Efflux-mediated fluoroquinolone resistance in the multidrug-resistant *Pseudomonas aeruginosa* clinical isolate PA7: identification of a novel MexS variant involved in upregulation of the *mexEF-oprN* multidrug efflux operon

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The emergence of multidrug-resistant *Pseudomonas aeruginosa* has become a serious problem in medical settings. *P. aeruginosa* clinical isolate PA7 is resistant to fluoroquinolones, aminoglycosides, and most β-lactams but not imipenem. In this study, enhanced efflux-mediated fluoroquinolone resistance of PA7 was shown to reflect increased expression of two resistance nodulation cell division (RND) -type multidrug efflux operons, *mexEF-oprN* and *mexXY-oprA*. Such a clinical isolate has rarely been reported because MexEF-OprN-overproducing mutants often increase susceptibility to aminoglycosides apparently owing to impairment of the MexXY system. A mutant of PA7 lacking three RND-type multidrug efflux operons (*mexAB-oprM*, *mexEF-oprN*, and *mexXY-oprA*) was susceptible to all anti-pseudomonas agents we tested, supporting an idea that these RND-type multidrug efflux transporters are molecular targets to overcome multidrug resistance in *P. aeruginosa*. *mexEF-oprN*-upregulation in *P. aeruginosa* PA7 was shown due to a MexS variant harboring the Valine-155 amino acid residue. This is the first genetic evidence shown that a MexS variant causes *mexEF-oprN*-upregulation in *P. aeruginosa* clinical isolates.

**Keywords:** *Pseudomonas aeruginosa*, efflux, *mexXY-oprA*, *mexEF-oprN*, MexS

**INTRODUCTION**

*Pseudomonas aeruginosa* is a metabolically versatile bacterium that can cause a wide range of severe opportunistic infections in patients with serious underlying medical conditions (Gellatly and Hancock, 2013). Infections caused by *P. aeruginosa* often are hard to treat; inappropriate chemotherapy readily selects multidrug-resistant *P. aeruginosa* against which very few agents are effective (Morita et al., 2014; Poole, 2014). This so-called "antibiotic resistance crisis" has been compounded by the lagging in antibiotic discovery and development programs occurred in recent years, and is jeopardizing the essential role played by antibiotics in current medical practices (Rossolini et al., 2014). To combat this organism, it is very useful to understand its antimicrobial resistance mechanisms (see reviews such as Breidenstein et al., 2011; Poole, 2011; Morita et al., 2015).

The complete genome sequence of the multiresistant *P. aeruginosa* PA7 has been determined (Roy et al., 2010). The sequence of this strain, which exhibits resistance to fluoroquinolones (FQs), aminoglycosides, and various β-lactams but is susceptible to carbapenems (imipenem), includes typical FQ-resistance mutations in gyrA (Thr83Ile) and parC (Ser87Leu) (Roy et al., 2010). PA7 has additionally acquired a mutated *aacA4* gene, the product of which (AAC(6’)-II) endows the strain with aminoglycoside (gentamicin and tobramycin) resistance (Roy et al., 2010). This clinical isolate is amenable to the construction of gene knock-out mutations, thereby facilitating the analysis of molecular mechanisms of multidrug antimicrobial resistance in this isolate. Previously we showed that the effect of the modifying enzyme is enhanced by the MexXY resistance nodulation cell division (RND) -type multidrug efflux system, especially in the presence of divalent cations, providing high-level aminoglycoside resistance in *P. aeruginosa* (Morita et al., 2012b). This observation emphasizes the importance of the MexXY multidrug efflux system for aminoglycoside resistance in multidrug-resistant *P. aeruginosa* clinical isolates (Morita et al., 2012a).

Four members of the RND family of multidrug efflux systems, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY have been implicated in FQ resistance in *P. aeruginosa* clinical isolates (Poole, 2011). As in other organisms, these RND family drug efflux transporters in *P. aeruginosa* operates as three-component pumps with an RND cytoplasmic membrane (CM) protein (MexB, MexD, MexF, and MexY) linked to an outer membrane channel-forming protein (OprM, OprJ, and OprN) by a CM-tethered periplasmic protein (MexA, MexC, MexE, and MexX) (Poole, 2013). Intriguingly PA7 possesses the *mexXY-oprA* multidrug efflux operon of which oprA is missing in most *P. aeruginosa* strains (Roy et al., 2010). We showed that the MexXY can utilize either the OprA or OprM as an outer membrane component of the tripartite efflux pump (Morita et al., 2012b). Multidrug-resistant *P. aeruginosa* clinical isolates,
including carbapenem-resistant *P. aeruginosa*, often have been reported to be MexXY and/or MexAB-OprM overproducers (e.g., Hocquet et al., 2007; Cabot et al., 2011; Fuste et al., 2013; Khuntayaporn et al., 2013; Vatcheva-Dobrevska et al., 2013). FQ efflux is mediated by the MexAB-OprM system constitutively produced at moderate levels in wild-type *P. aeruginosa* PAO1 (Morita et al., 2001; Poole, 2013). Expression of mexAB-oprM is controlled directly or indirectly by three repressors encoded by the mexR, naldC and nald genes, with inactivating mutations in any of these resulting in increased mexAB-oprM expression (Poole, 2011, 2013). In addition, nfxB and nfxC mutants (which overproduce components of the MexCD-OprF and MexEF-OprN systems, respectively) are well-known as efflux-mediated FQ-resistant mutants of laboratory and clinical isolates of *P. aeruginosa*, although nfxB mutations appear to be rare in clinical settings (Poole, 2011). nfxC mutants also show increased resistance to carbapenems such as imipenem, not because MexEF-OprN accommodates these agents but because of a coordinated, MexT-dependent reduction of OprD production in such mutants (Poole, 2011, 2013). Hyperexpression of mexEF-oprN (and reduction in oprD expression) is also seen in laboratory isolates disrupted in the mexS gene, which encodes a putative oxidoreductase (a.k.a qrh) of unknown function (Sobel et al., 2005; Lamarche and Deziel, 2011). There are, however, no reports (to our knowledge) with experimental evidence of mexEF-oprN-expressing clinical isolates harboring mutations in mexS (Poole, 2013), although some studies suggested possible mutation of mexS in nfxC-type clinical isolates (e.g., Llanes et al., 2011; Fournier et al., 2013). The incidence of MexXY over producers among clinical isolates has shown to be linked to the use of ciprofloxacin (Hocquet et al., 2008) and MexXY over producers have been often reported to be one of major phenotypes in multidrug resistant clinical isolates (Morita et al., 2012a; Poole, 2013). However, MexXY-expressing FQ-resistance or its contribution to FQ-resistance in clinical settings has not well been studied compared to the other three pumps. In this study, we used reverse genetics to investigate the role of efflux-mediated FQ resistance in multidrug-resistant *P. aeruginosa* clinical isolate PA7.

**MATERIALS AND METHODS**

**BACTERIAL STRAINS, PLASMIDS, AND GROWTH CONDITIONS**

The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cells were grown in Luria (L) broth and on LB agar (1.5%) under aerobic conditions at 37°C as previously described, unless otherwise indicated, with antibiotics as necessary (Morita et al., 2010). Bacterial growth was quantified by measuring the optical density at 600 nm on an Ultrospec 2100 Pro Spectrophotometer (GE Healthcare Corp., Tokyo, Japan), unless otherwise indicated. The plasmids pEX18Tc (Hoang et al., 1998), pYM101 (Morita et al., 2010), and their derivatives were maintained and selected using medium supplemented with 2.5-10 μg tetracycline ml⁻¹ for *E. coli* or 50–150 μg tetracycline ml⁻¹ for *P. aeruginosa*. The plasmids pUCP20T (Schweizer et al., 1996) and pFLP2 (Hoang et al., 1998) and their derivatives were maintained and selected using medium supplemented with 100 μg ampicillin ml⁻¹ for *E. coli* or 200 μg carbenicillin ml⁻¹ for *P. aeruginosa*.

**Table 1 | Bacterial strains and plasmids.**

| Strains or plasmids | Relevant characteristics | References |
|---------------------|--------------------------|------------|
| **E. COLI**         |                          |            |
| DH5α (PAGU9121)     | For recombinant DNA manipulation | Morita et al., 2010 |
| KAM3 (PAGU8846)     | For recombinant DNA manipulation | Morita et al., 1998 |
| S17-1 (PAGU8566)    | For conjugal transfer     | Morita et al., 2006 |
| **P. AERUGINOSA**   |                          |            |
| PAO1 (PAGU9741)     | Wild type                | Morita et al., 2006 |
| PA7 (PAGU1498)      | Multi-resistant clinical isolate | Roy et al., 2010 |
| PAGU1565            | PA7 ΔmexXYoprA           | Morita et al., 2012b |
| PAGU1603            | PA7 ΔmexAB-oprM          | Morita et al., 2012b |
| PAGU1748            | PA7 ΔmexEFopRN           | This study |
| PAGU1641            | PA7 ΔmexXYoprA ΔmexAB-oprM | Morita et al., 2012b |
| PAGU1751            | PA7 ΔmexXYoprA ΔmexEFopRN | This study |
| PAGU1753            | PA7 ΔmexAB-oprM ΔmexEFopRN | This study |
| PAGU1756            | PA7 ΔmexXYoprA ΔmexAB-oprM ΔmexEFopRN | This study |
| PAGU1793            | PA7 ΔmexS                | This study |
| PAGU1789            | PA7 ΔmexT                | This study |
| PAGU1867            | PA7 mexS(V155A)          | This study |
| DSM 1128            | Taxonomically PA7-related strain, wild-type | Kiewitz and Tummier, 2000 |
| K2153 (PAGU1741)    | Clinical isolate         | Sobel et al., 2003 |
| K2376 (PAGU1834)    | ΔmexS                    | Fetar et al., 2011 |
| PAGU1850            | K2153 ΔmexS attB::lacF-P₇₇ | This study |
| PAGU1851            | K2153 ΔmexS              | This study |
| PAGU1844            | K2153 ΔmexS              | This study |
| PAGU1854            | K2153 ΔmexS pUCP20T      | This study |
| PAGU1856            | K2153 ΔmexS pUCP20T::mexS₉₉₇ | This study |
| PAGU1856            | K2153 ΔmexS pUCP20T::mexS₉₉₇ | This study |
| PAGU1835            | K2153 ΔmexT              | Fetar et al., 2011 |
| PAGU1836            | K2153 ΔmexF              | Fetar et al., 2011 |
| PAGU1837            | K2153 ΔmexS ΔmexT         | Fetar et al., 2011 |
| PAGU1852            | K2153 ΔmexS ΔmexT         | This study |
| PAGU1846            | K2153 ΔmexS ΔmexT         | This study |
| PAGU1845            | K2153 ΔmexS ΔmexT         | This study |

**PLASMID**

pEX18Tc Broad-host-range gene replacement vector | Hoang et al., 1998 |

pYM133 pEX18Tc::ΔmexEFopN | This study |

pYM134 pEX18Tc::ΔmexS | This study |

(Continued)
Table 1 | Continued

| Strains or plasmids | Relevant characteristics | References |
|---------------------|--------------------------|------------|
| pYM135              | pEX18Tc::ΔmexT           | This study |
| pYM136              | pEX18Tc::ΔmexST          | This study |
| pYM137              | pEX18Tc::mexS<sub>PA7</sub> | This study |
| pYM138              | pEX18Tc::mexS<sub>PA7</sub>(V155A) | This study |
| pYM101              | P. aeruginosa chromosome integration vector | Morita et al., 2010 |
| pYM139              | pYM101::mexS<sub>PA7</sub> | This study |
| pYM140              | pYM101::mex<sub>D</sub>SM 1128 | This study |
| pYM141              | pYM101::mexT<sub>PA7</sub> | This study |
| pYM142              | pYM101::mexT<sub>D</sub>SM 1128 | This study |
| pUCP20T             | Broad-host-range cloning vector | Schweizer et al., 1996 |
| pYM143              | pUCP20T::mexS<sub>PA7</sub> | This study |
| pYM144              | pUCP20T::mex<sub>D</sub>SM 1128 | This study |
| pFLP2               | Flp recombinase-producing plasmid | Hoang et al., 1998 |

Molecular Biology Techniques

Plasmid DNA isolation from E. coli, DNA purification, measuring DNA concentration, DNA digestion with restriction enzymes, DNA dephosphorylation, DNA ligation, isolation of chromosomal DNA from P. aeruginosa, PCR conditions, nucleotide sequencing, competent cell preparation from E. coli, transformation of E. coli, and transfer of plasmids into P. aeruginosa via conjugation were performed as described previously (Morita et al., 2010), unless otherwise indicated. DNA sequences and amino acid sequences were analyzed through 2012 to 2013 with Pseudomonas Genome Database (Winsor et al., 2011), Basic Local Alignment Search Tool (BLAST) (Boratyn et al., 2013), SWISS-MODEL (Bordoli et al., 2009), Sorting Tolerant From Intolerant (SIFT) BLink (Kumar et al., 2009), and the software DNASIS Pro (Ver. 2.1; Hitachi, Japan).

Cloning of mexS and mexT from P. aeruginosa

mexS and mexT from P. aeruginosa (without endogenous promoters) were amplified by PCR using the primers listed in Table 2. The purified mexS PCR product, digested with BamHI and HindIII, was cloned into similarly digested, dephosphorylated pUCP20T and pYM101, yielding pUCP20T::mexS and pYM101::mexS. The purified mexT PCR product, digested with EcoRI and BamHI, was cloned into similarly digested, dephosphorylated pUCP20T and pYM101, yielding pUCP20T::mexT and pYM101::mexT.

Construction of In-frame Deletion Mutants from P. aeruginosa

In-frame deletion mutants of mexEF-oprN, mexS, and mexT from P. aeruginosa PA7 and its derivatives were constructed using the previously described sacB-based strategy (Morita et al., 2006, 2010). The plasmids and resulting P. aeruginosa mutants are listed in Table 1, while the primer pairs are listed in Table 2. The selection concentrations of tetracycline for the first homologous recombination event were adjusted to reflect the endogenous tetracycline MICs for the P. aeruginosa strains. These constructs were confirmed by colony PCR.

Construction of the ΔCTX-Based Site-Specific Integrants in P. aeruginosa

For gene complementation experiments in P. aeruginosa, ΔCTX phage-based site-specific integrants were constructed using the integration-proficient, tightly controlled expression vector pYM101 and associated techniques (conjugative transfer, gene replacement at the chromosomal attB site, and curing of the unwanted plasmid backbone from the chromosome via the pFLP2-encoded Flp recombinase) as previously described (Morita et al., 2010).

Site-Directed Mutagenesis of mexS<sub>PA7</sub> in the Chromosome of P. aeruginosa PA7

An amino acid substitution (Val155Ala) mutation was introduced into the mexS<sub>PA7</sub> gene of the insert in the plasmid pUC20T::mexS<sub>PA7</sub>. Site-directed mutagenesis within a target plasmid was carried out according to Geiser et al. (2001) and Morita et al. (2009). A 50-μl mixture consisting of 50 ng of plasmid DNA, 0.25 μM of each mutagenic primer pair (Table 2), 0.2 mM each deoxynucleoside triphosphate, 1 mM MgSO<sub>4</sub>, 2.5 U of KOD Hot Start DNA polymerase -Plus- Ver.2 (TOYOBO Co. Ltd., Osaka, Japan), 1× KOD buffer, and 4.0% (vol/vol) dimethyl sulfoxide was heated to 94°C for 2 min followed by 18 cycles of 0.5 min at 94°C, 1 min at 60°C, and 5 min at 68°C. The resulting DNA products were purified as above, digested overnight with 10 U DpnI (Roche Diagnostics K.K., Tokyo, Japan) to eliminate template plasmid, and used to transform E. coli DH5α. Plasmids were recovered from individual transformants, and the mexS insert was sequenced to identify plasmids bearing the desired mutation. The mexS insert carrying the mutation was digested with BamHI and HindIII and cloned into similarly digested and dephosphorylated pEX18Tc to yield pEX18Tc::mexS<sub>PA7</sub>(V155A). The mexS<sub>PA7</sub>(V155A) mutation was gene replaced onto the chromosome of P. aeruginosa PA7 using the previously described sacB-based strategy (Morita et al., 2009, 2012b).

Antibiotic Susceptibility Assay

The susceptibility of P. aeruginosa to antimicrobial agents in cation-adjusted Mueller–Hinton broth was assessed using the 2-fold serial microtiter broth dilution method described previously (Morita et al., 2012b). Minimal inhibitory concentrations (MICs) were defined as the lowest concentration of antibiotic resulting in visible inhibition of growth after 18 h of incubation at 37°C. MICs for the ΔCTX-based site-specific integrants were determined in the presence of the inducer 5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The categorization in susceptible, intermediate, and resistant was performed according to the interpretive standards of the Clinical and Laboratory Standards Institute (CLSI) (Patel et al., 2011).

Antimicrobial Agents

Amikacin, ampicillin, carbenicillin, chloramphenicol, ciprofloxacin, norfloxacin, and tetracycline were purchased from Wako Pure Chemicals Industries, Ltd (Osaka, Japan). Moxifloxacin and levofloxacin were purchased from LKT
### Table 2 | Primers used in this study.

| Primer       | Sequence(5’–3’)     | Purpose                                                                 | References |
|--------------|---------------------|------------------------------------------------------------------------|------------|
| EcoRI-mexS\_PA7-UF | GAT CGA ATT CTG GCC TCG GGG GAA ATC T | mexEFoprN genes disruption of *P. aeruginosa* PA7                     | This study |
| BamHI-mexS\_PA7-UR   | CTG AGG ATC CAT GTC TGA CTC CGC CAG TC | mexEFoprN genes disruption of *P. aeruginosa* PA7                     | This study |
| BamHI-opn\_PA7-DF    | CTG AGG ATC CTG ACG CGA TCA TCC CCG G | mexEFoprN genes disruption of *P. aeruginosa* PA7                     | This study |
| XhoI-opn\_PA7-DR     | GAT CCT CGA GGC CGA ACA AGC ATT GCC A | mexEFoprN genes disruption of *P. aeruginosa* PA7                     | This study |
| BamHI-mexS\_PA7-F     | GAT CGG ATC CGA TGC ACT GCA GAG GTT TGC | mexS gene cloning of *P. aeruginosa* PA7 and DSM 1128                 | This study |
| HindIII-mexS\_PA7-R   | CTA GAA GCT TAC ATC GGC GAC GTG GAT | mexS gene cloning of *P. aeruginosa* PA7 and DSM 1128                 | This study |
| EcoRI-mexS\_PA7-UF    | GCT AGA ATT CAG TTC GTC GTG GTA GCT GA | mexS gene disruption of *P. aeruginosa* PA7                          | This study |
| BamHI-mexS\_PA7-UR    | CTA GGG ATC CGG ACA TCG CAA ACC TCT G | mexS gene disruption of *P. aeruginosa* PA7                          | This study |
| BamHI-mexS\_PA7-DF    | GCT AGG ATC CTG CGC CGA TGG AGG AGC AC | mexS gene disruption of *P. aeruginosa* PA7                          | This study |
| HindIII-mexS\_PA7-DR  | CTA GAA GCT TAG TAC ATC CAC CGG CAC CT | mexS gene disruption of *P. aeruginosa* PA7                          | This study |
| EcoRI-mexT\_PA7-F     | GAT CGA ATT CCC CTG GAA AGC AGC AAC | mexT gene cloning of *P. aeruginosa* PA7 and DSM 1128                 | This study |
| BamHI-mexT\_PA7-R     | GAT CGG ATC CTA AGA GGC TGT CCG GGT C | mexT gene cloning of *P. aeruginosa* PA7 and DSM 1128                 | This study |
| SaeI-mexT\_PA7-UF     | GCT AGA GCT CTG GAA AGC ATC ACC CGG G | mexT gene disruption of *P. aeruginosa* PA7                          | This study |
| BamHI-mexT\_PA7-UR    | CTA GGG ATC CAT GGC GTT CCT CTT TTC C | mexT gene disruption of *P. aeruginosa* PA7                          | This study |
| BamHI-mexT\_PA7-DF    | GCT AGG ATC CGG ACA GCC TCT GAG TCA TCC AGC | mexT gene disruption of *P. aeruginosa* PA7                          | This study |
| HindIII-mexT\_PA7-DR  | CTA GAA GCT TGC GAC CGC CGC CCT GGC TT | mexT gene disruption of *P. aeruginosa* PA7                          | This study |
| mexS\_PA7(V155A)-F   | CTG ACC GAG GGC GCC CGC ATG TAC GGC CC | mexSV(155A) site directed mutagenesis of *P. aeruginosa* PA7         | This study |
| mexS\_PA7(V155A)-R   | GGC CGG TAC ATG CGC GCC GCC TCG GTG ATC AG | mexSV(155A) site directed mutagenesis of *P. aeruginosa* PA7         | This study |
| mexTS intergenic seq | GCA CCA GGA CTT CCC CTG | DNA sequencing of mexTmexS intergenic region                        | This study |
| uvrD-F           | ATCGACTTCTCCAGCTGCTGT | RT-PCR for uvrD gene of *P. aeruginosa*                               | Morita et al., 2012b |
| uvrD-R           | CTGAACTCGTCCACCCAGAT | RT-PCR for uvrD gene of *P. aeruginosa*                               | Morita et al., 2012b |
| mexE\_FA(RT)    | CCA CCC TGA TCA AGG ACG AGA | RT-PCR for mexE gene of *P. aeruginosa*                               | This study |
| mexE\_FB(RT)    | CGG TAG ACG GTG TGG TGG TGC | RT-PCR for mexE gene of *P. aeruginosa*                               | This study |
| mexS\_FA(RT)    | AGG GGG TCA ATG TCA TCC TC | RT-PCR for mexS gene of *P. aeruginosa*                               | This study |
| mexS\_FB(RT)    | CTG CAG GTG CTT CTT AAG AG | RT-PCR for mexS gene of *P. aeruginosa*                               | This study |
| mexT\_FA(RT)    | TAT TGA TGC CGA ACC TGC TG | RT-PCR for mexT gene of *P. aeruginosa*                               | This study |
| mexT\_FB(RT)    | GGA GGA TCT TCG GCT TGC TG | RT-PCR for mexT gene of *P. aeruginosa*                               | This study |
| oprD\_FA(RT)    | ATT GCA GTG CGG GTT TCC | RT-PCR for oprD gene of *P. aeruginosa*                               | This study |
| oprD\_FB(RT)    | ATG AAC CCC TCC TCG TGC | RT-PCR for oprD gene of *P. aeruginosa*                               | This study |
| mexA\_FA(RT)    | GAC AAC GTG ATG CAA CGA AGC | RT-PCR for mexA gene of *P. aeruginosa*                               | This study |
| mexA\_FB(RT)    | AGC TCG GTA TAT AGC GTG ACC | RT-PCR for mexA gene of *P. aeruginosa*                               | This study |

### REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTASE (qRT)-PCR

Overnight cultures of *P. aeruginosa* strains were diluted 1:100 in 10 ml, incubated with vigorous shaking at 37°C for 3–4 h, and harvested. Total RNA was stabilized with the RNA Protect Bacteria Reagent (Qiagen) and isolated with the RNeasy Mini Kit (Qiagen). The RNA samples were further treated with RQ1 RNase-Free DNase (Promega) and purified by using the RNeasy Mini Kit (Qiagen). Real-time qRT-PCR was performed with primer pairs internal to *uvrD*, *mexE*, *mexS*, *mexT*, and *oprD* (Table 2) using the One Step SYBR PrimeScript RT-PCR kit II (TaKaRa) in a Thermal Cycler Dice real-time system (TaKaRa). The transcript levels of the target gene in a given strain were normalized to levels of *uvrD* and expressed as a ratio (fold change) to that observed in the parental PA7 strain. Gene expression values were calculated based on triplicate experiments.

### NUCLEOTIDE SEQUENCE ACCESSION NUMBER

The nucleotide sequences of DNA regions containing *mexT* and *mexS* in *P. aeruginosa* DSM 1128 have been deposited in GenBank/EMBL/DDJB with the accession number AB889539.
RESULTS

CONTRIBUTION OF RND MULTIDRUG EFFLUX SYSTEMS TO FQ RESISTANCE IN P. aeruginosa PA7

FQ resistance in P. aeruginosa PA7 was compared with the two FQ-susceptible strains, PAO1 (the standard laboratory strain) and DSM 1128 (a strain taxonomically related to PA7) (Morita et al., 2012b). The MICs of the FQs (ciprofloxacin, levofloxacin, moxifloxacin, and norfloxacin) for P. aeruginosa PA7, PAO1, and DSM 1128 are shown in Table 3. P. aeruginosa PA7 exhibited increased resistance (ca. 256-fold) to these FQs compared to P. aeruginosa PAO1 and DSM 1128. P. aeruginosa DSM 1128 showed the same level of resistance to the FQs as P. aeruginosa PAO1. Based on the interpretive standards of the CLSI (Patel et al., 2011), P. aeruginosa PA7 was considered highly resistant to these FQs. This observation is not surprising, because P. aeruginosa PA7 possesses typical target mutations, one in gyrA (Thr83Ile) and one in parC (Ser87Leu), well-known to be associated with FQ resistance (Lomovskaya et al., 1999). However, target mutations alone are not sufficient to explain high-level FQ resistance in P. aeruginosa; the additional effect of up-regulation of one of the four RND efflux pumps (MexXY-OprA, MexAB-OprM, MexCD-OprJ, or MexEF-OprN) is necessary (Lomovskaya et al., 1999; Bruchmann et al., 2013).

Previously we showed that the ΔmexXY-oprA ΔmexAB-oprM double mutant of PA7 was slightly more susceptible (ca. 2- to 4-fold) to ciprofloxacin, while retaining high-level resistance (MIC = 32 μg/ml) to this antibiotic when compared to its parental strain (Morita et al., 2012b). That observation was inconsistent with data, derived via similar genetic analyses, in which a PAO1-derived ΔoprM gyrA parC triple mutant (i.e., a strain deleted for oprM and carrying the target mutations) exhibited increased susceptibility (64-fold) to levofloxacin compared to an isogenic MexAB-OprM overexpressing (nalB) mutant, while exhibiting an MIC (0.5 μg/ml) of levofloxacin similar to that of the wild-type PAO1 (0.25 μg/ml) (Lomovskaya et al., 1999). We noted that deletion of oprM in PAO1 was expected to inactivate any efflux pumps that require OprM as an outer membrane factor (e.g., MexAB-OprM, MexXY-OprM, MexVW-OprM) (Morita et al., 2001; Li et al., 2003). We therefore hypothesized that overexpression of other RND pumps such as MexCD-OprJ and MexEF-OprN was compensating for the effects of the mexAB-oprM mexXY-oprA double deficiency in PA7. As a first step in testing our hypothesis, we examined the primary sequences of the efflux system-encoding genes of PA7. Sequence analysis revealed the presence of mexCD-oprJ (PSPA7_0540–0539–0538) and mexEF-oprN (PSPA7_2745–2744–2743) homologs in P. aeruginosa PA7 (Roy et al., 2010), with predicted amino acid lengths (in PA7) and amino acid sequence identity and similarity [respectively; via BLAST of PA7 vs. PAO1-UW (Winsor et al., 2011)] as follows: MexC (387 aa; 88 and 95%), MexD (1043 aa; 95 and 98%), OprJ (475 aa; 89 and 93%); MexE (414 aa; 99 and 99%), MexF (1062 aa; 99 and 99%), and OprN (472 aa; 97 and 99%). As a second step in testing our hypothesis, we examined the regulation of these genes in PA7. qRT-PCR showed that expression of mexE was increased (ca. 22-fold) in PA7 compared to the wild-type strain DSM 1128. This observation indicated that FQ resistance in P. aeruginosa can be mediated by overexpression of the mexEF-oprN operon, in addition to the previously reported overexpression of mexXY-oprA due to mutation of the local repressor gene (mexZ) (Morita et al., 2012b).

We also observed that a ΔmexXY-oprA ΔmexAB-oprM ΔmexEF-oprN triple mutant derived from PA7 exhibited increased susceptibility to FQs (32–128-fold) compared to an isogenic ΔmexXY-oprA ΔmexAB-oprM double mutant, while exhibiting only mild elevation of FQ MIC (0.5–2 μg/ml) compared to wild-type PAO1 and DSM 1128 (0.125–1 μg/ml) (Table 3). Moreover, the triple mutant was much more sensitive (512-fold) to chloramphenicol than the double mutant, a further indicator of MexEF-OprN-mediated resistance (Sobel et al., 2005). (Table 3). We excluded a role for overexpression of mexCD-oprJ in PA7, as judged by the susceptibilities of the mutant (ΔmexXY-oprA ΔmexAB-oprM ΔmexEF-oprN) construct to not only FQs but also to tetracycline and chloramphenicol, which are substrates of MexCD-OprJ (Poole et al., 1996).

To clarify the roles of MexXY-OprA, MexAB-OprM and MexEF-OprN multidrug efflux pumps in the FQ resistance of P. aeruginosa PA7, we constructed a series of deletion mutants of the three mex operons and examined their drug susceptibility

| Strain          | Genotype                  | CIP (μg/ml) | NOR (μg/ml) | LVX (μg/ml) | MXF (μg/ml) | AMK (μg/ml) | CHL (μg/ml) | IPM (μg/ml) |
|-----------------|---------------------------|------------|------------|------------|------------|------------|------------|------------|
| PA7 (PAGU 1498) | Parent                    | 64         | 128        | 128        | 256        | 32         | 1024       | 2          |
| PAGU1565        | PA7 ΔmexXY-oprA           | 32         | 64         | 64         | 128        | 1          | 1024       | 2          |
| PAGU1603        | PA7 ΔmexAB-oprM           | 64         | 128        | 64         | 256        | 32         | 1024       | 2          |
| PAGU1748        | PA7 ΔmexEF-oprN           | 32         | 64         | 64         | 256        | 32         | 128        | 2          |
| PAGU1641        | PA7 ΔmexXY-oprA ΔmexAB-oprM | 32       | 64         | 32         | 64         | 1          | 1024       | 2          |
| PAGU1751        | PA7 ΔmexXY-oprA ΔmexEF-oprN | 8        | 16         | 8          | 32         | 1          | 256        | 2          |
| PAGU1753        | PA7 ΔmexAB-oprM ΔmexEF-oprN | 32       | 64         | 32         | 128        | 32         | 16         | 2          |
| PAGU1756        | PA7 ΔmexXY-oprA ΔmexAB-oprM ΔmexEF-oprN | 0.5     | 0.5        | 0.5        | 2          | 1          | 4          | 2          |
| DSM 1128 (PAGU 1504) | Wild type              | 0.125     | 0.25       | 0.25       | 1          | 0.5        | 32         | 1          |
| PAO1 (PAGU 974) | Wild type                | 0.125     | 0.25       | 0.25       | 1          | 0.5        | 32         | 1          |

AMK, amikacin; CHL, chloramphenicol; CIP, ciprofloxacin; IPM, imipenem; LVX, levofloxacin; MXF, moxifloxacin; NOR, norfloxacin.
profiles (Table 3). We observed that the ΔmexXY-oprA ΔmexEF-oprN double deletion mutant was more susceptible (4-fold) than the ΔmexAB-oprM ΔmexXY-oprA double mutant and the ΔmexAB-oprM ΔmexEF-oprN double mutant (Table 3). These data suggested that the MexXY-OpRA and MexEF-OpRN systems make similar contributions to FQ resistance in P. aeruginosa PA7, each having an effect larger than that of MexAB-OpRM. The apparent modest MexAB-OpRM contribution to resistance of FQs and trimethoprim, and chloramphenicol (Poole, 2013). The albeit against a rather narrow range of antimicrobials includ-
ing FQs, trimethoprim, and chloramphenicol (Poole, 2013). The induction of mexEF-oprN is regulated by a tran-
scriptional activator, MexT, a LysR family regulator (Kohler et al., 1999; Poole, 2013). mexT occurs upstream of mexEF-oprN and downstream of mexS, the latter gene also positively regulate by MexT (Kohler et al., 1999). Unusually, many so-called wild type strains possess inactive MexT (e.g., 8 bp insertion in mexT present in some PAO1 strains) (Maseda et al., 2000; Poole, 2013). The induction of mexEF-oprN contributes to multidrug resistance, albeit against a rather narrow range of antimicrobials including FQs, trimethoprim, and chloramphenicol (Poole, 2013). The enhanced resistance to imipenem of nfxC mutants or mexS deficient mutants results not from mexEF-oprN expression but the concomitant decrease in the level of outer membrane protein OprD (Poole, 2013), because OprD is an imipenem channel and serves as the primary route of entry of this antibiotic in P. aerugin-
osa (Trias and Nikaido, 1990). However, P. aeruginosa PA7 was reported to be susceptible to carbapenem, with MICs of 2 and 1 µg/ml for imipenem and meropenem, respectively (Roy et al., 2010). This observation of carbapenem susceptibility is somewhat paradoxical, given that a typical nfxC mutant or a mexS deficient mutant is expected to exhibit carbapenem resistance due to a coordinate, MexT-dependent reduction of OprD levels. We found that P. aeruginosa PA7 exhibited slight but reproducible elevation (2-fold) of resistance to imipenem compared to wild-type P. aeruginosa strains PAO1 and DSM 1128 (Table 4). Deletion of mexS in a PA7 background resulted in slightly increased expression of mexE (2-fold) and increased resistance to imipenem (4-fold) compared to PA7. Thus, PA7 appeared to exhibit sus-
ceptibility intermediate between that of wild type and a typical nfxC mutant. Deletion of mexT in a PA7 background resulted in drastically (>200-fold) decreased expression of mexE and mexS compared to PA7 (Table 4). Notably, expression of mexS in PA7 ΔmexT was more than 96-fold reduced compared to the wild-type DSM 1128 and PA7 mexS (V155A). This observation was consistent with a previous report (using β-galactosidase reporter assays) indicating that mexS (qrh) is constitutively expressed at a moderate level (Kohler et al., 1999; Poole, 2013).

We therefore determined the nucleotide sequences (ca. 2.4 kb) upstream of the mexEF-oprN operon in P. aeruginosa DSM 1128 (accession no. AB889539). This interval corresponds to the operon's cognate regulatory region, and includes the mexS and mexT loci. Comparison to the corresponding sequences from P. aeruginosa PA7 (data not shown) revealed that MexSPA7 is encoded as an A155V variant, and MexTPA7 as an A256T vari-
ant, compared to the DSM 1128 genome. In contrast, alanine-155 of MexS and alanine-256 of MexT are conserved among multiple laboratory and clinical strains, including PAO1, PA14, PAK, and K2153 (Sobel et al., 2005; Jin et al., 2011; Lamarche and Deziel, 2011). To test the function of the mexSPA7 and mexTPA7 loci, we amplified and cloned the individual mexS and mexT genes (without endogenous promoters) from P. aeruginosa PA7 and DSM 1128, and expressed the relevant genes in P. aeruginosa K2153 ΔmexS (Fetar et al., 2011) for a mexS complementation test or in K2153 ΔmexS ΔmexT (Sobel et al., 2005; Fetar et al., 2011) for a mexT complementation test (Table 5). [As demonstrated by other researchers, P. aeruginosa K2153 and its derivatives are useful for the analysis of mexEF-oprN-dependent antimicrobial resistance as regulated by the MexT activator and MexS function (Sobel et al., 2005; Fetar et al., 2011)]. Interestingly, introduction of mexS DSM 1128 or mexS PA7 into the chromosome of K2153 ΔmexS did not provide complementation of ΔmexS (Table 5). These data

### Table 4 | Relationship between antimicrobial resistance and mexS-mexit-mediated mexEF-oprN expression in P. aeruginosa.

| Strains (PA7) | Genotype | MIC (µg/ml) of | Relative mRNA level |
|--------------|----------|----------------|---------------------|
|              |          | CIP | CHL | IPM | mexE | mexS | mexT |
| PA7 (PAGU 1498) | Parent | 128 | 1024 | 2  | 1 | 1 | 1 |
| PAGU1793 | PA7 ΔmexS | >128 | >1024 | 8  | 2 | nd | 0.84 |
| PAGU1867 | PA7 mexS(V155A) | 64 | 256 | 1  | 0.053 | 0.57 | 0.74 |
| PAGU1789 | PA7 ΔmexT | 64 | 128 | 1  | <0.005 | <0.005 | nd |
| DSM 1128 (PAGU 1504) | Wild type | 0.125 | 32 | 1  | 0.046 | 0.48 | 1.1 |
| PAO1 (PAGU 974) | Wild type | 0.125 | 32 | 1  | nd | nd | nd |

CHL, chloramphenicol; CIP, ciprofloxacin; IPM, imipenem; nd, not done.
contrast previous instances in which we observed attB-site (i.e., single-copy) complementation for several other genes (Morita et al., 2010, 2012b). Presumably, in the present studies, failure to complement reflected insufficient mexS expression from the Pr7 promoter in PAGU1844, given that MexSDSM 1128 is expected to be functional. Introduction of PUCP20T::mexSDSM 1128 into P. aeruginosa K2153 ΔmexS yielded MICs identical to those of the K2153 mexS+ parent; introduction of PUCP20T::mexS7 yielded parent-like resistance to ciprofloxacin and chloramphenicol (Table 5). Taken together, these results suggested that MexS7 provides reduced function compared to MexSDSM 1128. Additionally it appears that the high levels of mexS expression are required to overcome the nfxC-type antimicrobial resistance.

Slightly increased expression of mexS was observed in P. aeruginosa PA7 compared to P. aeruginosa DSM 1128 (ca. 2-fold), while expression levels of mexT in P. aeruginosa PA7 were similar to those in P. aeruginosa DSM 1128 (ca. 0.9-fold) (Table 4). Introduction of mexTDSM 1128 or mexTPA7 into the chromosome of K2153 ΔmexS ΔmexT yielded the nfxC phenotype (Table 5), suggesting that the A256T substitution in MexT is not a primary reason for overexpression of mexEF-oprN in P. aeruginosa PA7. Using site-specific mutagenesis, we altered the plasmidborne mexS7 locus to encode a V155A version of the protein and replaced the endogenous PA7 chromosomal locus with the mutated gene. The PA7 mexS7(V155A) strain showed decreased mexE expression (0.053-fold compared to that of the PA7 parent), a value similar to that seen in DSM 1128 (0.034-fold compared to that of PA7) (Table 4). The PA7 mexS7(V155A) strain also showed decreased MICs for chloramphenicol (0.25-fold) (Table 4). Taken together with the complementation experiments, these data strongly suggested that the A155V substitution in MexS7PA7 is the primary reason for increased expression of mexEF-oprN and increased antimicrobial resistance in the PA7 strain.

DISCUSSION

In this study, the multidrug-resistant clinical isolate PA7 was shown to exhibit increased expression of mexEF-oprN as well as mexXY-oprA. Although multidrug-resistant P. aeruginosa clinical isolates have often been reported to be MexXY overproducers (Morita et al., 2012a), clinical strains of P. aeruginosa overproducing MexEF-OprN and MexXY-(OprA) efflux pumps simultaneously have rarely been reported. In fact, PA7 was not a typical nfxC mutant (i.e., a MexEF-OprN overproducer), and instead expressed intermediate levels of mexEF-oprN. We assume that simultaneous overproduction of MexEF-OprN and MexXY-(OprA) impairs P. aeruginosa growth, based on our observation that our PA7 ΔmexS construct (i.e., a simultaneous overproducer of MexEF-OprN and MexXY-OprA compared to the PA7 parent) was unstable even on L agar plates: colonies of the construct exhibited a non-uniform phenotype during growth on plates (data not shown). This observation is consistent with the increased susceptibility to aminoglycosides previously observed in MexEF-OprN-overproducing nfxC mutants, apparently owing to impairment of the MexXY system (Sobel et al., 2005).

Valine-155 of MexS7 also was shown as the likely primary reason for increased production of MexEF-OprN in PA7. With the exception of mexS7PA7, sequenced P. aeruginosa mexS genes (including those from PAO1, K2153, PA14, PAK, and DSM 1128) encode proteins with an alanine at residue 155 (Sobel et al., 2005; Jin et al., 2011; Lamarche and Deziel, 2011). The Ala155Val substitution is predicted by the SIFT algorithm (Kumar et al., 2009) to affect the protein’s function (data not shown). We hypothesize that MexS7PA7 retains function, albeit with decreased activity and/or altered regulation (e.g., allostery), compared with the other MexS orthologs. In fact, there were few differences among the whole structures of MexS7PA7, MexS7PA01, and MexSDSM 1128 models developed by using the SWISS-MODEL program (data not shown) (Bordoli et al., 2009). MexS is a member of the cd08268: MDR2 family of the Conserved Domains Database (CDD) of the National Center for Biotechnology Information (NCBI) (Fargier et al., 2012), and alanine-155 corresponds to one of the putative NAD(P) binding sites featured in the MDR2 family.

In addition to antibiotic resistance, an nfxC-type mutation has been linked to reduced levels of homoserine lactone-dependent quorum sensing (QS) -regulated virulence factors, including pyocyanin, elastase, rhamnolipids, and Pseudomonas Quinolone Signal (PQS), and to reduced expression of type-III secretion system (TTSS) effector proteins (Kohler et al., 2001; Linares et al., 2005). QS is a cell-to-cell communication mechanism employing diffusible signal molecules (Jimenez et al., 2012), and the TTSS is a mechanism by which bacterial pathogens can deliver effectors directly into the cytoplasm of eukaryotic host cells (Hauser, 2009). We found that PA7 had reduced level of pyocyanin production, rhamnolipid production, and swarming activity compared to DSM 1128 and PAO1 (data not shown), consistent with the typical nfxC mutant phenotype (Jin et al., 2011). However, PA7

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Table 5 | Functional characterization of mexS and mexT from P. aeruginosa PA7 and DSM 1128.

| Strains     | Genotype | MIC (µg/ml) |  |
|-------------|----------|-------------|---|
|             |          | CIP | CHL | IPM |  |
| K2153 (PAGU 1741) | Parent | 0.5 | 64 | 2 |  |
| K2376 (PAGU1834) | ΔmexS | 2 | 2048 | 8 |  |
| PAGU1850 | ΔmexS ΔattB::lacF-P7 | 2 | 2048 | 8 |  |
| PAGU1851 | ΔmexS ΔattB::lacF-P7-mexSPA7 | 2 | 2048 | 8 |  |
| PAGU1844 | ΔmexS | 2 | 1024 | 8 |  |
| PAGU1854 | ΔmexS ΔattB::lacF-P7-mexSDSM 1128 | 2 | 2048 | 8 |  |
| PAGU1855 | ΔmexS pUCP20T::mexS7 | 1 | 256 | 4 |  |
| PAGU1856 | ΔmexS pUCP20T::mexSDSM 1128 | 0.5 | 64 | 2 |  |
| K2942 (PAGU1837) | ΔmexSDS 7 | 0.5 | 64 | 2 |  |
| PAGU1852 | ΔmexS ΔmexT ΔattB::lacF-P7 | 0.5 | 64 | 2 |  |
| PAGU1846 | ΔmexS ΔmexT ΔattB::lacF-P7-mexTPA7 | 2 | 2048 | 8 |  |
| PAGU1845 | ΔmexS ΔmexT ΔattB::lacF-P7-mexTPA7 | 2 | 2048 | 8 |  |

CHL, chloramphenicol; CIP, ciprofloxacin; IPM, imipenem.

5 mM IPTG was added to allow expression controlled by the Pr7 promoter.
derivatives including PA7 mexSp$_{PA7}$ (V155A) also showed almost the same activities of the QS regulated virulence factors with PA7 (data not shown), which suggests that the impaired QS-related phenotype is not derived from the nfxC-like phenotype in PA7. We presume that this lack of correlation reflects the absence from PA7 of TTSS-encoding genes and of the mvrR (pgsR) gene, which is known to encode a LysR-type transcriptional regulator that modulates the expression of multiple QS-regulated virulence factors (Deziel et al., 2005; Roy et al., 2010). These deficiencies might be the source of the mexS mutation and increased mexEF-oprN expression under oxidative stress, sulfide stress, or nitrosative stress (Juhas et al., 2004; Fetar et al., 2011; Fargier et al., 2012).

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Morita et al. MexS of P. aeruginosa PA7

Frontiers in Microbiology | Antimicrobials, Resistance and Chemotherapy

January 2015 | Volume 6 | Article 8 | 8
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