
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<td>-</td>
</tr>
</tbody>
</table>

†Abbreviations: Nal, Nalidixic Acid; Cp, Ciprofloxacin; Nor, Norfloxacin; Cm, Chloramphenicol; Chx, Chlorhexidine

ª Not determined
or even PA4596 expression as assessed with RT-PCR (Figure 8, lane 7) indicating they too are spontaneous antibiotic-resistant mutants and do not carry the PA4596 cloning vector.

As mentioned before, K2951 (ΔnfxB) has a tendency to ‘revert’ to wild-type-like mexCD-oprJ expression and sensitivity to quinolones. Consequently, it was still unclear whether the deletion of PA4596 in strain K2951 during construction of strain K2955 led to the quinolone sensitivity or if the strain underwent the previously seen ‘reversion’.

3.5 Influence of NfxB on PA4596 expression

NfxB is known to be a repressor of both mexCD-oprJ and its own gene nfxB (193, 226). As PA4596 is surmised to be involved in this regulation system, there is a possibility that NfxB is also a repressor of PA4596 expression. In fact, PA4596 is derepressed in nfxB mutants or in the absence of nfxB (Figure 11). Conversely, loss of PA4596 in strain K2952 was shown to have no impact on nfxB expression indicating that PA4596 does not regulate nfxB (Figure 12).

3.6 Homodimerization of NfxB and its importance for repressor activity

Electrophoretic mobility shifts assays (EMSAs) and DNase 1 protection assays suggest that NfxB binds to the mexC promoter as a homodimer (226). To validate these claims, LexA 2-hybrid studies were used. The nfxB gene was cloned in-frame into the plasmid pMS604 creating pACP09, which expresses LexA1-87, the wild-type LexA DNA-binding domain, fused to NfxB. pACP09 was cloned into the reporter strain E. coli SU101, which contains a lacZ gene under the control of the wild-type LexA operator. The LexA DNA-binding domain is only able to interact
Figure 11. Impact of nfxB on PA4596 expression in K767-derivative P. aeruginosa.

RT-PCR was used to assess the expression of PA4596 and rpsL in K767 (wild-type; lane 1), K1536 (NfxB-like phenotype, mutation unknown; lane 2), K2951 (K767 ΔnfxB; lane 3), K372 (lane 4), K385 (K372 nfxB; lane 5). PA4596 was assessed at 24 and 26 cycles. rpsL was assessed at 20 and 22 cycles. All reactions were standardized using rpsL reactions as an internal control to ensure that equal amounts of RNA are added to all RT-PCR reactions. All RNA samples were checked for DNA contamination. Results presented are typical of at least two independent experiments.
Figure 12. Impact of PA4596 on \textit{nfxB} expression in \textit{P. aeruginosa}.

RT-PCR was used to assess the expression of \textit{nfxB} and \textit{rpsL} in K767 (wild-type; lane 1), K2952 (K767 \text{Δ}PA4596; lane 2). \textit{nfxB} was assessed at 28 and 30 cycles. \textit{rpsL} was assessed at 20 and 22 cycles. All reactions were standardized using \textit{rpsL} reactions as an internal control to ensure that equal amounts of RNA are added to all RT-PCR reactions. All RNA samples were checked for DNA contamination. Results presented are typical of at least two independent experiments.
with the LexA operator and cause repression of lacZ if the fused NfxB interact and cause dimerization. Thus, a decrease in β-galactosidase activity, as assessed with a β-galactosidase assay, is an excellent measure of NfxB self-association. As a negative control pMS604, expressing the non-homodimerizing Fos protein fused to LexA1-87, which is incapable of reducing β-galactosidase activity, was mobilized into SU101. Expression of this LexA1-87-NfxB fusion protein reduced β-galactosidase activity, indicating NfxB self-association (Figure 13).

*P. aeruginosa* K385 contains a missense mutation in its *nfxB* gene resulting in a protein product with an H87R amino acid substitution, thus coding for an inactive repressor leading to *mexCD-oprJ* hyperexpression (193). Similar to the wild-type *nfxB*, the mutated *nfxB* of strain K385 was cloned in-frame into pMS604 creating pACP10, which is capable of expressing a LexA1-87-NfxB<sub>H87R</sub> fusion protein. Expressed in SU101, LexA1-87-NfxB<sub>H87R</sub> did not reduce β-galactosidase expression compared to the LexA1-87-Fos negative control indicating that NfxB<sub>H87R</sub> is unable to self-associate (Figure 14). These results suggest that self-association of NfxB is required for repressor activity; however, an immunoblot using anti-NfxB antibodies will have to be used to ensure the mutant NfxB<sub>H87R</sub> is produced and not degraded.

### 3.7 Homodimerization of PA4596 and the interaction between PA4596 and NfxB

The amino acid sequence conservation of PA4596 and NfxB (61% identity) suggests that, like NfxB, PA4596 may also have the potential to self-associate. PA4596 was cloned in-frame into pMS604 to create pACP11, which is capable of expressing a LexA1-87-PA4596 fusion protein. When expressed in SU101, this LexA1-87-PA4596 fusion reduced β-galactosidase
Figure 13. Self-association of NfxB and PA4596.

β-galactosidase activity of E.coli SU101 strains expressing LexA fused to Fos, NfxB, or PA4596 from plasmid pMS604 or derivatives. A reduction in β-galactosidase activity indicates that the assayed protein is able to self-associate. Values presented indicate the mean of three repetitions of the experiment ± standard deviation.
Figure 14. Self-association of NfxB$_{H87R}$.

β-galactosidase activity of *E.coli* SU101 strains expressing LexA fused to Fos or NfxB$_{H87R}$ from plasmids pMS604 or pACP10, respectively. A reduction in β-galactosidase activity indicates that the assayed protein is able to self-associate. Values presented indicate the mean of three repetitions of the experiment ± standard deviation.
activity compared to the LexA\textsubscript{1-87}-Fos control indicating that PA4596 is able to self-associate (Figure 13).

As mentioned before, it was proposed that PA4596 and NfxB may interact even though it was unclear what the role of this interaction may be. The plasmid pDP804 expresses a mutated LexA\textsubscript{1-87}408 fused to the protein Jun. Reporter strain \textit{E. coli} SU202 contains a \textit{lacZ} gene under the control of a hybrid LexA operator. Only when the respective fused proteins of pMS604 and pDP804 interact can LexA\textsubscript{1-87} and LexA\textsubscript{1-87}408 bind to this hybrid LexA operator and cause repression of \textit{lacZ}. Proteins Fos and Jun are able to heterodimerize, and when they are simultaneously expressed from pMS604 and pDP804, they can reduce β-galactosidase activity in SU202. Thus, this reduction in β-galactosidase activity in SU202 represents an interaction between two proteins expressed on pMS604 and pDP804. The gene \textit{nfxB} was cloned in frame into the vector pDP804 to create pACP12, which can express a LexA\textsubscript{1-87}408-NfxB fusion protein. LexA\textsubscript{1-87}-PA4596 and LexA\textsubscript{1-87}408-NfxB co-expression resulted in a decrease in β-galactosidase activity comparable to the LexA\textsubscript{1-87}-Fos and LexA\textsubscript{1-87}408-Jun control, indicating that PA4596 and NfxB are able to interact (Figure 15).
**Figure 15. Interaction between NfxB and PA4596.**

β-galactosidase activity of *E. coli* SU202 strains expressing Fos or PA4596 from plasmids pMS604 or pACP11, respectively, and Jun or NfxB from plasmids pDP804 or pACP12, respectively. A reduction in β-galactosidase activity indicates that the assayed proteins are able to associate. Values presented indicate the mean of three repetitions of the experiment ± standard deviation.
Chapter 4: Discussion

4.1 Determination of the nfxB gene

The protein product of nfxB has been clearly demonstrated to be a repressor of mexCD-oprJ expression by binding to the overlapping promoter regions of nfxB and mexCD-oprJ, thereby preventing transcription (226). However, less is known about what comprises the functional nfxB gene and its protein product. The P. aeruginosa database annotates nfxB as a 564 bp gene that produces a ~21 kDa MW protein (236). However, 33 bp upstream of the annotated nfxB translation start site there is a second possible in-frame ATG start site; the translation of which would result in a ~22.5 kDa protein product. Additionally, both possible translational start sites have an adjacent putative Shine-Dalgarno sequence required for ribosomal binding. Interestingly, in a previous study, the cloned nfxB gene expressed in E. coli was shown to result in two protein products of ~21 kDa and ~23 kDa (226). N-terminal sequencing revealed that the amino terminus of these fragments correspond exactly to proteins translated from the two identified ATG start sites. Alternatively, these dual products could be an in vitro artifact of the E. coli expression system; however, anti-NfxB antibodies, generated against the 21 kDa product, detected both the 21 kDa and the 23 kDa product in P. aeruginosa indicating that both products are normally present in vivo. Otherwise, the ~21 kDa protein could be a degradation product of the ~23 kDa protein. In previous studies, nfxB strains had been complemented by large restriction fragments containing the nfxB gene, all of which extended at least 70 bp into the mexC gene (174, 175, 193). Unfortunately, this does little to help establish which ATG is the correct or at least dominant start site as all previously cloned nfxB-containing DNA fragments contained both start sites and their respective putative Shine-Dalgarno sequences. Additionally, the mRNA
start site of \textit{nfxB} was determined to occur at a cytosine 93 bp upstream of the originally annotated ATG start site indicating that the \textit{nfxB} transcript would include both ATG start sites and the putative Shine-Dalgarno sequence for each (226). In an attempt to determine the minimum sequence of \textit{nfxB} required for expression, three cloned \textit{nfxB} constructs were engineered, the first including only the downstream ATG start site, the second including both possible start sites, and the third including the entirety of the \textit{nfxB-mexC} intergenic region. Surprisingly, none of these cloned \textit{nfxB} constructs were able to complement \textit{nfxB} mutant or deletion strains. It is possible that the upstream ATG start site is used to initiate translation making the shortest \textit{nfxB} construct, containing only the downstream ATG start, unable to express \textit{nfxB}. However, the other two constructs that contain the entire putative \textit{nfxB} gene including both ATG start sites and their respective putative Shine-Dalgarno sequences were also unable to complement. It seems that additional upstream sequence extending into \textit{mexC} (included in previously cloned \textit{nfxB} constructs) is somehow required for \textit{nfxB} expression, although the nature of this requirement is not understood. Future studies should assess expression of \textit{nfxB} from the three \textit{nfxB}-containing vectors using RT-PCR to determine if transcription of \textit{nfxB} is, in fact, occurring. Additionally, engineering longer constructs containing additional DNA upstream of \textit{nfxB} (like pNFX-1) may help elucidate how much upstream sequence is required for \textit{nfxB} expression.

4.2 A novel mutation causing \textit{mexCD-oprJ} hyperexpression

The \textit{P. aeruginosa} mutant K1536 was obtained from the wild-type strain K767 after selection on LB-agar plates containing 0.4 µg/ml ciprofloxacin (82). This strain demonstrated a phenotype similar to strains carrying a mutation in the \textit{nfxB} gene including \textit{mexCD-oprJ}
hyperexpression; however, sequencing of the nfxB gene of strain K1536 revealed that it was wild-type. Additionally, no mutations were found in the mexCD-oprJ promoter of strain K1536. Consequently, mexCD-oprJ hyperexpression in strain K1536 must be the result of some other mutation. This mutation could occur in another, as of yet unidentified, (direct or indirect) regulator of mexCD-oprJ expression. Alternatively, the mutation could bring about physiological changes in the cell that simulate membrane stress, which is known to upregulate mexCD-oprJ expression. As such, it would be interesting to resolve the nature of the mutation in strain K1536 because it would help further define how mexCD-oprJ is regulated. One approach would include using transposon mutagenesis in an attempt to recreate the mutant. Once recreated, sequencing would allow the identification of the mutated gene and perhaps provide additional insights into mexCD-oprJ regulation.

4.3 Instability of antimicrobial resistance in the K767 nfxB deletion strain

Previously, nfxB mutants have been recovered that stably hyperexpress mexCD-oprJ due to the absence of NfxB repressor activity (143, 175, 191). Similarly, hyperexpression of mexCD-oprJ in the NfxB-phenotype strain K1536 (mutation unknown) is also stable (82). However, while the strain K767 ΔnfxB demonstrated mexCD-oprJ hyperexpression, it had a tendency to spontaneously lose its resistance to MexCD-OprJ-effluxed antimicrobials when stored on LB-agar plates. The nature of this loss of resistance is unclear; although a likely possibility is through downregulation of mexCD-oprJ, which could be assessed using RT-PCR. It has been shown that mexCD-oprJ hyperexpressors have a reduced growth rate compared to wild-type strains, which could provide a strong selective pressure against these strains (92). It is unclear, however, why
this selective pressure would affect an \( nfxB \) deletion, and yet have no impact on, for example, \( nfxB \) missense mutants. The genetic and phenotypic differences in the parent strain K767, from which the \( nfxB \) deletion was derived, and K372, from which the \( nfxB \) mutant strain K385 was obtained, could account for higher stability of the \( nfxB \) mutation in strain K385. The development of an \( nfxB \) deletion in strain K372 could be used to determine if the parent strain impacts the stability of \( mexCD-oprJ \) hyperexpressing \( \Delta nfxB \) mutants. Although stable \( nfxB \) mutants have been derived from a parent PA01 strain closely related to K767 (143), the observed mutations in \( nfxB \) all occur at the 3’ end of the gene leaving the 5’ end unchanged. A possibility is that their protein products are partially functional and that \( mexCD-oprJ \) is not fully derepressed as it is expected to be with the complete loss of NfxB. Alternatively, NfxB may have other cellular roles or may act on other genes and these activities, while lost in the \( \Delta nfxB \) strain, might be retained in the \( nfxB \) missense mutant strains. The \( nfxB \) gene in the \( mexCD-oprJ \)-hyperexpressing strain K1536 is not mutated and, so, functional. Therefore, the mutation in strain K1536 could entail a much smaller fitness cost than loss of NfxB. Although, these differences could account for the stability of \( mexCD-oprJ \) hyperexpression in \( nfxB \) mutants and the NfxB-phenotype strain, the instability of the K767 \( \Delta nfxB \) strain remains perplexing and little understood.

### 4.4 Role of PA4596 in \( mexCD-oprJ \) expression under wild-type conditions

Considering that expression of PA4596 is normally minimal in \( P. aeruginosa \) (compared to expression from cloned PA4596 as seen in figure 9), it was not entirely unexpected that deletion of PA4596 had no impact on the expression of \( mexCD-oprJ \). Like \( nfxB \), PA4596 has a
second upstream putative translational ATG start site in addition to the annotated one; therefore, in order to overexpress PA4596, two PA4596 cloning vectors were engineered, the first, pACP06, containing only the original ATG start site, and the second, pACP07, containing both possible ATG start sites. Overexpression of PA4596 from pACP06 (including only the downstream ATG start site) did not have an effect on resistance to antimicrobials effluxed by MexCD-OprJ.

Attempts to electroporate pACP07 (expressing PA4596 including both ATG start sites) into strain K767 or its derivative strains such as K1536 or K767 ΔPA4596 ΔnfxB resulted in generation of spontaneous resistant mutants. It is important to note that antimicrobials are used to select for plasmid transformants after electroporation. For example, Tc 100 µg/ml is used to select for pRK415 derivatives in *P. aeruginosa*. During successful electroporation, any spontaneous mutants would be greatly outnumbered by plasmid transformants. It may be that pACP07 was unable to be maintained in K767 derivative strains after electroporation leaving only spontaneous mutants after the tetracycline selection. The mutants recovered demonstrated considerably increased resistance to carbenicillin indicative of *mexAB-oprM* hyperexpression (145). Additionally, an increased susceptibility to paramomycin indicates MexXY downregulation, which occurs during MexAB-OprM hyperexpression (119,144,159). Moreover, tetracycline exposure has been shown to select for *mexAB-oprM* hyperexpressors in *P. aeruginosa* (10). In contrast to K767 derivative strains, pACP07 was able to be electroporated into and maintained in strain K372. pACP07 was also shown to successfully express PA4596 in K372 as was assessed by RT-PCR. It is unclear what genetic difference between these two strains determines if pACP07 can be maintained. Another vector pACP08, including PA4596 and the PA4596-PA4595 intergenic region was able to be electroporated into and maintained in the *P. aeruginosa* strain K767. pACP08, like pACP06, had no impact on resistance to antimicrobials effluxed by
MexCD-OprJ. Therefore, it is unlikely that PA4596 has a role in regulating *mexCD-oprJ* under wild-type conditions.

### 4.5 Role of PA4596 in *mexCD-oprJ* expression during the NfxB-phenotype

It was observed that *mexCD-oprJ* hyperexpression seen in the K767 Δ*nfxB* strain was lost upon deletion of PA4596. Oddly, deletion of PA4596 had no impact in the *nfxB* mutant K385 or the NfxB-phenotype strain K1536. The abrogation of *mexCD-oprJ* hyperexpression seen when PA4596 is deleted from K767 Δ*nfxB* may actually be the previously discussed mutation-related reduction of *mexCD-oprJ* expression seen in the unstable K767 Δ*nfxB* strain and unrelated to loss of PA4596. In order to support that loss of PA4596 abrogated *mexCD-oprJ* hyperexpression, PA4596 was expressed in the K767 ΔPA4596 Δ*nfxB* strain from the vectors pACP06 (including only the original annotated ATG start site) and pACP08 (including the PA4596-PA4595 intergenic region). However, neither of these vectors complemented loss of PA4596 by restoring *mexCD-oprJ* hyperexpression. Alternatively, PA4596 may not be properly expressed by either pACP06 or pACP08; as was done with pACP07 in K372, RT-PCR could be used to confirm transcription of PA4596. Interestingly, Aaron Campigotto created a PA4596 deletion of strain K1536 called K2879, which demonstrated a loss of *mexCD-oprJ* hyperexpression (unpublished). In his studies it was found that this hyperexpression could be restored by complementing with the PA4596 expressing plasmid pAJC02, which includes only the annotated (downstream) ATG start site. However, I found that K2879 transformed by pAJC02 did not demonstrate quinolone antibiotic resistance attributable to *mexCD-oprJ* hyperexpression. Overall, it seems unlikely that PA4596 is involved in *mexCD-oprJ* hyperexpression.
4.6 PA4596 and NfxB may functionally interact

Gel retardation experiments demonstrated that NfxB is capable of specifically binding to the overlapping \textit{nfxB} and \textit{mexCD-oprJ} regulatory region (226). The identified NfxB binding region contains two 39 bp repeats (59\% homology) each likely capable of binding an NfxB molecule. Moreover, the spacing of these 39 bp repeats suggests that NfxB may bind as a homodimer (226). Furthermore, in the gel retardation experiments multiple shifted bands were observed suggesting that NfxB binds this region at more than one place (226). Indeed, 2-hybrid analysis showed that wild-type NfxB self-associates, consistent with forming homodimers; whereas, the non-\textit{mexCD-oprJ}-repressing NfxB\textsubscript{H87R} was incapable of self-associating. Additionally, PA4596 was shown to self-associate and was able to interact with NfxB. Assuming that these interactions are conserved \textit{in vivo}, it is possible that PA4596 may have a natural regulatory role in combination with NfxB. Still, these results impart two possibilities. The first consists of only dimeric interactions resulting in three distinct species: a PA4596 homodimer, an NfxB homodimer, and a PA4596-NfxB heterodimer each possibly with independent targets and effects on gene regulation. There are examples where multiple protein dimer signals are integrated via differential operator binding to allow unique regulatory schemes. Restriction-modification (R-M) systems play a role in modulating horizontal transfer of genes and excising pathogenic DNA. These systems include a methyl-transferase, which protects host DNA and a restriction endonuclease capable of cleaving unprotected DNA. R-M genes are regulated by Controller (C) proteins, which act as homodimers and have an upstream high affinity binding site and a downstream low affinity binding site on the R-M regulatory region. When the R-M operator is unbound by C protein dimers, R-M genes are not transcribed. Conversely, low
concentrations of C protein dimers result in binding to only the upstream high affinity binding site and activate R-M gene transcription. However, an increase in the C protein dimer concentration allows secondary binding to the low affinity downstream binding site and results in repression of R-M genes (148). A similar system could be used for regulation with NfxB and PA4596 dimers where differential binding could modulate gene expression. Another possibility is that PA4596 and NfxB could interact to form a heteromultimer, which could have a regulon distinct from NfxB. An RNA microarray could be used to detect gene expression changes between a wild-type strain and an nfxB derivative, which would help establish the NfxB regulon. Moreover, this regulon could be assessed additionally with RT-PCR in a PA4596 deletion strain to see if PA4596 also has a role in modulating some of those genes.

Most cellular processes are controlled by simple gene regulation systems such as a single activator or repressor. Inherently, all regulation systems by nature demonstrate a certain degree of stochastic fluctuations, where small random events can cause some fluctuation in gene expression (67). Normally insignificant, stochastic fluctuations can be detrimental when regulating sensitive, potentially-toxic, cellular processes. For example, trace amounts of iron, copper, and zinc are essential for activity of several enzymes. However, slight increases in the concentrations of these trace elements such as could be caused by stochastic fluctuation are severely cytotoxic to the cell. Thus, elaborate regulatory mechanisms are used to minimize stochastic fluctuation and protect the cell. Interestingly, heterodimeric regulation has shown to be an important mechanism in reducing stochastic fluctuations in regulatory circuits, where the binding of regulatory proteins acts as a buffer that prevents the propagation of fluctuations in gene activity. This mechanism is known to often be associated with regulators that self-regulate (like nfxB), have a broad regulon, or regulate potentially toxic responses (overexpression of
mexCD-oprJ is detrimental to the cell) (67). PA4596 and nfxB are simultaneously regulated by NfxB, meaning derepression will result in upregulation of both genes. Consequently, PA4596 could have a role fine-tuning NfxB based regulation or modulate NfxB repression in certain cellular states.
Chapter 5: Conclusions

While there are many possibilities of how PA4596 may affect NfxB repression considering the PA4596-NfxB interaction, there is no evidence at this time of what the function of PA4596 may be. Electrophoretic mobility shift assays could be used to determine if PA4596, possibly as a dimer, binds to mexC/NfxB and PA4596 promoters, thereby suggesting a regulatory role. Additionally, RNA microarray comparison of the wild-type strain K767 and its PA4596 deletion may help identify functions of PA4596 or gene targets of PA4596.
References


111


113


