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MexXY efflux pump overexpression and aminoglycoside resistance in cystic fibrosis isolates of *Pseudomonas aeruginosa* from chronic infections.

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ABSTRACT

In this study, we analyzed 15 multidrug resistant cystic fibrosis isolates of *Pseudomonas aeruginosa* from chronic lung infections for expression of four different multidrug efflux systems, MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY, using quantitative reverse transcriptase-PCR. Overexpression of MexXY pump was observed in all of the isolates tested. Analysis of regulatory genes that control the expression of these four efflux pumps revealed a number of previously uncharacterized mutations. Our work shows that MexXY pump overexpression is common in cystic fibrosis isolates and could be contributing to their reduced aminoglycoside susceptibility. Further, we also identified novel mutations in the regulatory genes of four above-mentioned RND pumps that may be involved in the overexpression of these pumps.

Keywords: aminoglycoside resistance, MexZ, regulatory gene mutations

INTRODUCTION

Pseudomonas aeruginosa, a non-fermenting Gram negative organism, is notorious for causing multidrug resistant infections in immunocompromised individuals (Weinstein et al. 2005). Recently, the World Health Organization (WHO) classified *P. aeruginosa* as 'critical' among the list of twelve priority bacterial pathogens for which new antibiotics are urgently needed (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/). In Canada, it is one of the leading causes of respiratory tract infections in intensive care units (ICUs) (Zhanel et al. 2010; Zhanel et al. 2008). In cystic fibrosis (CF) patients particularly, multidrug resistance of *P. aeruginosa* poses a very significant problem. It is estimated that up to 80% of adult CF are colonized with *P. aeruginosa* (Canadian Cystic Fibrosis registry 2013 annual report, http://www.cysticfibrosis.ca/uploads/cf%20care/Canadian-CF-Registry-2013-FINAL.pdf). High rates of resistance displayed by *P. aeruginosa* to antibiotics makes treatment of these infections especially challenging. While *P. aeruginosa* uses a variety of mechanisms to evade the action of antibiotics, its intrinsic resistance to several antibiotics is primarily attributed to the activity of energy-dependent efflux systems belonging to the Resistance-Nodulation-Division superfamily (RND) (Kumar and Schweizer 2005; Li et al. 2015). These are tripartite systems that form a channel across the cell envelope of Gram-negative bacteria and facilitate the extrusion of compounds directly into the external environment using proton-gradient as energy source (Nikaido and Takatsuka 2009). Twelve different RND systems have been described in *P. aeruginosa*, at least four of which (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY) have been shown to be routinely overexpressed in various clinical isolates (Li et al. 2015).

Transcriptional regulation of RND pumps is generally mediated by a regulatory protein encoded upstream of the RND operon (Kumar and Schweizer 2005). However, it is becoming increasingly clear

that the transcriptional regulation of RND pumps in organisms like *P. aeruginosa* is quite complex (Li et al. 2015) and that RND pump overexpression can occur not only in response to the presence of antimicrobials but also to various environmental stresses (Alvarez-Ortega et al. 2013; Poole 2012). For example, in CF isolates of *P. aeruginosa*, overexpression of the MexXY pump has been linked to environmental conditions that the organism encounters in CF lungs (Morita et al. 2006). In order to gain more insights into the interplay between the antibiotic susceptibility, expression of RND pumps, and the underlying mechanisms of RND pump expression, we analyzed the expression of RND pumps in antibiotic resistant CF isolates of *P. aeruginosa* and sequenced their regulatory genes that may be responsible for their overexpression.

MATERIALS AND METHODS

Bacterial isolates and growth conditions

Bacterial isolates used in this study are listed in Table 1. These isolates were collected from sputum samples of CF patients (adult and paediatric) with chronic infection at the Hospital for Sick Children, St. Michael's Hospital, and Hamilton Health Sciences Center in Ontario from 2009 – 2013, as part of another research study (Yau et al. 2015). No information is available regarding the antibiotic usage in these patients. Lysogeny Broth (LB) growth medium (Becton, Dickinson and Company, Mississauga, ON, Canada) was used to culture cells at 37^{o}C with shaking, unless otherwise indicated.

Antibiotic susceptibility assays

Antibiotic susceptibility testing for all antibiotics, with an exception of chloramphenicol and colistin, was performed by broth microdilution using Sensititre GN2F panels (Trek Diagnostic Systems, Oakwood Village, Ohio, USA) according to manufacturer's instructions. For colistin, susceptibility was performed by disk diffusion method according to CLSI guidelines (CLSI 2006). Briefly, 3 to 5 colonies

of the *P. aeruginosa* isolate were inoculated into sterile saline and adjusted to a 0.5 McFarland turbidity standard using the Vitek Colorimeter. Ten µL of the 0.5 McFarland suspension was transferred into 11 mL of cation-adjusted Mueller Hinton broth with TES buffer. Fifty µL of the well mixed suspension was then dispensed into each well of the susceptibility panel. For disk diffusion testing, sterile cotton swab was dipped into the 0.5 McFarland suspension and streaked evenly in three directions over the entire surface of a Mueller Hinton Agar plate (Oxoid Inc., Napean, ON, Canada). Colistin (10 µg) disks (Oxoid) were applied to the inoculated plate. Each panel and plates were incubated aerobically at 37^{o}C for 24 hours. Panels were examined manually for growth in the wells and the inhibition zone size was measured for the disk diffusion testing. Susceptibility testing for chloramphenicol was carried out using the two-fold broth dilution method in a 96-well plate.

Results were interpreted using guidelines provided by CLSI (M100-27) (CLSI 2017). For chloramphenicol, guidelines used for interpretation is based on the non-Enterobacteriaceae interpretative criteria published in 2006 (CLSI M100-S16) (CLSI 2006).

RNA extraction and complementary DNA (cDNA) synthesis

Overnight culture of *P. aeruginosa* isolates was diluted (1:100) in LB medium (without antibiotics) and incubated at 37°C with shaking (200 rpm). One mL culture was then harvested at an OD_{600} of approximately 0.65 and pellets were frozen at -80°C overnight. RNA was extracted using commercially available RNeasy Mini Kit (Qiagen, Toronto, ON, Canada) using manufacturer's instructions. RNA was treated with DNAse I (Qiagen) to remove any genomic DNA carryover. Complementary DNA (cDNA) was synthesized using the iScript Reverse Transcriptase Supermix (BioRad, Mississauga, ON, Canada), using 200 ng of total RNA in a 20 µL reaction volume. Minus-RT controls were prepared, using the pool of RNA from all samples and by excluding the reverse transcriptase enzyme.

qRT-PCR

qRT-PCR reactions were performed using SsoFast Evagreen Supermix (BioRad). The RND pumps-encoding genes analyzed included *mexB, mexD, mexF* and *mexY* with *rpsL* used as housekeeping control. Primers, listed in Table 2, were designed using GeneScript online platform (www.genscript.com). Primer efficiencies were tested either by using cDNA or genomic DNA from PAO1 using protocols described previously (Fernando and Kumar 2012; Pfaffl 2001). qRT-PCR reactions were performed by diluting the cDNA mix 10-fold and using 1.5 µL of diluted cDNA as template in a total reaction volume of 5 µl that contained 300 nM of each primer. Relative quantification was carried out for at least two independent biological replicates with three technical replicates using Eco Real-Time PCR System (Illumina, San Diego, CA, USA). Expression of all target genes were normalized using the reference gene *rpsL*, and the expression was calculated relative to that of PAO1.

Sequencing of the regulatory genes

Regulatory genes associated with different RND pumps (*mexR,* and *nalD* for MexAB-OprM; *nfxB* for MexCD-OprJ; *mexT* and *mexS* for MexEF-OprN; *mexZ* and *parR* for MexXY) were amplified using the primers listed in Table 2. PCR products were purified using the Gene JET gel extraction kit (Thermo Scientific, Burlington, ON, Canada) by following the manufacturer's protocol. PCR products were sequenced at the sequencing facility of Genome Quebec at McGill University (Montreal, QC, Canada). All regulatory gene sequences were compared with the sequence of PAO1 (www.pseudomonas.com).

RESULTS

Antibiotic susceptibility of *P. aeruginosa* isolates

Results of antibiotic susceptibility testing are shown in Table 1. These isolates displayed resistance to various classes of antibiotics. Aminoglycoside resistance was prevalent in these isolates, with 11/15 isolates resistant to amikacin, 15/15 resistant to gentamicin, and 11/15 resistant to tobramycin. Two-third of the isolates (10/15) were resistant to ciprofloxacin (a fluoroquinolone). Five isolates were resistant to each of two cephalosporins tested (ceftazidime, a third-generation cephalosporin, and cefepime, a fourth-generation cephalosporin). Three isolates (PAAK088, PAAK089, and PAAK095) were resistant to both ceftazidime and cefepime. Aztreonam (a monobactam), meropenem (a carbapenem), and colistin had a relatively good activity against these isolates.

Expression of efflux pump-encoding genes

Results of expression analysis are shown in Table 1. We observed that MexXY pump was the most commonly expressed efflux pump with all but two isolates showing at least 4-fold higher expression of *mexY* than the control PAO1 strain. Eight isolates overexpressed (>4-fold) *mexF*, while five isolates overexpressed *mexB,* and three isolates *mexD*. Ten of the isolates tested concurrently overexpressed more than one kind of pump, for example six isolates (PAAK082, PAAK084, PAAK087, PAAK091, PAAK093, and PAAK094) overexpressed at least three different pumps. We were unable to detect (as confirmed by at least four independent assays) *mexB, mexD,* and *mexF* transcripts in PAAK088; and *mexF* transcript in PAAK082. However, PCR reactions using the DNA template confirmed the presence of the respective genes in these isolates (data not shown), suggesting either very low or no expression of respective pumps in these two isolates.

Analysis of regulator genes for mutations

A summary of the mutations found in the regulator gene(s) for various efflux pump operons is listed in Table 3. We also found several SNPs (single nucleotide polymorphisms) in various regulatory genes

that did not result in a change in the amino acid residues (Supplementary Table 1). Two regulators tested in our study that control the expression of the MexAB-OprM pump are MexR and NalD. In *mexR*, ^{126}V→E, was the most common mutation, present in five different isolates (PAAK083, PAAK084, PAAK087, PAAK092, and PAAK096). In the case of *nalD*, we observed a two nucleotide (^{397}GT) deletion in PAAK084 and PAAK087. Further PAAK083 also contained a two nucleotide deletion (^{398}TG). In addition, PAAK091 contained single amino acid change (^{187}D→H) in the C-terminal end of the NalD.

We found a number of mutations in the positive regulator *mexT* that encodes the activator of MexEF-OprN operon (Table 3). All isolates contained an 8-bp (cggccagc) deletion at 226 nucleotide position which is known to activate the MexT protein (Maseda et al. 2000). In addition, all isolates contained ^{172}F→I mutation. In the case of MexS, a MexT-dependent repressor of MexEF-OprN (Sobel et al. 2005), we observed ^{249}D→N mutation in the majority of isolates (8/15 isolates; PAAK082, PAAK083, PAAK084, PAAK085, PAAK087, PAAK090, PAAK091, and PAAK096).

With respect to *mexZ* that encodes the repressor of MexXY, we discovered 10 novel mutations. Most of these mutations would cause a shift in the reading frame or a premature termination of the protein synthesis. For example, a 17-bp deletion (^{440}gcggcgaactgccggcg) was found in PAAK082 would a shift in the reading frame. In ParR, the response regulator shown to regulate the expression of MexXY pump, we found ^{153}L→R and ^{170}S→N mutations as the most common ones.

DISCUSSION

The activity of RND efflux pumps is considered one of the most important contributors to the intrinsic antibiotic resistance of *P. aeruginosa* in CF lung infections (Mesaros et al. 2007). In this study, we analyzed the expression of RND pumps in multidrug resistant (MDR) CF isolates of *P. aeruginosa*

(Table 1) from patients with chronic infections. We used the antibiotic susceptibility testing method that is routinely used in clinical laboratories (Jones et al. 2015; Korting et al.). Even though the true MIC values for some of the antibiotics tested might be outside the concentrations range used in the Sensititre panel, the method effectively helped in determining the susceptibility of the isolates to respective antibiotics. Aminoglycoside resistance was prevalent in these isolates, which is not unexpected given that chronic *P. aeruginosa* infections in CF patients are routinely treated with long term tobramycin inhalation therapy (Ratjen et al. 2009; Young et al. 2013). Colistin was the most effective antibiotic against these isolates with all but two susceptible to the antibiotic, likely reflecting, at least partially, the fact it is less commonly used as therapy in CF patients in Canada.

We then analyzed our isolates for the expression of RND pumps in order to explore their role in antibiotic susceptibility. This was done by measuring the transcript levels of genes encoding the RND pump gene. While Western blots can provide a more definitive data on the expression of efflux pumps, lack of appropriate antibodies for analysis of large subset of proteins often hampers the use of this technique. Therefore, qRT-PCR is widely used to measure the expression of efflux pumps (Eaves et al. 2004; Fernando et al. 2013; Kumar et al. 2008; Poonsuk et al. 2014). All four pumps we studied have been shown to be overexpressed in clinical isolates displaying multidrug resistant phenotype (Hocquet et al. 2007; Jeannot et al. 2008; Llanes et al. 2004; Poonsuk et al. 2014).

Among the four pumps whose expression was tested in this study, MexAB-OprM is constitutively expressed (Li et al. 1995; Poole et al. 1993). MexAB-OprM pump is characterized by a remarkably wide substrate specificity and is capable of effluxing a wide range of antibiotics with little or no structural similarity (Kumar and Schweizer 2005). The MexXY system is capable of imparting inducible resistance to aminoglycosides, fluoroquinolones, macrolides, tetracyclines, tigecycline, as well as cephalosporins (Aires et al. 1999; Masuda et al. 2000a). The expression of MexXY pump has

been shown to be induced by agents that target ribosome (Jeannot et al. 2005). The MexCD-OprJ pump effluxes fluoroquinolones, macrolides, chloramphenicol and tetracyclines (Masuda et al. 2000b). MexCD-OprJ is generally not expressed in wild-type strains but its expression is considered to be response to membrane stress (Mine et al. 1999; Morita et al. 2009). MexEF-OprN, on the other hand, provides resistance to fluoroquinolones, chloramphenicol, trimethoprim, and carbapenem (Kohler et al. 1997). MexEF-OprN pump has not only been shown to be overexpressed in the CF isolates but also in response to metabolic stress (Kumar and Schweizer 2011; Wolter et al. 2009).

We observed that all of the isolates overexpressed at least one of the four RND pumps analyzed (Table 1). Notable was the MexXY pump that was found to be overexpressed in 13 isolates (>4-fold). The range of overexpression of *mexY* varied from 6.76- to 73-fold. It has been suggested that MexXY expression not only contributes to the antibiotic resistance of CF *P. aeruginosa* isolates but also to its adaptive strategy in the CF lung environment (Smith et al. 2006; Vettoretti et al. 2009). Widespread expression of MexXY pump in the CF isolates we tested does suggest that this pump may have a role to play that goes beyond their antibiotic resistance.

Overexpression of RND pumps in MDR CF isolates provided us an opportunity to study the mutational events in the regulatory gene responsible for the expression of respective efflux pump. We discovered several novel mutations in regulatory genes that may have an impact on the expression of their respective pumps. For example, with respect to MexAB-OprM, ^{83}R→C in PAAK091 (Table 3) is located within the proposed DNA-binding domain (37 to 99 amino acid residues) (21) of MexR, and therefore may play a role in the overexpression of this pump. The most common mutation in *mexR* that we observed (^{126}V→E) has also been described previously but is considered non-significant (Llanes et al. 2004). Similarly, ^{132}V→A mutation found in PAAK095 has been described previously (Quale et al. 2006) and is not believed to impact the expression of MexAB-OprM pump. None of the mutations

observed in *nalD* (tabta 3) have been described previously. Their role in the expression of MexAB-OprM pump remains unclear. For instance, both PAAK084 and PAAK087 contained a ^{397}GT deletion which would result in a frameshift mutation. However, *mexB* expression in PAAK084 was 1.8-fold and that in PAAK087 was 9.3-fold, compared to the wild-type control. This large difference in expression between these two isolates suggests that either these mutations are not significant and/or that there may be other regulatory element(s) involved. We would also like to point out that eight and 11 isolates that did not contain any mutations in *mexR* or *nalD,* respectively, still displayed overexpression of the MexAB-OprM, for example, PAAK082 or PAAK094. This suggests that other regulatory elements are involved in the expression of MexAB-OprM in these isolates.

MexCD-OprJ overexpression can result from mutations in *nfxB* (Purssell and Poole 2013). Of all the mutations we found in our isolates, only ^{21}R→H and ^{56}D→G have been described previously (Henrichfreise et al. 2007) and only ^{56}D→G is believed to impact the expression of *mexCD-oprJ* (Henrichfreise et al. 2007). We found these mutations in PAAK092 but intriguingly this isolate did not overexpress the MexCD-OprJ pump (Table 3). Both PAAK084 and PAAK087 contained ^{2}T →P, however overexpression of MexCD-OprJ was observed only in PAAK084. This indicates that ^{2}T →P mutation alone is not likely to have an effect on the expression of MexCD-OprJ pump. Both PAAK082 and PAAK083 contained ^{54}L→F mutation and both overexpressed the MexCD-OprJ pump (Table 3), suggesting that this mutation may be significant in the expression of MexCD-OprJ.

Unlike MexAB-OprM, MexCD-OprJ, and MexXY, whose expression is regulated by a repressor protein, the expression of MexEF-OprN is regulated by the transcriptional activator MexT (Kohler et al. 1997). Most strains of *P. aeruginosa* contain an 8-bp insertion in *mexT* that renders the protein inactive. Overexpression of the MexEF-OprN pump can be caused by the loss of this 8-bp insertion resulting in functional MexT which in turn activates the expression of *mexEF-oprN* operon (Maseda et al. 2000).

Our data suggest that all isolates expressed the active form of MexT, as none of the isolates tested contained the 8-bp insertion (Table 3). All of these isolates, with the exception of PAAK091, overexpressed MexEF-OprN pump.

Eight of the 15 isolates in our study showed at least one mutation in *mexS*, a gene that encodes an additional regulator of MexEF-OprN pump, deletion of which has been shown to cause MexEF-OprN overexpression (Sobel et al. 2005). MexEF-OprN regulation by MexS is believed to be MexT-dependent (Sobel et al. 2005; Uwate et al. 2013) i.e. an active MexT is needed for MexS to exert its function. A number of mutations have been reported previously in *mexS* that impact the expression of MexEF-OprN (Richardot et al. 2016; Sobel et al. 2005; Sole et al. 2015; Uwate et al. 2013). In our isolates, ^{249}D→N mutation was most common in *mexS*. Substitution of D to N at 249 position has been reported previously however, the MexS protein still maintains its activity in the presence of this mutation (Richardot et al. 2016; Sole et al. 2015). However, in our isolates there was no clear correlation between this mutation and the expression of MexEF-OprN pump (Tables 1 and 3).

Based on our data, MexXY was the most prevalent RND pump in our CF isolates. Further, all of these isolates were resistant to at least one of the three aminoglycosides tested (Table 1), antibiotics that are substrates of MexXY. Several mutations in *mexZ* have been reported that contribute to the overexpression of the MexXY pump (Guenard et al. 2014; Llanes et al. 2004; Quale et al. 2006; Sole et al. 2015). Mutations in *mexZ* have been shown to be common in chronic CF airway infections by *P. aeruginosa* (Smith et al. 2006). It has been suggested that these mutations may aid in the clonal expansion of *P. aeruginosa* in CF lungs (Smith et al. 2006). We were therefore particularly interested in the analysis of the regulatory genes controlling the expression of MexXY pump. We were able to identify a number of mutations in *mexZ* that have not been reported previously (Table 3). We mapped the mutations discovered in our isolates using the crystal structure of MexZ monomer (Alguel et al.

2010) and PyMOL (www.pymol.org) (data not shown). Mutations present in PAAK092 (6 bp insertion at 154), PAAK094 (^{37}L→P), and PAAK096 (^{50}G→S) are within the DNA-binding domain (Alguel et al. 2010) and are likely to impact the activity of the protein. A 17-bp deletion (from 440 to 456 nt.) was found in PAAK082. In addition to causing a frameshift in the downstream region, this 17-bp sequence is a part of the C-terminal domain of MexZ. It has been suggested from the way that MexZ dimerizes, changes in the C-terminal domain can be transmitted to the DNA-binding domain (Alguel et al. 2010), thus may affect MexZ binding to the target DNA sequence. Two other mutations (^{11}K→Stop in PAAK086 and ^{164}Q→Stop in PAAK090) will result in premature termination of the protein, presumably resulting in an inactive protein. While ^{164}Q→Stop has been reported before (Smith et al. 2006), ^{11}K→Stop mutation is novel and has not been reported before.

ParRS is a two-component regulatory system that has also been shown to play a role in the expression of MexXY pump (Muller et al. 2011). We found six different mutations in *parR* (Table 3) none of which have been previously described. Whether these mutations play a role in the expression of MexXY pump remains to be investigated.

In summary, even though our study was conducted in a limited number of CF isolates, we show in that MDR CF isolates of *P. aeruginosa* from chronic infections express multiple RND efflux pumps. MexXY overexpression was observed in all of the isolates, which may not only, at least partially, explain their aminoglycoside resistance but also suggest its role in the adaptation of *P. aeruginosa* to the CF lung environment. Further, we also found novel mutations in regulatory loci of RND pumps, in particular in *mexZ*, that may be responsible for the respective pump overexpression.

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Table 1. Antibiotic susceptibility (in µg/ml, with exception of colistin and chloramphenicol[†]) and expression of RND-protein encoding genes in *P. aeruginosa* isolates.

	Antibiotic Susceptibility												Relative Gene Expression[#]			
Isolate	**AMK**	**ATM**	**CAZ**	**CIP**	**FEP**	**GEN**	**MEM**	**PIP**	**TOB**	**TZP**	**CST[†]**	**CHL[††]**	***mexB***	***mexD***	***mexF***	***mexY***
PA01[a]	≤8 (S)	≤8 (S)	≤1 (S)	≤0.5 (S)	≤4 (S)	≤2 (S)	≤0.5 (S)	≤16 (S)	≤2 (S)	≤8/4	(S)	16 (I)	1[‡]	1[‡]	1[‡]	1[‡]
PAAK082[a]	64 (R)	≤8 (S)	16 (I)	32 (R)	1 (S)	>16 (R)	≤1 (S)	64 (I)	32/4 (I)	≤4 (S)	(S)	2 (S)	**14.07±1.05**	**4.21±0.98**	ND	**73.43±6.42**
PAAK083[b]	32 (I)	≤8 (S)	16 (I)	16 (I)	>4 (R)	>16 (R)	>8 (R)	>128 (R)	≤16/4 (S)	>8 (R)	(S)	128 (R)	0.63±0.04	2.09±0.36	2.72±1.00	**12.25±1.43**
PAAK084[a]	>64 (R)	≤8 (S)	16 (I)	2 (S)	2 (I)	>16 (R)	≤1 (S)	≤16 (S)	≤16/4 (S)	>8 (R)	(S)	4 (S)	1.83±0.12	**7.90±1.13**	**5.75±2.04**	**43.82±3.34**
PAAK085[b]	>64 (R)	≤8 (S)	32 (R)	4 (S)	4 (R)	>16 (R)	4 (I)	32 (I)	32/4 (I)	>8 (R)	(S)	2 (S)	0.76±0.01	0.36±0.04	2.07±0.50	**23.63±2.88**
PAAK086[a]	64 (R)	≤8 (S)	16 (I)	≤1 (S)	2 (I)	16 (R)	≤1 (S)	≤16 (S)	≤16/4 (S)	>8 (R)	(R)	2 (S)	0.87±0.02	0.63±0.13	**4.04±0.94**	**6.76±0.66**
PAAK087[a]	≤8 (S)	32 (R)	16 (I)	16 (I)	>4 (R)	>16 (R)	>8 (R)	>128 (R)	128/4 (R)	>8 (R)	(I)	≥512 (R)	**9.28±0.59**	0.87±0.23	**5.08±0.15**	**10.75±1.62**
PAAK088[a]	>64 (R)	>32 (R)	>32 (R)	>32 (R)	>4 (R)	>16 (R)	≤1 (S)	128 (R)	64/4 (I)	>8 (R)	(S)	8 (S)	ND	ND	ND	1.98±0.95
PAAK089[b]	≤8 (S)	16 (I)	32 (R)	>32 (R)	≤0.5 (S)	>16 (R)	4 (I)	64 (R)	64/4 (I)	>8 (R)	(S)	32 (R)	0.80±0.25	0.30±0.27	2.98±1.95	**11.78±0.2**8
PAAK090[b]	>64 (R)	≤8 (S)	≤4 (S)	≤1 (S)	4 (R)	>16 (R)	≤1 (S)	≤16 (S)	≤16/4 (S)	>8 (R)	(S)	4 (S)	0.15±0.04	0.83±0.25	2.15±0.61	1.91±0.08
PAAK091[a]	>64 (R)	≤8 (S)	>32 (R)	16 (I)	>4 (R)	>16 (R)	8 (R)	≤16 (S)	32/4 (I)	8 (I)	(S)	1 (S)	**6.31±2.01**	**13.30±1.64**	1.03±0.02	**9.09±1.40**
PAAK092[b]	64 (R)	≤8 (S)	8 (S)	≤1 (S)	4 (R)	16 (R)	≤1 (S)	≤16 (S)	≤16/4 (S)	≤4 (S)	(S)	8 (S)	2.77±0.16	0.61±0.22	**4.45±1.11**	**22.74±0.35**
PAAK093[a]	64 (R)	≤8 (S)	16 (I)	2 (S)	>4 (R)	16 (R)	≤1 (S)	≤16 (S)	≤16/4 (S)	8 (I)	(S)	8 (S)	**4.24±0.58**	0.18±0.07	**5.75±0.47**	**36.55±8.74**
PAAK094[a]	>64 (R)	≤8 (S)	16 (I)	8 (S)	1 (S)	>16 (R)	4 (I)	128 (R)	32/4 (I)	>8 (R)	(S)	8 (S)	**5.33±1.23**	1.12±0.19	**4.71±0.00**	**25.16±2.63**
PAAK095[a]	>64 (R)	>32 (R)	>32 (R)	>32 (R)	4 (R)	>16 (R)	>8 (R)	>128 (R)	>128/4 (R)	>8 (R)	(S)	8 (S)	2.67±0.35	0.43±0.18	**10.33±1.41**	**33.56±0.13**
PAAK096[a]	32 (I)	>32 (R)	8 (S)	32 (R)	4 (R)	>16 (R)	4 (I)	≤16 (S)	≤16/4 (S)	>8 (R)	(S)	64 (R)	1.94±0.88	0.71±0.25	**10.05±1.57**	**15.80±5.13**

[a]non mucoid; [b]mucoid

determined by [†]disc diffusion assay and [††]2-fold broth microdilution method in a 96-well plate.

R, resistant; I, intermediate; S, susceptible

AMK, amikacin; ATM, aztreonam; FEP, cefepime; CAZ, ceftazidime; CIP, ciprofloxacin; GEN, gentamicin; MEM, meropenem; PIP, piperacillin; TZP, piperacillin-tazobactam; TOB, tobramycin; CST, colistin; CHL, chloramphenicol; ND, not detected

[‡]Ct values for *mexB, mexD, mexF,* and *mexY* for PAO1 were 27.1±0.08, 29.61±0.07, 33.03±0.31, and 32.12±0.24, respectively.

[#]Expression of RND pump-encoding gene ≥4-fold is indicated in bold

Table 2. List of oligonucleotides used in this study.

Target gene	Primer	Sequence (5' → 3')	Amplicon size (bp)	Purpose
mexB	mexB_RT_F1	TTC AAC CGG ATG TTC CTT TC	100	mRNA detection
	mexB_RT_R1	CGA TCA CCA CGT AGA TCA GC		
mexD	mexD_RT_R1	CCGGTTTCCTGCTGATCTAT	105	mRNA detection
	mexD_RT_F1	GCAGCTCGTTGTTGATATTGC		
mexF	mexF_RT_F2	TCTACGACCCGACCATCTTC	100	mRNA detection
	mexF_RT_R2	AGGAACAGGATCACCACCAG		
mexY	mexY_RT_F1	TAATGGTCCTTGGCCACCT	96	mRNA detection
	mexY_RT_R1	GCCCAACGACATCTACTTCAA		
rpsL	N.rpsl F	TCTGACCAACGGTTTCGAGG	109	mRNA detection
	N.rpsl R	GCCCGGAAGGTCCTTTACAC		
mexR	mexR_F1	TTTTAGCTCGATGGCCGGTT	729	Sequencing
	mexR_R1	TCGGCATCAAGATGGACCTC		
	mexR_F2_int	GAGGGAAGAAACCTGGTCCG	298	
	mexR_R2_int	TTCGCCAGTAAGCGGATACC		
nalD	nalD_F1	AACCGCGGAATCCACGACT	868	Sequencing
	nalD_R1	CGGACGTCCAGGTGGATCTT		
nfxB	nfxB_F1	GACAGCTAATTCCTTTGGACGCG	745	Sequencing
	nfxB_R1	ATCTTCCCGAGTGTCGAGCA		
mexT	mexT_F1	GGCGGGCCAAACTGATGAAA	1260	Sequencing
	mexT_R1	CGATGGAATAAGCCGCACACC		
	mexT_F2_int	GAAACTGTTCCTCGGCCAGC	490	
	mexT_R2_int	AATAGTCGTCGAGGGTCAGCT		
mexS	mexS_F1	AGGGGCATAGGATCACTGACAG	1295	Sequencing
	mexS_R1	CGGTCAACGATCTGTGGATCTGA		
	mexS_F2int	CCCCGATCATTATCCGGCCTA	418	
	mexS_R2int	ATCGCCGAGCAGGGTCAT		
mexZ	mexZ_F1	TATGATCTGCGGCGCCTTTC	883	Sequencing
	mexZ_R1	TTCGGAACAAGGCGTCTGCA		
parR	parR_F1	ATCTCGAACGAGTCGCTGGAG	881	Sequencing
	parR_R1	GTAGAACGCGTCGATGACATGG		

Table 3. Mutation analysis of regulators of RND efflux pumps

	mexR		*nalD*		*nfxB*		*mexT*		*mexS*		*mexZ*		*parR*	
Isolate	**Nucleotide change**	**Impact**	**Nucleotide change**	**Impact**	**Nucleotide change**	**Impact**	**Nucleotide change**	**Impact**	**Nucleotide change**	**Impact**	**Nucleotide change**	**Impact**	**Nucleotide change**	**Impact**
PAAK082					^{160}C→T	^{54}L→F	^{226}CGGCCAGC deletion ^{514}T→A*	Frameshift	^{745}G→A	^{249}D→N	^{440}GCGGCGAACTG CCGGCG deletion	Frameshift	^{458}T→G ^{509}G→A	^{153}L→R ^{170}S→N
PAAK083	^{377}T→A	^{126}V→E	^{398}TG-del	Frameshift	^{160}C→T	^{54}L→F	^{226}CGGCCAGC deletion ^{506}C→T* ^{514}T→A* ^{654}G→T*	Frameshift	^{745}G→A	^{249}D→N	^{334}A deletion $^{413-414}$TC→CA*	Frameshift	^{458}T→G ^{509}G→A	^{153}L→R ^{170}S→N
PAAK084	^{377}T→A	^{126}V→E	^{397}GT-del	Frameshift	^{4}A→C	^{2}T→P	^{178}C→T ^{226}CGGCCAGC deletion ^{514}T→A*	^{60}P→S Frameshift	^{745}G→A	^{249}D→N	^{334}A deletion $^{413-414}$TC→CA*	Frameshift	^{458}T→G ^{509}G→A	^{153}L→R ^{170}S→N
PAAK085							^{226} CGGCCAGC deletion ^{514}T→A*	Frameshift	^{745}G→A	^{249}D→N	^{464}A→G	^{155}D→G		
PAAK086					^{46}G→A	^{16}V→I	^{178}C→T ^{226}CGGCCAGC deletion ^{514}T→A*	^{60}P→S Frameshift			^{31}A→T	^{11}K→Stop	^{458}T→G ^{509}G→A	^{153}L→R ^{170}S→N
PAAK087	^{377}T→A	^{126}V→E	^{397}GT-del	Frameshift	^{4}A→C	^{2}T→P	^{226}CGGCCAGC deletion ^{514}T→A*	Frameshift	^{745}G→A	^{249}D→N	^{334}A-deletion $^{413-414}$TC→CA*	Frameshift	^{458}T→G ^{509}G→A	^{153}L→R ^{170}S→N
PAAK088							^{226}CGGCCAGC deletion ^{514}T→A*	Frameshift						
PAAK089							^{178}C→T ^{226}CGGCCAGC deletion ^{514}T→A*	^{60}P→S Frameshift			^{605}C-deletion	Frameshift		
PAAK090							^{178}C→T ^{226}CGGCCAGC deletion ^{514}T→A*	^{60}P→S Frameshift	^{745}G→A ^{813}G→A	^{249}D→N ^{271}M→I	^{490}C→T	^{164}Q→Stop		
PAAK091	^{247}C→T	^{83}R→C	^{559}G→C	^{187}D→H			^{19}A→G ^{178}C→T ^{226}CGGCCAGC deletion ^{514}T→A*	^{7}M→V ^{60}P→S Frameshift	^{745}G→A ^{813}G→A	^{249}D→N ^{271}M→I			^{278}T→C	^{93}I→T
PAAK092	^{377}T→A	^{126}V→E			^{62}G→A ^{167}A→G	^{21}R→H ^{56}D→G	^{226}CGGCCAGC deletion ^{514}T→A*	Frameshift			^{154}T –ACAAGA insertion	Frameshift	^{268}C→A	^{90}L→I
PAAK093	^{317}A→G^{323}C→G	^{106}Q→R ^{108}A→G					^{226}CGGCCAGC deletion ^{514}T→A*	Frameshift			^{289}T-deletion	Frameshift		
PAAK094							^{226}CGGCCAGC deletion ^{514}T→A*	Frameshift			^{110}T→C	^{37}L→P		
PAAK095							^{226}CGGCCAGC deletion ^{514}T→A*	Frameshift					^{218}G→A ^{466}G→A	^{73}R→H ^{156}E→K
PAAK096	^{377}T→A^{395}T→C	^{126}V→E ^{132}V→A					^{19}A→G, ^{226}CGGCCAGC deletion ^{514}T→A*	^{7}M→V Frameshift	^{745}G→A	^{249}D→N	^{148}G→A ^{382}C→A	^{50}G→S ^{128}L→M		

*amino acid changes for mutations occurring past the frameshift mutation are not listed due to the change in the entire reading frame

Supplementary Table 1: Silent mutations in the regulatory genes of the CF isolates in this study

	mexR	*nalD*	*nfxB*	*mexT*	*mexS*	*mexZ*	*parR*
PAAK082				^{472}T→C ^{501}G→A	^{321}G→A ^{477}G→A ^{915}G→A ^{963}C→T	^{438}A→G	^{66}C→T ^{72}T→C ^{135}C→T ^{276}C→T ^{330}A→G ^{387}G→C ^{465}C→A ^{567}T→C ^{660}T→C ^{702}G→A
PAAK083	^{327}G→A ^{384}G→A ^{411}G→A	^{135}C→A		^{252}C→T ^{372}T→C ^{393}T→C ^{501}G→A ^{506}C→T ^{514}T→A ^{579}C→A ^{654}G→T	^{321}G→A ^{477}G→A ^{990}T→C	^{93}C→T ^{255}T→C ^{357}C→T ^{367}T→C $^{413\text{-}414}$TC→GA ^{438}A→G	^{72}T→C ^{135}C→T ^{270}C→T ^{387}G→C ^{465}C→A ^{567}T→C ^{660}T→C ^{702}G→A
PAAK084	^{223}C→T ^{327}G→A ^{384}G→A ^{411}G→A	^{135}C→A	^{555}T→G	^{321}G→A ^{514}T→A ^{936}G→C	^{321}G→A ^{477}G→A ^{990}T→C	^{93}C→T ^{255}T→C ^{357}C→T ^{367}T→C $^{413\text{-}414}$TC→GA ^{438}A→G	^{72}T→C ^{135}C→T ^{270}C→T 387G→C ^{465}C→A ^{567}T→C ^{660}T→C ^{702}G→A
PAAK085	^{210}G→A			^{514}T→A	^{990}T→C ^{1019}G→A	^{93}C→T ^{366}G→A ^{438}A→G	
PAAK086		^{555}T→C	^{66}G→A ^{330}C→T	^{514}T→A ^{936}G→C	^{111}G→T ^{657}C→T	^{93}C→T ^{255}T→C ^{367}T→C ^{438}A→G	^{66}C→T ^{72}T→C ^{135}C→T ^{270}C→T ^{375}G→A ^{387}G→C ^{465}C→A ^{567}T→C ^{660}T→C ^{702}G→A
PAAK087	^{223}C→T ^{327}G→A ^{384}G→A ^{411}G→A	^{135}C→A	^{555}T→G	^{514}T→A	^{321}G→A ^{477}G→A ^{990}T→C	^{357}C→T ^{367}T→C $^{413\rightarrow414}$TC→GA ^{438}A→G	^{72}T→C ^{135}C→T ^{270}C→T ^{387}G→C ^{465}C→A ^{567}T→C ^{660}T→C ^{702}G→A
PAAK088			^{330}C→T ^{555}T→G	^{514}T→A		^{93}C→T ^{438}A→G	
PAAK089	^{378}G→T		^{330}C→T	^{372}T→C ^{393}T→C ^{514}T→A ^{684}G→A ^{702}T→C ^{765}G→A ^{771}A→G	^{321}G→A ^{477}G→A	^{438}A→G ^{605}C-del	^{150}G→A ^{387}G→C ^{528}T→C ^{567}T→C ^{660}T→C

PAAK090			^{330}C→T ^{555}T→G	^{372}T→C ^{393}T→C ^{514}T→A	^{321}G→A ^{477}G→A	^{93}C→T ^{177}C→T ^{282}C→T ^{438}A→G	^{150}G→A ^{387}G→C ^{528}T→C ^{567}T→C ^{660}T→C
PAAK091		^{555}T→C	^{480}T→C ^{537}T→C ^{543}C→T ^{555}T→G	^{321}G→A ^{510}G→A ^{514}T→A ^{735}C→A ^{936}G→C	^{309}G→A	^{438}A→G	^{150}G→A ^{387}G→C
PAAK092	^{264}C→T ^{327}G→A ^{384}G→A ^{411}G→A		^{51}T→C ^{81}G→A ^{423}G→A ^{537}T→C ^{555}T→G	^{514}T→A	^{321}G→A	^{93}C→T ^{438}A→G	^{150}G→A ^{387}G→C ^{567}T→C ^{660}T→C
PAAK093		^{21}T→C ^{295}T→C	^{330}C→T ^{555}T→G	^{514}T→A		^{93}C→T ^{438}A→G	^{387}G→C ^{462}G→A ^{660}T→C
PAAK094			^{546}C→T	^{472}T→C ^{501}G→A ^{514}T→A ^{765}G→A ^{771}A→G		^{438}A→G	^{150}G→A ^{387}G→C ^{660}T→C
PAAK095			^{555}T→G	^{514}T→A ^{936}G→C		^{93}C→T ^{438}A→G	^{387}G→C ^{660}T→C
PAAK096	^{384}G→A ^{411}G→A		^{312}C→T ^{555}T→G	^{514}T→A ^{936}G→C	^{831}G→A ^{990}T→C	^{93}C→T ^{438}A→G ^{552}C→T	^{660}T→C