Evidence of MexT-Independent Overexpression of MexEF-OprN Multidrug Efflux Pump of Pseudomonas aeruginosa in Presence of Metabolic Stress

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Abstract

Background: The Pseudomonas aeruginosa MexEF-OprN efflux pump confers resistance to clinically significant antibiotics. Regulation of mexEF-oprN operon expression is multifaceted with the MexT activator being one of the most prominent regulatory proteins.

Methodology: We have exploited the impaired metabolic fitness of a P. aeruginosa mutant strain lacking several efflux pumps of the resistance nodulation cell division superfamily and the TolC homolog OpmH, and isolated derivatives (large colony variants) that regained fitness by incubation on nutrient-rich medium in the absence of antibiotics. Although the mexEF-oprN operon is uninducible in this mutant due to a 8-bp mexT insertion present in some P. aeruginosa PAO1 strains, the large colony variants expressed high levels of MexEF-OprN. Unlike large colony variants obtained after plating on antibiotic containing medium which expressed mexEF-oprN in a MexT-dependent fashion as evidenced by clean excision of the 8-bp insertion from mexT, mexEF-oprN expression was MexT-independent in the large colony variants obtained by plating on LB alone since the mexT gene remained inactivated. A search for possible regulators of mexEF-oprN expression using transposon mutagenesis and genomic library expression approaches yielded several candidates but proved inconclusive.

Significance: Our results show that antibiotic and metabolic stress lead to up-regulation of MexEF-OprN expression via different mechanisms and that MexEF-OprN does not only extrude antimicrobials but rather serves other important metabolic functions.

Introduction

Pseudomonas aeruginosa is a non-fermentative Gram-negative nosocomial pathogen of significant clinical relevance. It is known to cause a variety of infections including pneumonia, bloodstream infections, urinary tract infections, endocarditis, and burn wound infections [1,2,3,4,5,6,7]. Infections caused by P. aeruginosa pose a considerable challenge in the clinical settings owing to its high intrinsic resistance to almost all antibiotics in clinical use [8,9,10,11,12,13,14]. Energy-mediated efflux of antibiotics by Resistance-Nodulation-Cell Division (RND) pumps in P. aeruginosa is considered the major factor responsible for its high antibiotic resistance. To date, 11 different RND pumps, capable of effluxing antibiotics/antimicrobial products, have been characterized in P. aeruginosa that include MexAB-OprM [15], MexCD-OprJ [16], MexEF-OprN [17], MexGH-OpmD [18], MexJK-OpmM/OpmH [19], MexMN [20], MexPQ-OpmE [20], MexVW-OprM [21], MexXY-OprM [22], TriABC-OpmH [23], and MusABC-OpmB [24]. Expression of these pumps is usually under tight regulatory control, however molecular mechanisms that regulate the expression of a number of these pumps are not fully understood. Elucidating the mechanisms of regulation of RND pump will not only aid in a better understanding of the antibiotic resistance of P. aeruginosa but will also provide valuable insights into their natural function.

The MexEF-OprN pump is the only positively regulated RND pump of P. aeruginosa and its expression is activated by a LysR family protein, MexT, encoded by a gene located upstream of the mexEF-oprN operon [25]. It has been shown to efflux fluoroquinolones, trimethoprim, and chloramphenicol [17,26]. MexEF-OprN overexpression has also been shown to be associated with a concurrent downregulation of the outer membrane protein OpmD, which in turn results in decreased susceptibility to imipenem [17,27]. Interestingly, a number of isolates of P. aeruginosa have been reported that contain a 8-bp insertion in the mexT gene that results in an inactive MexT protein that is unable to activate the expression of mexEF-oprN operon [28]. In addition, the regulation of mexEF-oprN operon has been shown to be...
controlled by MexS, an oxidoreductase, as well [27]. MvaT, a global regulator of virulence genes in *P. aeruginosa*, has also shown to be able to repress the expression of the MexEF-OprN pump [29]. In addition to resistance to antibiotics, the overexpression of the MexEF-OprN pump was linked to reduced production of extracellular virulence factors like pyocyanin, elastase, and rhamnolipids [30]. Recent studies also link MexT with the expression of the type III secretion system in *P. aeruginosa* [31]. MexEF-OprN overexpression has also been observed in response to nitrosative stress in absence of any antibiotics [32,33]. These studies indicate involvement of a complex mexEF-oprN regulatory network which largely remains to be elucidated.

The present study was performed to advance our understanding of the regulatory mechanisms involved in the expression of MexEF-OprN. We show that, in the absence of other RND pumps, the expression of MexEF-OprN pump can be activated in a nutrient rich-medium in absence of any antibiotics. We also identify putative novel proteins that could be involved in the regulation of this RND independent of the MexT protein. We show that, in the absence of other RND pumps, the expression of MexEF-OprN pump can be activated in a nutrient rich-medium in absence of any antibiotics. We also identify putative novel proteins that could be involved in the regulation of this RND pump.

**Results**

A *P. aeruginosa* multiple efflux pump-deficient mutant regains fitness by overexpressing MexEF-OprN

*P. aeruginosa* PAO386 (Table 1) was used for our studies. This strain contains deletions in four different RND pump encoding operons, namely mexAB-oprM, mexCD-oprJ, mexJK, and mexXY. In addition, it also lacks the structural gene for the outer membrane protein OpmH, the *P. aeruginosa* TolC homolog. The MexEF-OprN pump is not expressed in PAO386 as a result of an 8-bp insertion in the mexT gene. This strain exhibits a slow growing phenotype and the colonies on LB agar (without supplementation of any antibiotics) have a pin-point morphology after an overnight incubation at 37°C. However, following an extended incubation (5–7 days) at room temperature, a few large-colony variants (with stable phenotype, for example unsectored) were observed (Fig. 1). One such colony was isolated, designated PAO573, and retained for further characterization.

To assess whether the increased fitness of PAO573 was due to expression of an efflux pump, we assessed the antibiotic susceptibility profile of this strain. PAO573 was highly resistant to chloramphenicol (MIC >1024 μg/mL) and trimethoprim (MIC >1024 μg/mL) when compared to PAO386 (in this strain both chloramphenicol and trimethoprim MICs were 16 μg/mL). Since both of these antibiotics are MexEF-OprN pump substrates [17] we suspected possible expression of this efflux system in PAO573.

To assess this notion, a ciprofloxacin resistant derivative was isolated by plating PAO386 onto LB agar supplemented with 0.05 μg/mL of ciprofloxacin, a condition known to select for MexEF-OprC expressing NfxC-type mutants. One colony growing after incubation at 37°C for 48 h was selected and the strain designated as PAO393. This mutant was also highly resistant to chloramphenicol and trimethoprim, exhibiting the same MIC values as PAO573 (>1024 μg/mL).

**Confirmation of MexEF-OprN expression in PAO393 and PAO573**

To confirm MexEF-OprN expression in PAO393 and PAO573, we first performed Western blot analysis of whole cell lysates using anti-OprN antibodies (Fig. 2). OprN could not be detected in the

### Table 1. *P. aeruginosa* strains used in this study.

| Strain       | Relevant characteristics                                                                 | Source/Reference |
|--------------|------------------------------------------------------------------------------------------|------------------|
| PAO1         | *P. aeruginosa* prototroph. Contains the 8-bp insertion in the mexT gene, and thus the mexEF-oprN operon is uninducible. | [46]             |
| PAO327       | PAO1 with Δ(mexAB-oprM) Δ(mexCD-oprJ) Δ(mexXY)                                           | [19]             |
| PAO386       | PAO1 with Δ(mexAB-oprM) Δ(mexCD-oprJ) Δ(mexXY) Δ(opmH)                                   | This study       |
| PAO393       | Cp' mutant expressing mexEF-oprN derived from PAO386 plated on Cp-supplemented LB agar   | This study       |
| PAO573       | Large colony variant expressing mexEF-oprN derived from PAO386 plated on LB agar         | This study       |
| PAO599       | Gm'; PAO386::mini-Tn-T-Gm-mexElacZ                                                      | This study       |
| PAO600       | Gm'; PAO393::mini-Tn-T-Gm-mexElacZ                                                      | This study       |
| PAO601       | Gm'; PAO573::mini-Tn-T-Gm-mexElacZ                                                      | This study       |
| PAO602       | Gm', PAO1::mini-Tn-T-Gm-mexElacZ                                                        | This study       |
| PAO604       | PAO1::mini-Tn-T-mexElacZ; Gm-cassette deleted from PAO602                                | This study       |
| PAO605       | PAO386::mini-Tn-T-mexElacZ; Gm-cassette deleted from PAO599                              | This study       |
| PAO606       | PAO393::mini-Tn-T-mexElacZ; Gm-cassette deleted from PAO600                              | This study       |
| PAO607       | PAO573::mini-Tn-T-mexElacZ; Gm-cassette deleted from PAO601                              | This study       |
| PAO706       | Gm'; PAO327::mini-Tn-T-Gm-mexElacZ                                                      | This study       |
| PAO707       | PAO327::mini-Tn-T-Gm-mexElacZ; Gm-cassette deleted from PAO706                           | This study       |
| PAO709       | Gm', PAO707 with ΔPAO2050::Gm                                                        | This study       |
| PAO712       | Gm', PAO707 with ΔPAO487::Gm                                                        | This study       |
| PAO719       | PAO707 with ΔPAO487; Gm-cassette deleted from PAO712                                   | This study       |
| 8485         | Tc'; mvaT::ISlacZ/hah (transposon insertion at nucleotide 57 relative to the start codon) | UWGC             |
| PAO1081      | Gm'; 8485::mini-Tn-T-Gm-lacZ                                                           | This study       |
| PAO1084      | Gm', 8485::mini-Tn-T-Gm-mexElacZ                                                      | This study       |

Abbreviations: Cp, ciprofloxacin; Gm, gentamicin; mexElacZ, mexE promoter; *, resistant; Tc, tetracycline; UWGC, University of Washington Genome Center.

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MexE-OprN expression in PAO573 is independent of MexT

Since lacZ fusion and Western blot experiments confirmed transcriptional and translational expression of MexE-OprN, we next probed the status of mexT in the two mutant strains since the parental strain PAO393 was derived from a PAO1 strain with an 8-bp insertion in mexT. PCR amplification and sequencing of the mexT gene from PAO1, PAO393 and PAO573 revealed that the 8-bp insertion was lost from PAO393 (the mutant isolated from ciprofloxacin-supplemented media) but not from PAO573 (the mutant strain isolated as a large colony variant on LB agar) (Fig. 4). This indicates MexT-dependent MexE-OprN expression in PAO393 but MexT-independent expression of this efflux pump in PAO573.

Attempts at identification of mexEF-oprN regulatory proteins other than MexT

Since the mexEF-oprN operon expression was constitutively expressed in a MexT-independent manner in PAO573 we hypothesized that additional regulatory protein(s) may be involved in regulation of its expression. We attempted two experimental approaches to test this hypothesis, random mutagenesis of mexEF-oprN promoter–lacZ bearing strains and identification of clones in a P. aeruginosa expression library that either up- or down-regulate β-galactosidase expression in mexEF-oprN promoter–lacZ bearing strains.

Random mutagenesis approaches

In order to identify elements involved in the regulation of MexEF-OprN pump, our first approach involved random mutagenesis using the Mariner transposon delivery vector pBT20 [34], of PAO604 (PAO1::mexE promoter–lacZ) in order to identify potential repressor protein(s). Since colonies of PAO604 are white on LB agar plates supplemented with X-gal, we screened for blue colonies following the transposon mutagenesis as an insertion in a repressor gene should result in increased expression of the β-galactosidase gene under the control of mexE promoter. However, in spite of several attempts, we were unable to find any mutants that showed up-regulation of β-galactosidase expression.

In an attempt to find potential activator(s), we attempted random mutagenesis screen in PAO607 (PAO573::mexE promoter–lacZ fusion). We assumed that if the expression of mexEF-oprN in this strain was due to an altered activity of an activator molecule then disruption of such molecule would result in white colonies on LB-agar supplemented with X-gal. However, our attempts repeatedly identified insertions in the lacZ gene only.

Screening of a P. aeruginosa expression library

Since transposon mutagenesis did not identify any regulatory protein candidates, we decided to search for a potential repressor.

MexE-OprN expression in PAO573 is independent of MexT
using an alternative approach. We transformed PAO607 (PAO373::mexE_{promoter}-lacZ) with a PAO1 plasmid library and identified four colonies harboring recombinant plasmids that could repress the expression of a β-galactosidase gene under the control of the mexE promoter (Fig. 5). Sequencing of the four recombinant plasmids thus identified – pPS1640, pPS1642, pPS1643 and pPS1644 – revealed several possible regulatory protein candidates. Plasmid pPS1640 harbored PA2050 that encodes for a probable sigma factor. A recent study has speculated on the role of a yet unidentified sigma-factor in the expression of MexEF-OprN pump (negative expression control) and PAO607 is PAO327::mvaT strain with or without plasmids containing various PA genes. PAO604 is PAO1::mvaT strain with or without plasmids containing various PA genes. PAO573 contains the ORFs PA2486 and PA4047. PA2486 encodes a protein with homology to serine/threonine kinases and PA4047 encodes a probable molybdenum transport regulator. We decided to investigate its role in the expression of MexEF-OprN operon. For reasons detailed above, we used again the PAO327 background and created PAO719 by deleting PA0487 from PAO707 which is an unmarked derivative of PAO327 containing the mvaT gene, the product of which has been reported fusion in this strain (PAO1084), however the strain did not show any increased promoter activity compared to the derivative (PAO1081) containing the promoter-less lacZ insertion (data not shown).

The plasmid pPS1643 contains the ORFs PA2486 and PA4047. PA2486 encodes a protein with homology to serine/threonine kinases and PA4047 encodes a probable molybdenum transport regulator. We decided to investigate its role in the expression of MexEF-OprN operon. For reasons detailed above, we used again the PAO327 background and created PAO719 by deleting PA0487 from PAO707 which is an unmarked derivative of PAO327 containing the mvaT gene, the product of which has been reported fusion in this strain (PAO1084), however the strain did not show any increased promoter activity compared to the derivative (PAO1081) containing the promoter-less lacZ insertion (data not shown).

The expression of MexEF-OprN pump in the wild type strain P. aeruginosa PAO1. Additionally, we did not find any mutations in the PA2050 gene in PAO573 (data not shown).

Plasmid pPS1642 was also shown to repress the β-galactosidase activity in our reporter strain. This plasmid contains a 1,710 bp insert that includes a partial sequence of the mvaT gene, missing 124 bp of the 3’-end. Even though the insert contained only a partial sequence of the mvaT gene, the product of which has been implicated in the expression of mexEF-oprN operon [29], we used a mvaT insertion mutant available from the University of Washington Genome Center in order to investigate its role in the expression of MexEF-OprN system in our strain. We inserted the mini-Tn71\(\text{mexE}_{\text{promoter-}}\text{lacZ}\) reported fusion in this strain (PAO1084), however the strain did not show any increased promoter activity compared to the derivative (PAO1081) containing the promoter-less lacZ insertion (data not shown).

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for a AraC family transcriptional regulator, and PA2490 encoding a hypothetical protein. Since MexS was previously shown to be involved in the regulation of mexEF-oprN operon expression [27], we sequenced the mexS genes from PAO386 and PAO573. Sequence comparisons did not reveal any mutations in the mexS gene of PAO573 (data not shown). Also, since MexS activity is MexT-dependent and since mexT gene product in PAO573 remains inactive as a result of the 8-bp insertion, we do not believe that MexS plays a role in the repression of mexE_promoter-lacZ encoded β-galactosidase activity by pPS1644. In order to

**Figure 6. Effect of PA2050, PA0487, and PA2489 on the expression of mexEF-oprN operon.**

**A. Deletion of PA2050 in PAO707**. PAO709 contains a gene deletion of PA2050 introduced into PAO707 which is PAO1 with Δ(mexAB-oprM) Δ(mexCD-oprJ) Δ(mexXY) and chromosomally-integrated mexE_promoter-lacZ; pPS1640 contains the gene PA2050 cloned in pUCP20. **B. Deletion of PA0487 in PAO707**. PAO719 contains a gene deletion of PA0487 in PAO707; pPS1643 contains the gene PA0487 in pUCP20. **C. Expression of PA2489**. pPS1648 containing the PA2489 gene was electroporated into PAO607 (PAO573::mexE_promoter-lacZ); pUCP20 was used as the vector control. PAO604 is the PAO1::mexE_promoter-lacZ negative control and PAO607 is the PAO573::mexE_promoter-lacZ positive control.

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investigate the possible role of PA2489 (encoding an AraC family transcriptional regulator), we deleted a 2,291 fragment that contained mexT and 180 bp of the 3'-end of the PA2490 gene thus effectively removing all three (mexT, mexS, and PA2490) genes from pPS1644 but leaving PA2489 intact. However, transformation of the resulting pPS1648 into PAO607 did not result in any decrease in β-galactosidase activity (Fig. 6C).

Discussion

In this study, we attempted to identify factors that control the expression of MexEF-OprN pump in the absence of antibiotic substrates. To avoid interference by other efflux systems we employed a P. aeruginosa strain that is lacking four different RND pumps [MexAB-OprM, MexCD-OprJ, MexJ, and MexXY]. In addition, the strain also lacks the outer membrane protein OpmH, a TolC homolog, that has been shown to function with at least two different RND pumps in P. aeruginosa, MexJ [36] and TriABC [23]. We were readily able to isolate MexEF-OprN overexpressing mutants of this strain in the presence of media supplemented with ciprofloxacin. These mutants consistently showed a loss of the 8-bp insertion in the mexT gene resulting in expression of active MexT which induces the expression of the MexEF-OprN pump. However, in absence of any antibiotics in the media, colonies of PAO386 appear very small and have a pin-point morphology, some of which upon longer incubation give rise to a large colony variant. These large colony variants showed an increased expression of MexEF-OprN pump as confirmed in the representative strain PAO573 by increased MICs to MexEF-OprN substrates, detection of OprN by immunoblotting with anti-OprN antibodies (Fig. 2), β-galactosidase assays with strains expressing mexEpromoter-lacZ transcriptional fusions (Fig. 3), and also qRT-PCR using mexEF-oprN operon specific primers (data not shown). Since the 8-bp insertion was still present in the mexT gene of PAO573, the overexpression of the MexEF-OprN operon was independent of MexT protein suggesting a role of other regulator(s) in the expression of this pump.

A search for such regulator(s) using a transposon mutagenesis approach was unsuccessful, but screening of a PAO1 expression library for clones capable of repressing β-galactosidase expression in a PAO573mexEpromoter-lacZ host revealed several candidates. We identified and sequenced four different recombinant plasmids from the screening of the PAO1 library, namely pPS1640 (containing the ORF PA2050), pPS1642 (containing the partial sequence of PA2053), pPS1643 (containing the ORFs PA0486-PA0487), and pPS1644 (containing PA2489-mexS-mexT), that were found to repress the expression of the reporter β-galactosidase gene in the host strain (Fig. 5). PA0487 encodes a probable molybdenum transporter regulator, PA2050 encodes a probable sigma factor, while PA4315 encodes for the transcriptional regulator MvaT. The insert in pPS1644 contains a gene (PA2489) that encodes an AraC family transcriptional regulator, a gene (PA2490) that encodes for a hypothetical protein, and also the mexS and mexT genes. While these genes repress mexE promoter activity in the PAO573 background, deletion and re-transformation analyses gave inconsistent results, especially PA0487 and PA2050, and did not yield the same phenotypes, i.e. mexEF-oprN expression, when the respective genes were mutated in PAO1. Furthermore, when PCR-amplified from PAO573 the genes contained on and presumably expressed by the respective plasmids did not contain any mutations.

The other reasons for inconsistent observations for deletion phenotypes for PA0487 and PA2050 could be the unstable nature of our mutants. One notable point is that the phenotype that we observed in PAO573 results from the deletion of OpmH, which may indicate that the possible physiological stress on this strain could be a cumulative effect of hampered activity of other yet uncharacterized pumps that may require OpmH for function, and the mexEF-oprN over-expression phenotype is likely to be a result of accumulation of metabolic by-products or secondary metabolites. Also, our study is difficult to repeat in the wild-type strain since the activity of other pumps (that were deleted in PAO386) would most likely prevent the over-expression of MexEF-OprN pump.

In conclusion, our study shows that the expression of MexEF-OprN pump can be derepressed in absence of antibiotic stress as shown by some of the previous studies [32,33]. We also provide further evidence for regulation of the expression of mexEF-oprN pump being under control of a very complex regulatory network and that additional studies are required to understand the underlying mechanisms. In support of this notion, a recent study postulated transcriptional regulation of the mexEF-oprN multidrug efflux operon by an unidentified repressor [34] but it remains pure speculation whether inactivation of this repressor might be the root cause for the observed mexEF-oprN operon over-expression in PAO573. In this context, however, it is interesting to note that just as deletion of the putative repressor-binding site caused high-level mexEF-oprN expression [34], the unknown mutation(s) present in PAO573 also caused a level of MexT-independent mexEF-oprN expression that was significantly higher than that observed in PAO939 where mexEF-oprN expression was MexT-dependent (Fig. 3). The most striking discovery of the present study is that antibiotic and metabolic stressors lead to MexEF-OprN over-expression by independent mechanism(s). Antibiotic stress caused mexEF-oprN over-expression via a MexT dependent mechanism whereas metabolic stress caused expression of this operon via a MexT-independent mechanism. While the nature of the metabolic signal(s) causing MexEF-OprN expression remain unknown, our results provide further evidence that RND efflux pumps not only extrude antimicrobials but rather serve other important metabolic functions.

Materials and Methods

Bacterial strains, plasmids and growth conditions

P. aeruginosa strains used in this study are listed in Table 1. E. coli DH5α (Invitrogen, Carlsbad, CA) was used for the gene cloning experiments. LB medium (EM Sciences, Gibbstown, NJ) was used for the growth of E. coli and P. aeruginosa strains. When required, the growth medium was supplemented with the following antibiotics: ampicillin (Ap) (Sigma, St. Louis, MO) (100 μg/mL, E. coli), carbenicillin (Cb) (Gemini Bioproducts, Sacramento, CA) (200 μg/mL, P. aeruginosa), and gentamicin (Gm) (Sigma) (30 μg/mL, P. aeruginosa).

MexEF-OprN overexpressing strains were isolated by streaking PAO386 on LB agar or LB agar supplemented with 0.05 μg/mL of ciprofloxacin (Cp; Sigma). A list of plasmids used in this study is provided in Table S1.

Antibiotic susceptibility assays

Antibiotic susceptibility testing was performed for chloramphenicol and trimethoprim using the two-fold microdilution method as specified by the Clinical Laboratory Standards Institute [37]. Both antibiotics were purchased from Sigma.

Polymerase chain Reactions, DNA manipulations, and genetic techniques

PCR reactions were performed using either Taq DNA polymerase (New England Biolabs, Beverly, MA) or HiFi high-fidelity Taq polymerase (Invitrogen, Carlsbad, CA).
Extraction of plasmid and genomic DNA and gel purification of the DNA was performed using kits available from Qiagen (Qiagen, Valencia, CA). Transfer of DNA into P. aeruginosa strains was achieved either by using tri-parental mating [39] or by the 10-minute rapid electroporation [39] methods described previously. Construction of the reporter fusion using the promoter region of the mexEF-oprN operon (mexE\_promoter) and the promoter-less E. coli lac\_Z gene was accomplished as follows. The promoter region of the mexEF-oprN operon was amplified on a 683-bp fragment from PAO1 genomic DNA using Taq polymerase, primers 5’-GGCGAGTGCGACTCGAGATATTGCC (a PstI site is underlined) and 583’-TTTCCGAGCTTGCGCGGAGGCGCTCA (a HindIII site is underlined) and cloned into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) to obtain pPS1453 [40] digested with the same enzymes to derive pPS1519. Assembly of the mexE\_promoter-lac\_Z fusion construct in a mini-Tn7 vector allows the insertion of the reporter fusion in single copy in the P. aeruginosa chromosome, which was achieved using the helper plasmid pTNS2 [40] and a previously published protocol [40]. Confirmation of the insertion and the subsequent removal of the Gm\_Z cassette was performed by protocols described previously [40].

Creation of unmarked gene deletions in P. aeruginosa was performed using previously described methods [41].

Immunodetection of OprN
Western blot detection of the OprN protein (the outer membrane component of the MexEF-OprN pump) was performed using an anti-OprN polyclonal antiserum. One mL of bacterial cells grown in LB broth to a density of A_{600} \sim 1.0 were harvested and normalized using 2× sample buffer (0.0025 M v/v bromphenol blue, 20% v/v glycerol, 6% v/v β-mercaptoethanol, 2.5% v/v SDS in Tris Buffer). Normalization was carried out as follows, A_{600} x 0.08 = μL 2× sample buffer. The cell suspension was boiled for 5 minutes and an equal volume of each sample (3-5 μL) was loaded on a 10% SDS-polyacrylamide gel. Immunodetection of the OprN protein was carried out using previously described protocols [42].

β-galactosidase assays
Reporter strains containing the mexE\_promoter-lac\_Z fusion inserted in the genome were constructed as described above for PAO1, PAO386, PAO393, and PAO573 to derive PAO604, PAO605, PAO606, and PAO607, respectively.

β-galactosidase assays were performed using a previously described method [43]. Briefly, overnight cultures of bacterial strains grown in LB-broth at 37°C were subcultured in fresh LB using a 1:200 inoculum and cultures were grown until they reached an optical density of approximately one at 600 nm (A_{600} = 1.0), at which point, one mL aliquots were removed and cells harvested at 13,000 rpm in a microcentrifuge. Cell pellets were resuspended in 0.1 M phosphate buffer (pH 7.0). Permeabilization of cells was achieved by addition of SDS/chloroform. Assays of β-galactosidase activity and activity unit calculations were performed as described previously [44].

Construction of P. aeruginosa library
A P. aeruginosa library was constructed by partially digesting genomic DNA from the wild-type PAO1 with EcoRI and EcoRI+BamHI and purifying the DNA fragments ranging from approximately 1.5 Kb to 4 Kb from an agarose gel. The DNA fragments were dephosphorylated using alkaline phosphatase (New England Biolabs) following the manufacturer’s instructions and then ligated into the broad-host range cloning and expression vector pUCP20 [45] digested with EcoRI and EcoRI+BamHI, respectively. The ligation mixtures were transformed into E. coli DH5α cells and plated on LB agar supplemented with ampicillin (100 μg/mL) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/mL) (Gold Biotechnology, St. Louis, MO). Following an overnight incubation at 37°C, 825 white colonies were selected and patched on fresh LB agar plates supplemented with ampicillin (100 μg/mL) and X-gal (40 μg/mL). The patches were washed off using saline and inoculated into LB broth supplemented with ampicillin, plasmids were extracted following an overnight incubation at 37°C with shaking, and electroporated into PAO607 (the derivative of PAO573 containing the chromosomally-integrated mini-Tn7-mexE\_promoter-lac\_Z fusion). Cells were plated on LB agar supplemented with Cb (200 μg/mL) and X-gal (40 μg/mL). Following an overnight incubation at 37°C, four white to light blue colonies were observed and selected for further analysis. Plasmids were extracted from these four P. aeruginosa PAO607 transformants and sequenced at Colorado State University’s Proteomics and Metabolomics Facility.

Supporting Information
Table S1 List of plasmids used in this study.

(DOCX)

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Author Contributions
Conceived and designed the experiments: AK HPS. Performed the experiments: AK. Analyzed the data: AK HPS. Contributed reagents/materials/analysis tools: AK. Wrote the paper: AK HPS.
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