Primary mechanisms mediating aminoglycoside resistance in the multidrug-resistant Pseudomonas aeruginosa clinical isolate PA7

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The multiresistant taxonomic outlier Pseudomonas aeruginosa PA7 possesses the conserved efflux genes, mexXY; however these are linked to a unique gene encoding an outer membrane channel, dubbed oprA, that is absent in most P. aeruginosa strains. Using genetic knockouts and single copy chromosomal complementation, we showed that aminoglycoside resistance in PA7 is mediated in part by the MexXY-OprA pump, and intriguingly that MexXY in this strain can utilize either the OprA or OprM outer membrane channel, linked to the mexAB efflux genes. We also identified a small portion of the oprA gene immediately downstream of the mexY gene in PAO1, suggesting that non-PA7 P. aeruginosa strains might have possessed, but lost, the intact mexXY-oprA efflux pump locus. Consistent with this, most of a panel of serotype strains possessed the truncated oprA but the serotype O12 isolate had an intact mexXY-oprA locus, similar to PA7 and the related strain DSM 1128. We also showed that the mexZ repressor gene upstream of mexXY-oprA in PA7 is mutated, leading to overexpression of mexXY-oprA, using sequencing, homologous replacement and real-time quantitative reverse transcriptase PCR. Finally we assessed the contribution of MexXY and aminoglycoside modifying enzymes AAC together to resistance in PA7 and the AAC(6’)-Iaε-mediated amikacin-resistant clinical isolate IMCJ2.S1, concluding that the effect of the modifying enzymes is enhanced by functional efflux, especially in the presence of divalent cations, to develop high-level aminoglycoside resistance in P. aeruginosa.

INTRODUCTION

Pseudomonas aeruginosa is a common nosocomial pathogen that causes a broad range of infections with a high mortality rate (Mahar et al., 2010; Lida et al., 2010; Lambert et al., 2011). A major factor in its prominence as a pathogen is, in part, its intrinsic resistance to a number of antibacterial agents (Poole et al., 1993; Hancock, 1998; Poole, 2002) and, particularly, the development of increased multidrug resistance in healthcare settings (Giamarellos-Bourboulis et al., 2006; Kirikae et al., 2008; Kallen et al., 2010; Keen et al., 2010). This organism readily acquires resistance via chromosomal mutations (e.g. overexpression of the efflux pump) and lateral gene transfer (e.g. acquisition of metallo-β-lactamase) (Lister et al., 2009; Poole, 2011). The emergence and spread of multidrug-, extensive drug- and pan-drug-resistant P. aeruginosa infections are very serious and of great concern, as few agents are effective against these organisms. In addition, antibiotic-resistant Gram-negative bacteria, including P. aeruginosa, increase the hospital costs and length of stay associated with healthcare-associated infections (Mauldin et al., 2010).

Aminoglycosides such as amikacin, gentamicin and tobramycin are a vital component of antipseudomonal chemotherapy for a variety of infections, particularly pulmonary infections in cystic fibrosis (CF) patients (Poole, 2005, 2011). Aminoglycoside resistance in P. aeruginosa has often arisen via acquired aminoglycoside-modifying enzymes (AMEs) and 16S rRNA methylases (RMTs), typically involving the MexXY endogenous efflux system (Poole, 2011). In particular, upregulation of the MexXY efflux pump is common, and MexXY has been implicated in aminoglycoside resistance in clinical isolates, particularly CF isolates (Sobel et al., 2003; Vogne et al., 2004; Henrichfreise et al., 2007; Islam et al., 2004; Hocquet et al., 2007; Vettoretti et al., 2009). A significant relationship between antibiotic use, for example, aminoglycoside, fluoroquinolone and cefepime, and the incidence of MexXY-overproducing P. aeruginosa has been reported (Hocquet et al., 2008). AMEs are also common determinants of aminoglycoside resistance in P. aeruginosa, except for CF isolates (Vogne et al., 2004;
Henrichfreise et al., 2007; Sekiguchi et al., 2007; Samuelsen et al., 2010; Poole, 2011). Each AME has limited substrate specificity, but individual aminoglycoside-resistant *P. aeruginosa* isolates can carry multiple (2–5) AMEs and thus exhibit broad-spectrum aminoglycoside resistance (Poole, 2005). Among the AMEs, AAC(6′)-II and ANT(2′)-I are the most prevalent mechanisms for aminoglycoside resistance in *P. aeruginosa* (Armstrong & Miller, 2010; Poole, 2005). RMTs were recently discovered in *P. aeruginosa* (Yokoyama et al., 2003) and these promote high-level resistance to clinically used aminoglycosides, such as gentamicin, tobramycin and amikacin (Doi & Arakawa, 2007; Doi & Arakawa, 2007; Poole, 2011).

The complete genome sequence of *P. aeruginosa* PA7, which exhibits resistance to multiple antibiotics, including amikacin, was recently reported (Roy et al., 2010). The identification of mexXY-oprA (PSPA7_3269-3270-3271), encoding a multidrug efflux pump, and aacC4 (PSPA7_3724.1), encoding an AAC(6′)-II, as aminoglycoside resistance determinants from the PA7 genome was very interesting, although there are several other AMEs in the PA7 genome that are not considered to be clinically relevant judging from their putative substrate specificities. MexXY-OprA of PA7 is a ‘hybrid’ system in that the PA7-specific oprA gene that is absent from the other known *P. aeruginosa* strains such as PAO1, PA14 and LESB58, and is linked to mexXY (Roy et al., 2010). MexX (397 aa) and MexY (1045 aa) of PA7 showed strong sequence similarities to MexXY (www.pseudomonas.com) of PAO1 [95 (98)% and 97 (99)% identity (similarity), respectively] and other *P. aeruginosa* strains, while OprA (467 aa) of PA7 showed higher sequence similarity to OprA of *Burkholderia* species [56–58 (70–71)% identity (positives)], such as *Burkholderia thailandensis*, *Burkholderia pseudomallei*, *Burkholderia mallei*, *Burkholderia multivorans* and *Burkholderia cenocepacia*, than to OprM encoded by the mexAB-oprM multidrug efflux operon of *P. aeruginosa* PAO1 [44 (59)% identity (similarity)], which MexXY of *P. aeruginosa* PAO1 requires as an outer membrane component to function (Mine et al., 1999; Aires et al., 1999). OprA of *B. pseudomallei* is an outer membrane component of AmrAB-OprA, which is a major multidrug efflux pump that is implicated in the pan-aminoglycoside resistance of the organism and is closely related to MexXY of *P. aeruginosa* (Moore et al., 1999; Mima & Schweizer, 2010). The cognate regulator mexZ (PSPA7_3268) located upstream of mexXY-oprA but divergently transcribed in PA7 is intact (Roy et al., 2010). Aac4 of PA7 functions as an AAC(6′)-II due to the substitution of a serine for a leucine at position 117 in PSPA7_3724.1 (Roy et al., 2010). This type of Aac4 variant is also called the AAC(6′)-Ib variant and AAC(6′)-Ib9 (Mugnier et al., 1998). This amino acid change has been associated with a shift from amikacin to gentamicin resistance in vitro after site-directed mutagenesis (Rather et al., 1992) and in various clinical isolates (Lambert et al., 1994; Mugnier et al., 1998; Casin et al., 2003; Poole, 2005). A recombinant *Escherichia coli* strain carrying AAC(6′)-Ib9 is strongly resistant to tobramycin and gentamicin and moderately (or slightly) resistant to amikacin (Rather et al., 1992; Casin et al., 2003).

It has been reported that at least 135 genes are involved in low-level tobramycin resistance in *P. aeruginosa* PA14 through random transposon mutagenesis, emphasizing the complexity of aminoglycoside resistance (Schurek et al., 2008). In fact, the cumulative effects of several non-enzymic mechanisms on the resistance of *P. aeruginosa* to aminoglycosides have previously been shown (El’Garch et al., 2007). *P. aeruginosa* PA7 might accumulate several low-level aminoglycoside resistance mechanisms in addition to MexXY-OprA and AAC(6′)-II [AAC(6′)-Ib9] to achieve high-level aminoglycoside resistance. In this study, we used a reverse genetic approach to evaluate the interplay between the two resistance mechanisms for clinically relevant aminoglycosides in *P. aeruginosa* PA7 and found that these two mechanisms are sufficient to explain the high level of aminoglycoside resistance in PA7. In addition, the efflux pump is necessary to acquire high-level aminoglycoside resistance in the form of AMEs in *P. aeruginosa*.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study are listed in Supplementary Table S1 (available with the online version of this paper). Bacterial cells were grown in Luria broth (1 % tryptone, 0.5 % yeast extract and 0.5 % NaCl) under aerobic conditions at 37 °C, unless otherwise indicated. All procedures for bacteria in which infectious aerosols or splashes may have been created were conducted in biological safety cabinets (NSC-II B2-1200; Dalton) in our biosafety level 2 laboratory facilities. Bacterial growth was quantified by measuring the optical density at 600 nm on an Ultraspec 2100 Pro Spectrophotometer (GE Healthcare), unless otherwise indicated. The plasmids pEX18Tc (Hoang et al., 1998), pYMI01 (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 pSPT26 (Masaoka et al., 2000), pBR322 (Morita et al., 1998), 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*. The plasmids pSTY28 (Masaoka et al., 2000), pKO3 [identical to pK03 (Link et al., 1997), except for the addition of a 3 kb stutter sequence in the multiple cloning site (http://arep.med.harvard.edu/labgc/pko3.html)], and their derivatives were maintained and selected using medium supplemented with 2–10 μg chloramphenicol ml⁻¹ for *E. coli*.

**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 were analysed with the Pseudomonas Genome Database v2 (www.pseudomonas.com), BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), CLUSTAL W of Kyoto University Bioinformatics Center (http://www.genome.jf/tools/clustalw/) and DNASIS Pro (Ver. 2.1; Hitachi).

**Cloning of oprA, mexXY, mexXY-oprA and aac4 from *P. aeruginosa PA7*.** The oprA, mexXY, mexXY-oprA and aac4 genes
from *P. aeruginosa* PA7 were amplified by PCR using the primers listed in Supplementary Table S2. The purified oprA PCR product, digested with EcoRI and BamHI, was cloned into pSPorl, digested with the same enzymes and dephosphorylated. The oprA genes were then released via digestion with EcoRI and BamHI and cloned into pYM101 and pSTV28 to yield pYM123 and pYM117, respectively.

The purified *mexXY*-oprA PCR products, digested with EcoRI, were cloned into pSTV28, digested with EcoRI and Smal, and dephosphorylated to yield pYM116 and pYM118, respectively. The mexXY-(oprA) genes were then released via digestion with EcoRI and HindIII and cloned into pYM101 to yield pYM121 and pYM122, respectively. The purified *aacA4* PCR product, digested with EcoRI and HindIII, was cloned into pYM101, digested with the same enzymes and dephosphorylated, to yield pYM124.

**Cloning of mexXY from *P. aeruginosa* PAO1.** The mexXY genes from PAO1 (Mine et al., 1999) were released via the digestion of pSPorl::mexXY (Morita et al., 2006) with EcoRI and cloned into pSTV28 and pYM101, digested with EcoRI, and dephosphorylated to yield pYM115 and pYM120, respectively.

**Construction of an in-frame tolC deletion mutant from *E. coli* KAM3.** To introduce an in-frame *tolc* gene deletion into *E. coli* KAM3 (Morita et al., 1998), deletion constructs were first prepared in pKOV by cloning PCR-amplified DNA fragments (~0.75 kb) corresponding to the upstream and downstream regions of the gene sequences to be deleted. These regions were amplified by PCR from the chromosomal DNA of *E. coli* KAM3 using the primer pairs listed in Supplementary Table S2 and the previously described reaction mixtures and conditions (Morita et al., 2010), except DMSO was not added. The resulting plasmid pYM119 was introduced into *E. coli* KAM3 cells, replacing the chromosomal *tolC* with Δtolc by homologous recombination (Chen et al., 2002). KAM3ΔtolC was selected as a strain that did not grow in the presence of 200 μg sodium deoxycholate ml⁻¹ (Hirakawa et al., 2003). This construct was confirmed by colony PCR.

**Construction of in-frame deletion mutants from *P. aeruginosa*.** In-frame deletion mutants of the oprA (PSPA7_3271), mexXY (PSPA7_3269-3270), mexXY-oprA (PSPA7_3269-3270-3271), oprM (PSPA7_0527), mexAb-oprm (PSPA7_0525-0526-0527) and/or *aacA4* (PSPA7_3724) genes from *P. aeruginosa* PA7 and their derivatives were constructed using the previously described sacB-based strategy (Morita et al., 2006, 2010). The plasmids and *P. aeruginosa* mutants are listed in Supplementary Table S1, while the primer pairs are listed in Supplementary Table S2. The *AmexXY* mutant from IMCJ2.S1 was constructed using pCSV05-01 (Sobel et al., 2003). The selection concentrations of tetracycline during the first homologous recombination event were variable depending on the tetacycline MICs for the *P. aeruginosa* strains. These constructs were confirmed by colony PCR.

**Construction of the *φCTX*-based site-specific integrants in *P. aeruginosa*.** For gene complementation experiments in *P. aeruginosa*, *φCTX* phage-based site-specific integrants were constructed using the integration-proficient, tightly controlled expression vector pYM101 as described previously (Morita et al., 2010). For the constructs generated from *P. aeruginosa* PA7 and their derivatives, Flp-recombinase-mediated excision of unwanted plasmid sequences with pFlp2 was not performed because those strains were resistant to carbenicillin (Roy et al., 2010), which is used to select pFlP-containing *P. aeruginosa*. The primer pairs are listed in Supplementary Table S2, while the plasmids and *P. aeruginosa* mutant constructs are listed in Supplementary Table S1. These constructs were confirmed by colony PCR.

**Detection and sequencing of oprA genes in *P. aeruginosa* strains.** DNA regions downstream from the mexY genes in the *P. aeruginosa* strains PAO1, PA7, DSM 1128 (Kiewitz & Tümmler, 2000) and O12 (King et al., 2008), the multidrug-resistant clinical isolates IMC798, IMC799 and IMCJ2.S1 (Kitao et al., 2009), the pan-aminoglycoside-resistant clinical isolate K2162 (Sobel et al., 2003), and subsets of the international antibiotic typimg scheme serotype strains (Hancock, 1998) (Supplementary Table S1) were amplified by PCR with the primers *EcoRI*-oprA-F2 and BamHI-oprA-R2 (Supplementary Table S2). After separation by using 1.0 % agarose gel electrophoresis using Mupid-exU (TaKaRa), the purified PCR products were sequenced with the same primers. Agarose I was purchased from Amresco (Solon).

**Construction of mexZ* mutant from *P. aeruginosa* PA7 and PA7-ΔaacA4.** The DNA region encompassing the mexZ gene and the intergenic region between the mexZX genes from *P. aeruginosa* DSM 1128 was amplified by PCR with the primers *EcoRI*-mexZ-mexXp-F and BamHI-mexZ-mexXp-R (Supplementary Table S2). The purified mexZ-mexXp PCR product, digested with EcoRI and BamHI, was cloned into mini-CTX-λacZ (Becher & Schweizer, 2000), digested with the same enzymes and dephosphorylated, to yield pYM125. mexZ-mexXp was released via digestion with EcoRI and BamHI and cloned into pEX18Tc to yield pYM132. The mexZ gene from *P. aeruginosa* DSM 1128 in pYM132 was replaced with the PSPA7_3268 gene of *P. aeruginosa* PA7 and PA7 ΔAAC by the same sacB-based recombination method described above, yielding *P. aeruginosa* PA7 Z⁺ and PA7 ΔZ⁺ ΔAAC, respectively. These constructs were confirmed by PCR amplification and sequencing.

**Antibiotic susceptibility testing.** The MICs of selected antimicrobial agents in cation-adjusted Mueller–Hinton broth (CAMHB) were determined in Falcon 3077 Microtest U-Bottom plates (Becton Dickinson) by using the broth microdilution method (Jorgensen & Turnidge, 2007). Adjustment of the cations Ca²⁺ (20 μg ml⁻¹) and Mg²⁺ (10 μg ml⁻¹) was accomplished by the addition of 3.68 % CaCl₂·2H₂O (Wako Pure Chemicals) solution and 8.36 % MgCl₂·6H₂O (Wako) solution, respectively, into Mueller–Hinton Broth (Becton Dickinson) to ensure acceptable results in *P. aeruginosa* isolates, unless otherwise indicated (Jorgensen & Turnidge, 2007). The MICs for the *φCTX* phage-based site-specific integrants were determined in the presence of the inducer isopropyl-β-D-1-thiogalactopyranoside (IPTG; 5 mM) (Morita et al., 2009). Cultures of *P. aeruginosa* grown overnight at 37 °C without shaking in L medium containing 50 mM KNO₃ (Wako) diluted with 0.85 % NaCl (Wako) to OD₆₀₀ 0.1, corresponding to ~1.8 × 10⁶ c.f.u. ml⁻¹ (Morita et al., 2010). A portion of this suspension was diluted 1 : 180 (1.0 × 10⁷ c.f.u. ml⁻¹) with CAMHB, and 0.05 ml of this dilution was added to each well containing 0.05 ml of the antimicrobial agents diluted in CAMHB to achieve a final inoculum of 5.0 × 10⁶ c.f.u. ml⁻¹. The results were determined after incubation at 37 °C for 18–20 h.

**Antimicrobial agents.** Gentamicin, amikacin, ciperoxacin, tetacycline, ampicillin, chloramphenicol and carbenicillin were purchased from Wako Pure Chemicals. Tobramycin was purchased from Towa Pharmaceuticals. Arbekacin was purchased from Shinonochim. Cefepime was purchased from Sandoz.

**Real-time quantitative reverse transcriptase (RT)-PCR.** Overnight cultures of *P. aeruginosa* strains in Bacto Tryptic Soy Broth (Becton Dickinson) were diluted 1 : 100 in 10 ml CAMHB, 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 using the RNeasy Mini Kit (Qiagen). Real-time quantitative RT-PCR was performed with primer pairs internal to */uvrD* and *mexX* (Supplementary Table S2) using the One Step SYBR PrimeScript RT-PCR kit II (TaKaRa) in a Thermal Cycler Dice real-time system (TaKaRa). The
transcript levels of mexX in a given strain were normalized with those of
uvrD (the primer pairs used are listed in Supplementary Table S2) and
expressed as a ratio (fold change) to that observed in the parental PA7
strain. Gene expression values were calculated from three independent
bacterial cultures, each of which was tested in triplicate.

RESULTS

Involvement of the MexXY-OprA/OprM in
aminoglycoside resistance of P. aeruginosa PA7

Aminoglycoside resistance in P. aeruginosa PA7 was
compared with the two aminoglycoside-sensitive strains
PAO1, the most well-known strain (Sobel et al., 2003;
Morita et al., 2006), and DSM 1128, a taxonomically
related PA7 strain (Martin & Beveridge, 1986; Kiewitz &
Tümmler, 2000; Roy et al., 2010). The MICs of the
antisepsudomonal aminoglycosides (amikacin, arbekacin,
gentamicin and tobramycin) for antipseudomonal aminoglycosides (amikacin, arbekacin,
much more resistant (et al., 1999). In fact, the ΔoprA ΔoprM double mutant (PA7
ΔA ΔM) showed the same susceptibility to the aminoglyco-
sides, although the ΔoprM mutant (PA7 ΔM) did not;
therefore, MexXY utilizes OprM in addition to OprA as one
of its outer membrane components in PA7. This result
indicates that MexXY-OprA/OprM contributes to the action
of antipseudomonal aminoglycosides in PA7. We noticed
that the ΔoprA ΔoprM double mutant (PA7 ΔA ΔM) showed
the same phenotype as the ΔmexXY-oprA ΔmexAB-oprM
double mutant (PA7 ΔXYA ΔAMB). These two mutants
(PA7 ΔA ΔM and PA7 ΔXYA ΔAMB) were more sensitive
to ciprofloxacin, cefpirome and tetracycline than the ΔmexXY-oprA or ΔmexAB-oprM mutant (PA7 ΔXYA or PA7 ΔAMB,
respectively) (Table 1). MexXY-OprA and MexAB-OprM
seem to be major antimicrobial efflux pumps in PA7, in a
similar manner as in PAO1 (Morita et al., 2001).

Functional expression of MexXY-OprA in E. coli
and P. aeruginosa

To examine the function of the mexXY-oprA genes in P.
aeruginosa PA7, we cloned conserved genes (mexXY), a
unique gene (oprA) and whole genes (mexXY-oprA), and
expressed each of them in E. coli KAM3 (Morita et al., 1998)
lacking the acrB gene, which is an RND-family transporter
component of the major multidrug efflux pump AcrAB-
TolC in E. coli. This strain is very useful for the analysis of
various multidrug efflux pumps from micro-organisms
(Morita et al., 1998; Mine et al., 1999; Masaoka et al., 2000)
and plants (Li et al., 2002). E. coli KAM3 containing the
mexXY or mexXY-oprA genes of PA7 showed increased
resistance to a number of antibiotics, including erythromy-
cin, ciprofloxacin, cefpirome, tetracycline and gentamicin
(Table 2) to the same extent as E. coli KAM3 containing the
mexXY genes of P. aeruginosa PAO1 (data not shown). We
did not find any functional difference between the two
MexXY pumps of PA7 and PAO1 expressed in E. coli KAM3,
suggesting that MexXY of P. aeruginosa PA7 is a functional
homologue of MexXY from P. aeruginosa PAO1 and that
MexXY appears to utilize TolC as its outer membrane
component in E. coli. To see if the oprA of PA7 is functional,
we expressed the mexXY, oprA or mexXY-oprA genes of P.
aeruginosa PA7 in E. coli KAM3 ΔtolC cells lacking not only
the acrB gene but also the tolC gene, which encodes the outer
membrane component of AcrAB-TolC (Table 2). Only E.
coli KAM3 ΔtolC containing mexXY-oprA showed increased
resistance to the same antimicrobials listed above, suggesting
that OprA can also associate with the MexXY system in E.
coli.

Next, we characterized the mexXY, oprA and mexXY-oprA
genes of PA7 in P. aeruginosa PAO1 derivatives (Table 1).
Interestingly, the introduction of mexXY into the chro-
omosome of PAO1 ΔmexXY (PAO1 ΔXY attB::LacI-XYPAO1
and PAO1 ΔXY attB::LacI-XY) yielded the PAO1 phenotype,
and the introduction of mexXY into the chromosome
of PAO1 ΔZXY (PAO1 ΔZXY attB::LacI-XYPAO1 and
PAO1 ΔZXY attB::LacI-XY) yielded the PAO1 ΔmexZ
et al., 1999). In fact, the ΔoprA ΔoprM double mutant (PA7
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resistance to the same antimicrobials listed above, suggesting
that OprA can also associate with the MexXY system in E.
coli.
phenotype. Likewise, we could not find any significant difference between MexXY of PA7 and MexXY of PAO1. On the other hand, the introduction of mexXY-oprA into PAO1 ΔmexZ (PAO1 ΔmexZXY, attB::LacI-XY) yielded slightly more (two- to four-fold) than the ΔmexZ phenotype and the introduction of mexXY-oprA into PAO1 ΔmexZXY

**Table 1.** Change in the MICs of aminoglycosides and other antimicrobial agents against various constructs from *P. aeruginosa* PA7, PAO1, IMCJ2.S1, and K2162

MICs were determined in Mueller–Hinton broth with (+) or without (−) adjustment of cations Mg\(^{2+}\) and Ca\(^{2+}\) at least three times. MICs for the ΔCTX-based site-specific integrants were determined in the presence of the inducer 5 mM IPTG (isopropyl-β-D-1-thiogalactopyranoside). Abbreviations for genes or plasmids: XYA, mexXY-oprA; XY, mexXY; A, oprA; M, oprM; ABM, mexAB-oprM; Z, mexZ; AAC, aacA4; pV, pYM101 (Morita *et al.*, 2010); pXYA, pYM101::mexXY-oprA; pAAC, pYM101::aacA4; LacI, lacI, attB, a chromosomal site which ΔCTX elements integrate in the 3′ end of a tRNA\(^{Ser}\) gene of *P. aeruginosa* (Morita *et al.*, 2010). Abbreviations for antimicrobial agents: AMK, amikacin; GEN, gentamicin, TOB, tobramycin; ABK, arbekacin; CIP, ciprofloxacin; FEP, cefepime; TET, tetracycline.

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<tr>
<th>Strain</th>
<th>MIC (μg ml(^{-1})) for:</th>
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<td></td>
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<tr>
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<td>8</td>
</tr>
<tr>
<td>PA7 ΔZ ΔAAC</td>
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</tr>
<tr>
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</tr>
<tr>
<td>PA7 ΔXYA ΔAAC, attB::pXYA</td>
<td>16</td>
</tr>
<tr>
<td>PA7 ΔXYA ΔAAC attB::pAAC</td>
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<tr>
<td>PAO1</td>
<td>2</td>
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<tr>
<td>PAO1, attB::LacI</td>
<td>2</td>
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<tr>
<td>PAO1, attB::LacI-AAC</td>
<td>8</td>
</tr>
<tr>
<td>PAO1 ΔZ</td>
<td>4</td>
</tr>
<tr>
<td>PAO1 ΔZ, attB::LacI</td>
<td>4</td>
</tr>
<tr>
<td>PAO1 ΔZ, attB::LacI-AAC</td>
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<tr>
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</tr>
<tr>
<td>PAO1 ΔXY attB::LacI-XPAO1</td>
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</tr>
<tr>
<td>PAO1 ΔXY, attB::LacI-X</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>PAO1 ΔZXY, attB::LacI-Y</td>
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<tr>
<td>IMCJ2.S1</td>
<td>256</td>
</tr>
<tr>
<td>IMCJ2.S1 ΔXY</td>
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<tr>
<td>K2162</td>
<td>256</td>
</tr>
<tr>
<td>K2162 ΔXY</td>
<td>32</td>
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</table>
Table 2. Functional expression of MexXY-OprA in E. coli
KAM3 or KAM3 ΔtolC

MICs were determined in CAMHB at least three times in the presence of the inducer 0.1 mM IPTG. Abbreviations for a strain or plasmids: ΔtolC, KAM3 ΔtolC; pXY, pSTV28::mexXY; pA, pSTV28::oprA; pXYA, pSTV28::mexXY-oprA. Abbreviations for antimicrobial agents are as in the legend for Table 1; ERY, erythromycin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant pump(s)</th>
<th>MIC (µg ml⁻¹) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FEP</td>
</tr>
<tr>
<td>KAM3/ pSTV28</td>
<td>TolC</td>
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</tr>
<tr>
<td>KAM3/pXY</td>
<td>MexXY-TolC</td>
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<tr>
<td>KAM3/pXYA</td>
<td>MexXY-TolC</td>
<td>0.063</td>
</tr>
<tr>
<td>ΔtolC/ pSTV28</td>
<td>OprA</td>
<td>0.015</td>
</tr>
<tr>
<td>ΔtolC/pXY</td>
<td>MexXY</td>
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</tr>
<tr>
<td>ΔtolC/pA</td>
<td>OprA</td>
<td>0.015</td>
</tr>
<tr>
<td>ΔtolC/pXYA</td>
<td>MexXY-OprA</td>
<td>0.031</td>
</tr>
</tbody>
</table>

(PAO1 ΔZXY attB:: LacI-XYA) also yielded the same phenotype as PAO1 ΔXY attB:: LacI-XYA. These results might demonstrate that the ΔmexZ phenotype could not be explained by the overexpression of mexXY alone. The molecular mechanisms underlying the ΔmexZ phenotype will be reported in a separate paper. Because neither MexXY nor MexAB in PAO1 was functional as a multidrug efflux pump or extruded its substrate drugs in the absence of OprM, PAO1 ΔoprM was more susceptible than PAO1 to MexXY-specific substrates (aminoglycosides and gentamicin), MexAB-specific/preferable substrates (carbenicillin and chloramphenicol) and shared substrates (tetracycline) (Table 3), which was consistent with previously described results (Morita et al., 2001). The introduction of oprA restored PAO1 phenotypes for the MexXY substrates but not enough for MexAB substrates (Table 3). OprA did not seem to complex with MexAB as well as it did with MexXY.

Table 3. Functional expression of OprA in the PAO1 ΔoprM background

MICs were determined in CAMHB at least three times in the presence of the inducer 5 mM IPTG. Abbreviations for strains/plasmids and antimicrobial agents are given in the legend to Table 1; CAR, carbenicillin; CHL, chloramphenicol.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant pump(s)</th>
<th>MIC (µg ml⁻¹) for:</th>
</tr>
</thead>
<tbody>
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<td></td>
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<td>PAO1</td>
<td>MexXY/MexAB-OprM</td>
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<td>MexAB-OprM</td>
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<tr>
<td>ΔoprM</td>
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<td>0.5</td>
</tr>
<tr>
<td>ΔoprM attB:: lacI</td>
<td>MexXY/MexAB-OprA</td>
<td>2</td>
</tr>
</tbody>
</table>

Prevalence of OprA in P. aeruginosa

We isolated the mexY (PA2018)-PA2017 intergenic region (240 bp) in PAO1 and identified a small gene encoding an ORF of 72 aa (Fig. 1a). The majority (residues 1–64) of the gene product showed very strong similarity to the OprA N-terminal region (residues 1–72) of PA7 [61 (68)% identity (similarity); Fig. 1b]. The intergenic regions of DSM 1128 and 16 serotype international antigenic typing scheme P. aeruginosa strains were detected and sequenced. Most of them possessed the truncated oprA gene, very similar to PAO1 [397 bp DNA fragment was amplified (lane 1 in Fig. 1c)], and only DSM 1128 and O12 had an intact oprA gene, very similar to PA7 [a 1569 bp DNA fragment was amplified (lane 2 in Fig. 1c); the nucleic acids were sequenced, translated and compared (Supplementary Fig. S1)]. Other clinical strains (IMCJ2.S1, IMCJ798, IMCJ797 and K2162) also did not have a complete oprA gene (data not shown). It seemed that the original P. aeruginosa (ancestor) possessed an intact oprA gene, and strains such as PAO1, PA14, LESB58, etc. that possessed a cryptic oprA gene became more dominant in the environment once some strains had lost it. All of the strains possessing a complete oprA gene (PA7, DSM 1128, and O12) were of serotype O12.

P. aeruginosa PA7 is an agrZ-type mutant

Transcriptional expression of mexX in PA7 was measured by real-time quantitative RT-PCR. mexX expression in PA7 was much higher (~50-fold) than in DSM 1128 (0.017 ± 0.0104 as a ratio to PA7), indicating that P. aeruginosa PA7 is a mexXY-oprA-upregulated strain. To date, three kinds of mutants (agrZ, agrW1 and agrW2) have been recognized as MexXY-overproducers as a result of genetic mechanisms: mutants with alterations in mexZ, the cognate repressor gene located upstream of, but transcribed divergently from, mexXY (type agrZ); mutants with impaired protein synthesis (type agrW1); and mutants with alterations in parRS, a recently discovered two-component regulatory system (type agrW2) (Llanes et al., 2004; Muller et al., 2011; de Bentzmann & Pleisiat, 2011). Although Roy et al. (2010) reported that the regulatory gene mexZ (PSPA7_3268) of mexXY-oprA in P.
*Pseudomonas aeruginosa* PA7 was intact, we found an obvious inconsistency in the N-terminal portion in comparison with the MexZs of *P. aeruginosa* PAO1 and PA14, while there was significantly high similarity in the C-terminal portion. MexZ of *P. aeruginosa* PAO1 contains a characteristic N-terminal helix–turn–helix DNA-binding domain that is conserved among the TetR family of transcriptional regulators (Aires et al., 1999; Alguel et al., 2010). When the nucleic acid sequence of PSPA7_3268 was compared with mexZ in *P. aeruginosa* PAO1, a strong similarity was observed, but a gap region was also identified; moreover, the putative start codon of PSPA_3268 is TTG, which is very rare. Sequencing of *P. aeruginosa* DSM 1128 revealed a deleted region in MexZ of *P. aeruginosa* PA7 because of the high similarity between DSM 1128 and PA7. Differences between them include (1) the 10 bp deletion at the residues 228–237 causing a frameshift mutation within mexZ of PA7 and (2) T to C substitution at residue 257 causing a silent mutation (underlined in Fig. 2a). In addition, the start codon of mexZ in DSM 1128 seems to be GTG at position 1–3 according to the mexZ so of PAO1, PA14 etc. and not TTG at position −31 to −29, which corresponds with the putative start codon of PSPA_3268 in PA7 (Fig. 2a). MexZ (210 aa) of DSM 1128 showed strong sequence similarities, but also some discrepancies, with MexZ of PAO1 and PA14 [89 (93)% and 88 (93)% identity (similarity), respectively; Fig. 2b]. Replacement of PSPA7_3268 with mexZ of DSM 1128, yielding PA7_mexZ + (PA7 Z +), showed decreased mexX expression (0.019 ± 0.0026 as a ratio to PA7) in PA7 Z +, which was nearly identical to DSM 1128, and decreased MICs for the antipseudomonal aminoglycosides. The ability to complement the apparent mexZ mutation with a wild-type mexZ supports the notion that PA7 is an agrZ-type mutant.

**Aminoglycoside resistance in *P. aeruginosa* PA7 via the combination of the efflux pump and modifying enzymes**

We evaluated the interplay between MexXY-OprA and AacA4 on aminoglycoside resistance in *P. aeruginosa* PA7.

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**Fig. 1.** Prevalence of OprA homologues. (a) Localization of an ORF (oprA') encoding a gene product (OprA') of 72 aa between PA2018 (mexY) and PA2017 in PAO1. Nucleotide sequences of oprA' and amino acid sequences are shown in red. For MexY and PA2018 only the 39 and 58 carboxy-terminal amino acid sequences are shown. (b) Comparison of OprA' of PAO1 (OprA'_PAO1) with the N-terminal 72 aa of OprA of PA7 (OprA_PA7) with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). (c) PCR detection of oprA' and oprA in PAO1 (lane 1) and PA7 (lane 2). M, 100 bp DNA ladder (TaKaRa). PCR products corresponding to full-length (1569 bp) and truncated (397 bp) oprA are indicated.
The ΔaacA4 mutant of *P. aeruginosa* PA7 (PA7 ΔAAC) was much more sensitive to gentamicin (>128-fold) and tobramycin (128-fold), but only slightly more sensitive to arbekacin (fourfold) and amikacin (twofold) (Table 1). PA7 *mexZ* ΔaacA4 (PA7 Z+ ΔAAC) and ΔmexXY-oprA ΔaacA4 (PA7 ΔXYA ΔAAC) demonstrated almost the same sensitivity to the antipseudomonal aminoglycosides as PAO1 and its ΔmexXY mutant, respectively (Table 1). Gene complementation experiments with the pCTX phage-based site-specific integration system confirmed the results from the gene knockout experiments (PA7 ΔXYA attB::pXYA, PA7 ΔAAC attB::pAAC, PA7 ΔXYA ΔAAC attB::pAAC, and PA7 ΔXYA ΔAAC attB::pAAC; Table 1). Moreover, aacA4-introduced PAO1 (PAO1 attB::laci-AAC), PAO1 ΔmexZ (PAO1 ΔZ attB::Laci-AAC) and PAO1 ΔmexXY (PAO1 ΔXY attB::Laci-AAC) showed similar MICs for the antipseudomonal aminoglycosides to PA7 ΔmexZ+ (PA7 Z+), PA7 and PA7 ΔmexXY-oprA (PA7 ΔXYA), respectively (Table 1). Thus, the combination of the efflux pump and modifying enzymes was necessary and sufficient to explain the high level of resistance to antipseudomonal aminoglycosides in *P. aeruginosa* PA7 (illustrated in Fig. 3).

**Fig. 2.** PA7 possesses a mutated *mexZ* gene. (a) Sequence of a *mexZ* gene in DSM 1128. The numbers refer to the position of the nearest residue on each line. Changes (underlined) in the *mexZ* gene of PA7 include: (1) residues 228–237 are deleted causing a frame shift that results in a premature stop at codon TGA at the position 419–421, and (2) the T at position 255 (underlined) is changed to a C without altering the MexZ amino acid sequence. In addition, the start codon of *mexZ* in DSM 1128 seems to be GTG at the position 1–3 according to the *mexZ*s of PAO1, PA14 etc. and not TTG to the position –31 to –29 which corresponds to the putative start codon of PSPA_3268 in PA7. (b) Comparison of MexZ of PAO1 (MexZ _ PAO1) with MexZ of DSM 1128 (MexZ _ DSM1128) using CLUSTAL W of Kyoto University Bioinformatics Center (http://www.genome.jp/tools/clustalw/). Asterisks (*) and dots (. or :) indicate residues that were identical and similar, respectively.

**Contribution of MexXY to aminoglycoside resistance in the other clinical *P. aeruginosa* isolates**

Recently, a multidrug-resistant *P. aeruginosa* carrying the aac(6′)-Iae gene, namely IMCJ2.S1 (Sekiguchi et al., 2005), was shown to have caused an outbreak in Japanese
community hospitals (Sekiguchi et al., 2007). A recombinant E. coli containing AAC(6’)-Iae is resistant to amikacin and tobramycin, but not to gentamicin or arbekacin (Sekiguchi et al., 2005). We assessed the impact of mexXY genes on aminoglycoside resistance in IMCJ2.S1 (Table 1). Interestingly, a large decline in aminoglycoside MICs was found upon deletion of mexXY (32- to 64-fold) in this strain (Table 1). As shown previously (Sobel et al., 2003), this decline in aminoglycoside MICs upon deletion of mexXY was also found in the pan-aminoglycoside-resistant K2162 strain (Table 1).

**Requirement of MexXY, but not aminoglycoside-modifying enzymes (AMEs), for the antagonism of aminoglycosides by divalent cations in P. aeruginosa**

Because divalent cations (Mg$^{2+}$ and Ca$^{2+}$) have been shown to enhance MexXY-mediated antimicrobial resistance in P. aeruginosa PAO1, possibly by enhancing the activity of the efflux system (Mao et al., 2001), we investigated the effect of divalent cations on aminoglycoside resistance and its requirement for MexXY in the clinical strains PA7, IMCJ2.S1 and K2162 (Table 1). As expected, all of these strains showed decreased resistance (4- to 16-fold) to aminoglycosides without the addition of divalent cations; however, the mexXY isogenic mutants did not (see PA7 ΔXYA, IMCJ2.S1 ΔXY and K2162 ΔXY in Table 1), irrespective of the presence of AMEs [AacA4 for PA7 ΔXYA and Aac(6’)-Iae for IMCJ2.S1 ΔXY].

**DISCUSSION**

Most of the previous studies on aminoglycoside resistance in P. aeruginosa focused on only one of the two mechanisms, especially the existence of AMEs (e.g. Sekiguchi et al., 2007; Samuelsen et al., 2010), while others pointed out that upregulation of the MexXY efflux system and acquisition of AMEs co-existed in the same clinical P. aeruginosa strains (e.g. Henrichfreise et al., 2007; Hocquet et al., 2007). In this study, we demonstrated that interplay between the MexXY efflux pump and the AAC modifying enzyme in P. aeruginosa provides high-level aminoglycoside resistance in clinical use. In fact, efflux (and/or membrane impermeability)-mediated aminoglycoside resistance is much more prominent in P. aeruginosa than in Enterobacteriaceae (Armstrong & Miller, 2010). Although recently surfaced RMTs provide high-level aminoglycoside resistance even to recombinant E. coli (Yokoyama et al., 2003), we speculated that RMT-encoding genes spread, in theory, more slowly than Enterobacteriaceae because P. aeruginosa showed natural resistance to aminoglycoside due to the MexXY efflux pump (Aires et al., 1999) and acquired sufficient high-level aminoglycoside resistance via a combination of AMEs (e.g. PA7, IMCJ2.S1) or impermeability (Sobel et al., 2003; El’Garch et al., 2007). Of course, RMT-encoding genes can also spread among P. aeruginosa strains by selection of other antimicrobial agents (e.g. carbapenem) in the form of accompanying another acquired resistant gene (e.g. carbapenemase-encoding genes) (e.g. Doi et al., 2007). Recently, novel endogenous aminoglycoside resistance determinants were reported (Schurek et al., 2008; Lee

**Fig. 3.** Primary mechanisms mediating aminoglycoside (e.g. gentamicin) resistance in the multidrug-resistant P. aeruginosa clinical isolate PA7. The main findings in this study, namely the primary mechanisms mediating antipseudomonal aminoglycosides (e.g. gentamicin) resistance in PA7, are shown schematically. According to the interpretive Clinical and Laboratory Standards Institute standard, MICs of gentamicin were categorized as sensitive (≤4 μg ml$^{-1}$), intermediate (8 μg ml$^{-1}$) and resistant (≥16 μg ml$^{-1}$). The very high resistance (>1024 μg ml$^{-1}$) in PA7 and high resistance (128 μg ml$^{-1}$) in PA7 Z+, in which expression of the pump was repressed by MexZ, were due to the efflux pump MexXY-OprA and the modifying enzymes AacA4. Resistances around or a little bit below the breakpoint (16, 8 and 2 μg ml$^{-1}$) in PA7 ΔXYA, PA7 ΔAAC and PA7 Z+ ΔAAC, respectively, were due to the absence of one of the two determinants. Hyper-sensitivity (0.25 μg ml$^{-1}$) in PA7 ΔXYA ΔAAC was due to absence of both the determinants. The genes are abbreviated: XYA, mexXY-oprA; Z, mexZ; AAC, aacA4.
et al., 2009; Strube & Gill, 2009; Kindrachuk et al., 2011; Hinz et al., 2011) in addition to previous ones (Poole, 2005). It might be interesting to examine the relationship between the MexXY pump and each determinant, or combinatorial determinants, in order to eradicate aminoglycoside resistance in *P. aeruginosa*.

We showed that the O12 serotype strains possessed complete oprA genes. O12 is a predominant serotype associated with multidrug resistance to a number of antibiotic classes, including aminoglycosides and β-lactams, although it represents a minor serotype in the environment (Legakis et al., 1982; Pitt et al., 1990; Bert et al., 1996; Samuelsen et al., 2010). The presence of the oprA gene in *P. aeruginosa* might be more advantageous for its survival in the presence of aminoglycosides, other antimicrobials such as β-lactams (e.g. cefepime, ceftobiprole; Hocquet et al., 2006; Baum et al., 2009), tigecycline (Dean et al., 2003), LBM415 (peptide deformylase inhibitor; Caughlan et al., 2009) and/or some other sources of stress (e.g. oxidative stress; Fraud & Poole, 2011). In fact, *P. aeruginosa* is exposed to high levels of reactive oxygen species in cystic fibrosis lungs, where mexXY overexpression mutants accumulate due to DNA damage resulting from the inflammatory response (Smith et al., 2006; Fraud & Poole, 2011). The multidrug-resistant O12 clone clusters very tightly, includes only clinical isolates and emerged during the 1980s, perhaps by selection from heavy antibiotic use (Pirnay et al., 2009; Woodford et al., 2011). O12 might be more dominant due, in part, to the presence of oprA in hospitals in which antimicrobials, such as aminoglycosides, promoting MexXY-OpRA-mediated multidrug resistance were used. *P. aeruginosa* PA7 isolated before 1984 from a wound infection in Buenos Aires, Argentina (Pirnay et al., 2009; Roy et al., 2010), might have also acquired multidrug resistance via the heavy use of antibiotics, including gentamicin or tobramycin, to treat wounds at that time. Apparently, a slightly increased resistance (two- to fourfold) to amikacin, ciprofloxacin and cefpirome was observed with or without the presence of oprA (PA01 ΔZXY attB::LacI-XY and PA01 ΔZXY attB::LacI-XYA in Table 1). Such a small difference might be significant during antibiotic treatment or in the presence of subinhibitory concentration of antibiotics. Examination of the importance of oprA in *P. aeruginosa* when exposed to various stresses is currently in progress at our laboratory.

MexXY seems to be promiscuous and able to form active drug efflux complexes with OprA, OprM and TolC; it might be interesting to study the functional interaction or assembly of MexXY with the outer-membrane channels. From this point of view, it is noteworthy that it seems to be the upregulation of MexXY rather than the presence of OprA that is important for aminoglycoside resistance. In Enterobacteriaceae such as *E. coli*, *Salmonella* sp. and *Klebsiella* sp. etc., the AcrAB-TolC multidrug efflux pump is a major contributor to the multidrug resistance (Piddock, 2006). Upregulation of acrAB genes strongly correlated with fluoroquinolone and multidrug resistance in clinical *E. coli* isolates, while tolC expression did not (Swick et al., 2011). Considering that the AcrAB-ToLC complex of *E. coli* assembles between pre-assembled inner-membrane complex AcrAB and outer-membrane channel TolC (Tikhonova et al., 2011), how many pre-assembled inner-membrane complexes (i.e. AcrAB or MexXY) are produced might influence how many substrates the bacterial cell pumps out and it might be the reason why upregulation of acrAB or mexXY is relevant in the emergence of fluoroquinolone or aminoglycoside resistance in the pathogens.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


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