INTRODUCTION

Pseudomonas aeruginosa has been recognized as an increasingly important and worrisome species in health care-associated infections (Poole, 2011). This bacterium possesses intrinsic resistance to many antimicrobials because of the low permeability of its outer membrane barrier and the presence of multidrug efflux transporters (Nikaido, 1994; Hancock, 1998). Although fluoroquinolones (e.g., ciprofloxacin), broad-spectrum β-lactams (e.g., imipenem), and anti-pseudomonas aminoglycosides (e.g., amikacin) are often available for treatment, β-lactamases (e.g., TEM, SHV, and CTX-M) are widespread. 

The emergence and spread of multidrug-, extensive drug-, and pandrug-resistant P. aeruginosa infections is of great concern as multidrug-resistant clinical isolate PA7, serotype O12 deserves special attention. Moreover, the discovery of the cognate outer membrane component (OprA) of MexXY in the multidrug-resistant clinical isolate PA7, serotype O12 deserves special attention.

Keywords: aminoglycoside resistance, Pseudomonas aeruginosa, efflux, MexXY, PA5471, OprA
of these pumps and discuss how to combat efflux-mediated aminoglycoside resistance.

PRE-MexXY DISCOVERY ERA: RND MULTIDRUG EFFLUX PUMPS AS DETERMINANTS OF RESISTANT TO A WIDE RANGE OF ANTIMICROBIALS, BUT NOT AMINOGLYCOSIDES

In 1993, the first RND-type multidrug efflux system of \textit{P. aeruginosa}, MexAB-OprM (OprM was called Opm at that time), was discovered at approximately the same time as the AcrAB (AcrB was called Acr at that time) system of \textit{E. coli} (Poole et al., 1993b). It was the first genetic evidence that an efflux operon was involved in multiple antibiotic resistance in \textit{P. aeruginosa} (Poole et al., 1993b). The following year, the efflux activity of tetracycline, chloramphenicol, norfloxacin, and benzylpenicillin was shown using antibiotic accumulation assays in intact cells, which was the first biochemical evidence of the role of efflux in intrinsic multidrug resistance in \textit{P. aeruginosa} (Li et al., 1994a,b, Li et al., 1995). Taken together, the MexAB-OprM system was shown to contribute to the intrinsic resistance of \textit{P. aeruginosa} to a wide range of antimicrobial compounds including fluoroquinolones, tetracycline, chloramphenicol, and \(\beta\)-lactams such as carbenicillin (Poole et al., 1993b, Li et al., 1995). Its homologs (MexCD-OprJ and MexEF-OprN) were then discovered as determinants of multidrug resistance in \textit{P. aeruginosa} (e.g., Morita et al., 2003; Feter et al., 2011). An unidentified efflux system that requires OprM was shown to contribute to resistance to quinolones and cephalosporins, such as cefpirome, erythromycin, and tetracycline, but not \(\beta\)-lactams, such as ceftizoxime and carbenicillin, in the \textit{P. aeruginosa} PAO1 background (Zhuo et al., 1999). More details on these three pumps can be found in recent reviews (e.g., Li and Nikaido, 2004, 2009; Lister et al., 2009).

In those days, RND multidrug efflux systems such as MexAB-OprM and AcrAB-OprM, which can confer a wide variety of drugs that appear to contain hydrophobic domains of significant sizes (Nikaido, 1996), were considered to be similar to the \(\beta\)-galactosidase multidrug efflux pump of mammalian cells, which extrudes not only basic compounds but also neutral and weakly acidic compounds (Nikaido, 1994). However, there was no evidence for the efflux of aminoglycosides, which are very hydrophilic drugs, among the antibiotics used to treat \textit{P. aeruginosa} infections (Li et al., 1994a; Nikaido, 1996).

\textbf{MexXY SYSTEM OF \textit{P. aeruginosa} WAS IDENTIFIED BY THREE DIFFERENT GROUPS}

The MexXY system was discovered in 1999 in Japan as the fourth RND-type multidrug efflux system of \textit{P. aeruginosa} PAO1 (Mine et al., 1999). This system was functionally expressed and conferred resistance to fluoroquinolones, tetracycline, erythromycin, etc. in the \textit{E. coli} KAM3 mutant (Morita et al., 1998) lacking the acrB gene, which is an RND transporter component of the major multidrug efflux pump (AcrAB-ToIC) in \textit{E. coli}. Interestingly, unlike the other three already known systems, no open-reading frame encoding the outer membrane component, such as OprM, was found in the region downstream from the mexY gene (Mine et al., 1999). However, this system was found to function cooperatively with OprM of \textit{P. aeruginosa} and ToIC of \textit{E. coli} (Mine et al., 1999).

Nine months after the discovery described above, a French group showed that MexXY was involved in the natural resistance of \textit{P. aeruginosa} PAO1 to aminoglycosides as well as tetracycline and erythromycin (Aires et al., 1999). Although the overexpression of MexXY increased the level of resistance to fluoroquinolones in \textit{P. aeruginosa} PAO1 cells, disruption of mexXY from PAO1 had no detectable effect on susceptibility to these agents (Aires et al., 1999). mexZ, which is located upstream of but transcribed separately from mexX, was identified (Aires et al., 1999). Its product, MexZ, contains a helix-turn-helix motif, which is characteristic of DNA-binding proteins, at its N-terminal, similar to the repressors of RND-type multidrug efflux genes (e.g., AcrR, a repressor of \textit{acrB} in \textit{E. coli}), supporting the notion that mexZ negatively controls the expression of the operon (Aires et al., 1999).

The following month (10 months after the first discovery), a group in the USA showed that MexXY, which they called AmrAB, was an aminoglycoside impermeability factor in spontaneous aminoglycoside-resistant mutants of the impermeability phenotype from \textit{P. aeruginosa} PAO1 (Westbrook-Wadman et al., 1999). Interestingly, a dramatic decrease in the amount of OprM was observed in the mutants compared to wild-type PAO1, indicating that OprM is unlikely to be the outer membrane component associated with this efflux system in the mutants (Westbrook-Wadman et al., 1999). In addition, MexXY was shown to be upregulated in clinical \textit{P. aeruginosa} isolates displaying aminoglycoside impermeability, suggesting that the pump is a clinically relevant mechanism of aminoglycoside resistance in \textit{P. aeruginosa} (Westbrook-Wadman et al., 1999).

The following year, the complete genomic sequence of \textit{P. aeruginosa} strain PAO1 (PAO1-UW) was published in Nature (Stover et al., 2000). Although the locus IDs PA2019-18 of the PAO1-UW genome sequence correspond to the mexXY genes, the nucleotide sequences of PA2019-18 were not identical with those of previously published mexXY (Aires et al., 1999; Mine et al., 1999) findings. This is probably because the DNA sequencing technology at that time was unable to analyze GC-rich bacteria such as \textit{P. aeruginosa} (66–67% GC content; Winsor et al., 2011). Therefore, we analyzed MexXY using the nucleotide sequences from the PAO1-UW complete genome (Winsor et al., 2011) because we live in the post-genome era.

\textbf{STRUCTURE AND FUNCTION OF MexY}

The RND components of RND-type tripartite multidrug efflux pumps determine substrate specificity (e.g., Srikumar et al., 1997; Eda et al., 2003); therefore, we focused on the structure and function of MexY rather than MexX or OprM. Very recently, the crystal structure of the RND-type multidrug efflux pump AcrB of \textit{E. coli} revealed the presence of two discrete, high-volume multisite binding pockets that contribute to the remarkably broad substrate recognition of AcrB and its homologs (Nakashima et al., 2011). Although we basically assume that MexY pumps out antimicrobials in a similar manner as AcrB, it will be intriguing to uncover the molecular basis of how MexY accommodates aminoglycosides.
because they are strongly hydrophilic molecules that are completely different from the relatively hydrophobic compounds (e.g., minocycline, doxycycline, rifampicin, and erythromycin) used as substrates of AcrB (Nakashima et al., 2011).

Generally speaking, the function of a transporter (e.g., substrate specificity and energy coupling) should be determined by its time course efflux assay and evaluated using kinetic constants (e.g., Yerushalmi et al., 1995; Edgar and Bibi, 1997; Mite et al., 1998; Morita et al., 2000). However, it is difficult to conduct such an assessment in a small bacteriology laboratory. The reconstitution of proteoliposomes revealed that AcrB, AcrD, and MexB of E. coli were H+/drug antiporters (Zgurskaya and Nikaiko, 1999; Ares and Nikaiko, 2005; Kim et al., 2005), and we assume that MexY pumps out antimicrobials coupled with the same energy. In addition, five charged and polar amino acid residues that are involved in the proton translocation pathway are conserved between MexY and AcrB of E. coli (Takatsuka and Nikaiko, 2006). Unfortunately, the purification, reconstitution, and characterization of the MexXY pump remain to be established, and the energy coupling and substrate specificity of MexXY has not been examined through its efflux activity. On the other hand, the MexXY-mediated energy-dependent efflux activity of ethidium (Mite et al., 1999), aminoglycosides (Aires et al., 1999; Vogne et al., 2004), tetracycline (Aires et al., 1999), Ala-Nap (MC-005,356) (Ma et al., 2001), and fluoresein-di-β-D-galactopyranoside (Matsumoto et al., 2011) has been measured in whole cells.

It is conventional to use differences in minimum inhibitory concentrations (MICs) between bacterial cells with and without a multidrug efflux transporter to estimate substrate specificity (e.g., Nishino and Yamaguchi, 2001; Nishino et al., 2003). Although the comparison of MICs can sometimes be a misleading indicator of pump function (e.g., Nagano and Nikaido, 2009), it can still indicate the possible clinical relevance of a pump (e.g., Poole, 2004, 2012; Palock 2006). Mutant strains lacking major multidrug efflux pump(s) have been used to determine substrate specificity (e.g., Nishino and Yamaguchi, 2001; Morita et al., 2001b). The substrate specificity of MexXY-OprM was determined using a mutant from PAO1 that overproduced MexXY-OprM, but not MexAB (and AmpC in the case of β-lactams), and were compared with a mutant lacking MexXY/MexAB-OprM (and AmpC in the case of β-lactams; Manola et al., 2006b). MexXY-OprM-mediated resistance was then observed for quinolones, macrolides, tetracyclines, aminoglycosides, chloramphenicol, lincomycin, and most β-lactams, but not for novobiocin, polymyxin B, and some β-lactams (carbenicillin, sulbactam, cefadroxil, cefazidime, oxacillin, imipenem, and aztreonam) among a wide variety of antimicrobial agents (Manola et al., 2008b). In conclusion, MexXY-OprM is a multidrug efflux transporter whose specificity is extraordinary broad, but different compared with MexAB-OprM, MexCD-OprJ, MexEF-OprN, and other RND efflux transporters in P. aeruginosa. In addition, MexXY-OprM was the only pump to mediate aminoglycoside resistance and was thus considered to recognize aminoglycosides as substrates (Manola et al., 2008b).

Basic local alignment search tool (BLAST) analysis showed that MexY was highly conserved in P. aeruginosa strains: more than 99% (99%) identity (positive) for most strains and 97% (98%) identity (positive) for PA7 (Morita et al., 2012). There was no functional difference between the MexYs of PAO1 and PA7 when they were expressed in either E. coli or P. aeruginosa (Morita et al., 2012). MexY was more similar [70%-73% (83-86%) identity (positive)] to orthologs of R. pseudomallei and various R. cepacia complexes than other RND pumps of P. aeruginosa and other Pseudomonas species (Morita et al., 2012). These Burkholderia species, except for B. mallei, are intrinsically resistant to aminoglycosides (e.g., Kenny et al., 1999; Thibault et al., 2004; Vermis et al., 2003; Jassim et al., 2011). R. gladioli is also known to be involved in human infections (Segonds et al., 2009); however, no MexY (AmrB) ortholog exists in R. gladioli (Seo et al., 2011), consistent with the fact that all isolates tested were susceptible to aminoglycosides (Segonds et al., 2009). Interestingly, the most similar functional ortholog to MexY exists in Achromobacter xylosoxidans and has a 74% identity (86% positive); this pump was named AxyY in strain AXN-A (Bador et al., 2012). A. xylosoxidans is also an opportunistic human pathogen capable of causing a wide range of infections (Gilpuchinsky et al., 1988; Bador et al., 2011). Most A. xylosoxidans clinical isolates were resistant to the tested aminoglycosides, including amikacin (Ngow and Puthucheary, 1985; Gilpuchinsky et al., 1988). The AxyY pump contributes to aminoglycoside resistance in a similar manner to MexY and AmrBs (Bador et al., 2012).

COBALT analysis is a multiple sequence alignment tool for finding a collection of pairwise constraints. Such constraints are derived from data of the conserved domain database, protein motif database, and sequence similarity of RND pumps (Papadopoulos and Agarwala, 2007), including all pumps from P. aeruginosa (Sinoi-UY and E. coli K12 (MG1655). The exception is heavy metal efflux pumps, which are characterized by their relationships. Therefore, we focused on the four branches containing the four Mex pumps in P. aeruginosa (Figure 1). The MexY branch is located next to the MexD branch and includes the AmrBs of Burkholderia species (e.g., Mima and Schweizer, 2010) and AxyY of A.xylosoxidans (Bador et al., 2012). The MexD branch includes the AdeE pump of A. baumannii (Magnet et al., 2001), MtrD of Neisseria gonorrhoeae (Hagman et al., 1997), and BdelB of Bradyrhizobium japonicum (Lindemann et al., 2010). The SmcZ pump of S. maltophilia, which can mediate aminoglycoside resistance (Crossman et al., 2008), also belongs to the MexD branch. Many pumps in the MexY/MexD branches can mediate aminoglycoside resistance (e.g., Magnet et al., 2001; Crossman et al., 2008; Lindemann et al., 2010; Mima and Schweizer, 2010), which hints at the structure-function relationship of pumps involved in aminoglycoside resistance. MexF is located in the branch that contains the AcrB/D and MexF pumps of E. coli (Nishino and Yamaguchi, 2001; Nishino et al., 2003). AdE of A. baumannii (Daniez-Podie et al., 2008), BpelB of B. pseudomallei (Mima and Schweizer, 2010), AxyB of A. xylosoxidans (Bador et al., 2011), and Vmbl of Vibrio parahaemolyticus (Matsuo et al., 2007). Among them, some pumps (e.g., AcrD and MexF) were reported to be involved in aminoglycoside resistance under some conditions (Li et al., 2003; Ares and Nikaiko, 2005). The MexF branch includes AdeG of A. baumannii (Coyne et al., 2010) and MdB of Salmonella enterica (Nishino et al., 2006).
Morita et al. Efflux-mediated aminoglycoside resistance

FIGURE 1 | Phylogenetic trees for representative RND transporters. According to the COBALT program, the trees were constructed using the Fast evolution method and rendered with (A) Rectangle and (B) Radical. Protein names are abbreviated; for example, "MexY_PAER" stands for "MexY of P. aeruginosa." Accession numbers are shown in parentheses. The four branches shown in red are: (a) MexY, (b) MexD, (c) MexB, and (d) MexF.

GENE EXPRESSION OF THE MexXY SYSTEM

MexXY was shown to be induced by sub-inhibitory concentrations of tetracycline, erythromycin, aminoglycosides, tigecycline, and LMB415 (a peptide deformylase inhibitor), but not ofloxacin in P. aeruginosa PAO1 (Masuda et al., 2000a; Dean et al., 2003; Caughlan et al., 2009). Moreover, ofloxacin and cefpirome were also shown to be inducers, but only in a PAO1 mutant lacking MexAB (and AmpC in the case of cefpirome; Masuda et al., 2000a,b). MexZ was shown to bind an inverted repeat region located in the mexZ-mexX intergenic region directly as a homodimer, which encompasses the putative mexXY promoter, but the inducers failed to alter the MexZ-operator interactions (Matsuo et al., 2004). The crystal structure of MexZ has since been solved (Alguel et al., 2010). The antibiotic inducibility of the MexXY multidrug efflux system of P. aeruginosa was shown to be involved in the modulation of MexZ activity by the antibiotic-inducible PA5471 gene product (Morita et al., 2006; Table 1). PA5471 encodes a predicted product of 43.5 kDa, which was identified as a hypothetical protein conserved between bacteria and archaea, and is a representative of the uncharacterized protein family UFP0027 in the Pfam protein families database (Morita et al., 2006) or the PRK09588 cluster in ProtClustDB (NCBI Protein Clusters Database; Klimke et al., 2009). Recently, it was demonstrated that RctB of E. coli, which is related to members of this family, is a novel RNA ligase and functions as a bona fide RNA repair protein in vivo (Tanaka and Shuman, 2011). PA5471 is found upstream of and in a possible operon with an open-reading frame dubbed PAS470; RT-PCR confirmed both the drug inducibility of PAS470 and its expression from a polycistronic message that also contains PA5471 (Morita et al., 2006). PAS470 is predicted to encode a peptide chain release factor of 22.3 kDa (Morita et al., 2006). A homolog of PA5471 from E. coli K12, ykfJ (b0235), which was, however, C-terminally truncated (approximately 1 kb; Baranov et al., 2006), was also shown to be inducible by 4-azaleucine, which is known...
to interfere with translation, and it too is linked to a putative peptide release factor gene (Morita et al., 2006). P. aeruginosa senses antibiotic-mediated ribosomal disruption and links it to PA5471 gene expression by monitoring the translation of a 13-amino-acid leader peptide region (PA5471.1) found ∼250 bp upstream of the PA5471 coding sequence on PA5471 mRNA (Morita et al., 2009). The antimicrobial-inducible PA5471 gene product has been shown to interact with the repressor MexZ and interfere with its DNA binding activity in vitro (Yamamoto et al., 2009), and this finding contributed to elucidating the molecular mechanisms of the MexXY induction. However, PA5471 is not sufficient for MexXY recruitment in response to antibiotic exposure, and additional antibiotic-dependent effects are needed in P. aeruginosa (Morita et al., 2009). Exposure to reactive oxygen species (ROS; e.g., peroxide) induces the expression of the PA5471 gene, leading to MexXY-dependent aminoglycoside resistance (Fraud and Poole, 2011). Moreover, long-term (8-day) exposure of P. aeruginosa to peroxide (mimicking chronic in vivo ROS exposure) increased the frequency of PA5471- and mexXY-dependent aminoglycoside resistance (Fraud and Poole, 2011). Recently, reduced (approximately twofold) expression of the ppsU-pomA operon (encoding the SOS ribosomal proteins L21 and L27) was shown to promote mexXY expression via the PA5471 gene in pan-aminoglycoside resistant mutants from PAO1 and a CF clinical isolate (Luo et al., 2012). Such expression was in the form of ribosomal protein mutations that influence mexXY expression, including rpgY (encoding ribosomal protein L25; Hill et al., 2007) and rplp (encoding ribosomal protein L7; Wotring-Wadman et al., 1999). Transcriptome profiling revealed that significantly increased expression was observed for the mexXY and PA5471 genes in both the PA2572 and PA2573 mutants compared with the wild-type PAO1 strain during exponential growth in Luria-Bertani media (McLaughlin et al., 2012). PA2572 encodes a putative response regulator of a two-component system required for full virulence to Galleria mellonella (Wax moth) and PA2573 also encodes an orphan chemotaxis sensor which seems to function in part through signal transduction involving PA2572 (McLaughlin et al., 2012).

A recent study identified a gene, parR, encoding the response regulator of a two-component system, ParR, which promotes either induced or constitutive mexXY upregulation, thereby activating the MexXY efflux system as well as OprD porin loss and lipopolysaccharide modification in a MexZ-independent manner (Muller et al., 2011). Overexpression of ParR, a small non-coding RNA between PA5305 and PA5336 in the genome of P. aeruginosa PAO1, in the stationary phase increased the expression of the mexXY and mexZ genes as well as type III secretion genes, while reducing the expression of genes for arginine metabolism (Goldberg et al., 2008).

**MexXY SYSTEM AS AN ANTIMICROBIAL RESISTANCE DETERMINANT IN P. aeruginosa**

Pseudomonas aeruginosa shows intrinsic resistance against many antimicrobials because of the low permeability of its outer membrane and the presence of efflux systems (Nikaido, 1994; Hancock, 1998). MexXY was shown to be involved in natural resistance to aminoglycosides, tetracycline, tigecycline, erythromycin, and LMB415 in P. aeruginosa PAO1 (Aires et al., 1999; Masuda et al., 2000a; Morita et al., 2001a; Dean et al., 2003; Caughlan et al., 2009). MexXY was also shown to be necessary for the adaptive resistance of P. aeruginosa PAO1 to aminoglycosides (Hocquet et al., 2003). It is of note that MexXY is the only pump of the 12 identified RND systems that mediates aminoglycoside resistance in P. aeruginosa PAO1 (Poole, 2011). The antagonism of aminoglycosides by the divalent cations Mg2+ and Ca2+ is well documented (Medeiros et al., 1971), and culture in cation-adjusted Mueller–Hinton broth is recommended as a susceptibility test to ensure acceptable results when P. aeruginosa isolates are tested (Barry et al., 1992). MexXY was shown to be required for the antagonism of aminoglycosides by divalent cations in P. aeruginosa PAO1 (Mao et al., 2001). Although Phe-Amp-β-naphthylamide (PAβN, MC-207,110) is known as a non-specific inhibitor against RND-type multidrug efflux pumps (Lomovskaya et al., 2001), this inhibitor, as observed for divalent cations, antagonized the activity of aminoglycosides (amikacin and netilmicin) in a MexXY-dependent manner, even though it also inhibited MexXY-dependent fluoroquinolone (levofloxacin) resistance (Mao et al., 2001). Conversely, PAβN inhibited MexXY-mediated aminoglycoside (gentamicin) resistance (Mesias et al., 2007). The reason for the discrepancy between these two results remains unknown. Increased susceptibility to aminoglycosides in mexRβ mutants, which upregulate mexCD-oprF expression, was correlated with increased resistance to fluoroquinolones and some β-lactams, such as cefepime, concomitant with a higher susceptibility to aminoglycosides and some β-lactams, such as ticarcillin, aztreonam, and imipenem. This was shown to be partly due to the impaired activity of MexXY-OprM because of major changes in cell physiology, but not the expression/production of mexXY/MexY and oprM/OprM (Jeannot et al., 2008; Mulet et al., 2011). The increased susceptibility to aminoglycosides in MexEF-OprN-overproducing mexRβ mutants was also observed, apparently owing to impairment of the MexXY system (Sobel et al., 2005). MexXY expression (and so MexXY-mediated resistance) was independent of the AmgRS two-component system in which mutations enhanced aminoglycoside action to control an adaptive response to membrane stress (Lee et al., 2009).

Multidrug-resistant P. aeruginosa clinical isolates have often been reported to be MexXY overproducers (e.g., Lianes et al., 2004, 2006; Wolter et al., 2004; Deplano et al., 2005; Henrichsfeise et al., 2007; Hocquet et al., 2007; Maniati et al., 2007; Vetorretti et al., 2009a; Beaudoin et al., 2010; Xavier et al., 2010; Fehlberg et al., 2012; Pasca et al., 2012). Time series analysis (January 1999 to January 2005) revealed a significant relationship between antibiotic use (aminoglycosides, fluoroquinolones, and cephalosporins, but not carbapenems) and the incidence of MexXY-overproducing P. aeruginosa in a French hospital (Hocquet et al., 2008). MexXY (n = 39) and MexAB (n = 31) were the most frequently overproduced pumps in 85 non-CF P. aeruginosa strains with low-level ciprofloxacin resistance (MICs ranging from 0.25 to 2 μg/mL, which are still susceptible or intermediate according to the CLSI breakpoints; Lianes et al., 2011). A large proportion of the strains were MexXY overproducers in genotypically distinct P. aeruginosa clinical isolates that were less susceptible to cefepime than to cefazidime, and these were identified in Europe (Hocquet et al., 2006; Penna et al., 2009; Campo Equisabel et al., 2011) and the USA (Lavo- haveson et al., 2008). In contrast, both cefepime and cefazidime...
MexXY in this strain utilizes either the OprA or OprM outer membrane pump.
P. aeruginosa is the most frequently mutated genes during chronic infection by P. aeruginosa (Miller et al., 2012), although overproduced OpmB (PA2525) can function as OprM of PAO1 (Aires et al., 1999; Masuda et al., 2000a; Morita et al., 2004, 2009).

MexXY was also shown to be necessary in subpopulations of P. aeruginosa CF isolates that are hypersensitive to ticarcillin (called Tac<sup>T</sup>; Venturini et al., 2009). mexZ was shown to be one of the most frequently mutated genes during chronic infection by P. aeruginosa in CF patients (Smith et al., 2006; Feliziani et al., 2009).

However, a number of studies highlighted the absence of mutations in mexZ or the mexXY promoter region in MexXY-overproducing P. aeruginosa CF isolates (Sobel et al., 2003; Vogne et al., 2004; Islam et al., 2009). To date, three kinds of mutants (argZ, argV1, and argV2) have been recognized as MexXY overproducers as a result of genetic mechanisms: mutants with impaired binding or unbinding of MexZ due to alterations in the mexZ or mexZ-mexC intergenic region (type argZ); mutants with impaired protein synthesis (type argV); and mutants with alterations in parRS (type argV2; de Bentzmann and Plesiat, 2011).

Oxidative stress, a component of the host’s immune system in the CF lung, induced mexXY expression via PA3471 and promoted aminoglycoside resistance (Fraud and Poole, 2011). Under conditions of oxidative stress, P. aeruginosa can develop aminoglycoside resistance, even in the absence of aminoglycosides (Poole, 2012). It is also very plausible that the routine use of aminoglycosides (e.g., tobramycin) might simply select for MexXY-overproducing P. aeruginosa in the CF lung (Smith et al., 2006).

Although it is obvious that MexXY is one of the determinants of antimicrobial resistance in P. aeruginosa in the clinical setting (Poole, 2011), only a few reports have assessed the in vivo impact of the MexXY system on antibiotic therapy for P. aeruginosa infections (e.g., Martin et al., 2016).

**COGNATE OUTER-MEMBRANE COMPONENT OprA OF THE MexXY PUMP IS FOUND IN SEROTYPE O12 BUT IS LOST IN OTHERS**

The mexXY operon lacks a gene coding for the outer membrane protein in P. aeruginosa PA01 (Mine et al., 1999). OprM is necessary for the function of MexXY and MexAB in P. aeruginosa PAO1 (Aires et al., 1999; Masuda et al., 2000a; Morita et al., 2001a), although overproduced OpmB (PA2525) can function as an outer membrane component of MexXY/MexAB, and MexCD (Murray et al., 2002). Intriguingly, the multidrug resistant taxonomic outlier P. aeruginosa PA7 possesses a unique gene (opmA) downstream of mexXY encoding an outer membrane channel that is absent in most P. aeruginosa strains (Roy et al., 2010). MexXY in this strain utilizes either the OprA or OprM outer membrane channel (Morita et al., 2012; Table 1). While OprM is functional with both MexXY and MexAB, OprA did not associate as strongly with MexAB as it did with MexXY (Morita et al., 2012). We compared the OprA of P. aeruginosa PA7 with the OprM family (Remans et al., 2010) from P. aeruginosa PA01 as well as TolC of E. coli K12 (Mine et al., 1999), OprA of B. pseudomallei 1026b (Mima and Schweizer, 2010), OprZ of A. xylosoxidans 2XX-A (Bador et al., 2012), and AdeC of A. baumannii AYE (Magniet et al., 2011; Figure 2). COBALT analysis showed that OprA of P. aeruginosa PA7 and its close orthologs (OprA of B. pseudomallei 1026b and OprZ of A. xylosoxidans 2XX-A) is located close to OpeI and is followed by OprM of the OprM outer membrane family of P. aeruginosa PA01 (Figure 2).

Interestingly, a small portion of the oprA gene immediately downstream of the mexY gene in PA01 was identified, suggesting that non-PA7 P. aeruginosa strains might have possessed, but lost, the intact mexXY-oprA efflux pump locus (Morita et al., 2012; Table 1). Consistent with this, the majority 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 (Morita et al., 2012). 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 (Pirnay et al., 2009; Roy et al., 2010). O12 might be more dominant due, in part, to the presence of oprA in hospitals in which antimicrobials promoting MexXY/OprA-mediated multidrug resistance, such as aminoglycosides, 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 also have 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 shown in the presence and absence of oprA (Morita et al., 2012). Such a small difference might be significant during antibiotic treatment or in the presence of sub-inhibitory concentrations of antibiotics.

**AcR IS AN AMINOGLYCOSIDE EFFLUX PUMP THAT IS THE MOST SIMILAR TO MexXY AMONG THE RND PUMPS IN E. coli K12**

AcR has the highest similarity score at the amino acid level to MexY of the E. coli K12 RND pumps and was shown to be an aminoglycoside efflux pump as judged by MIC determination and the aminoglycoside efflux assay (Rosenberg et al., 2000). However, differences in aminoglycoside resistance (uptake) between the parent strain IC6723 and its acrD-deletion mutant IC6723ΔacrD was possibly not limited to AcrD function because IC6723 was constructed by inserting the tet gene from PBR322 into acrD (Rosenberg et al., 2000). The increased aminoglycoside uptake might be due to not only AcrD deficiency but also to the production of an aberrant cytoplasmic membrane protein (the product of acrD with the inserted tet) and/or the tetracycline<sup>H</sup><sup>+</sup> antiporter itself (Merlin et al., 1989a,b; Wyka and St John, 1990). While disruption of tolC or acrA did not increase the susceptibility of K12 to aminoglycosides (Rosenberg et al., 2000), both of them were...
Morita et al. Efflux-mediated aminoglycoside resistance

FIGURE 2 | Phylogenetic trees for representative OMPs. According to the COBALT program, the trees were constructed using the Fast evolution method and rendered with (A) Rectangle and (B) Radical. Protein names are abbreviated; for example, “OprA_PAER” stands for “OprA of P. aeruginosa.” Accession numbers are shown in parentheses.

necessary for the function of acrD against various antimicrobials; however, no aminoglycosides were used in the study (Hirakawa et al., 2003). We do not rule out the hypothesis of Rosenberg et al. (2000) that AcrD protein can perhaps function without the participation of AcrA and TolC in the case of aminoglycoside efflux.

It is evident that purified AcrD can function as an H\(^{+}\)-driven aminoglycoside efflux pump (Aires and Nikaido, 2005). Especially, strong stimulation of proton efflux was observed when aminoglycosides (e.g., streptomycin) were added to the more acidic intra-vesicular space of reconstituted AcrD proteoliposomes containing AcrA and Mg\(^{2+}\) (Aires and Nikaido, 2005), indicating that AcrD captures aminoglycosides exclusively from the periplasm in E. coli (Nikaido, 2011). The difference in the MICs of amikacin and gentamicin between a parent strain and its in-frame acrD-deletion mutant or between an acrBD-deletion mutant and its acrD-overexpressing complementation mutant was approximately twofold (Elkins and Nikaido, 2002; Aires and Nikaido, 2005). There was no significant difference in kanamycin resistance in the case of an in-frame deletion (Hirakawa et al., 2003), and a twofold difference was observed in the case of overproduction (Nishino and Yamaguchi, 2001; Nishino et al., 2010). An acrA in-frame deletion mutant also showed an approximately twofold increased susceptibility to aminoglycosides (Aires and Nikaido, 2005). However, similar observations were not seen for AcrB (Nishino and Yamaguchi, 2001; Elkins and Nikaido, 2002; Aires and Nikaido, 2005). A comparison of the entrances of the vestibules, which are found in the central cavities (Murakami et al., 2002) of AcrD (which transports aminoglycosides) and AcrB (which does not) crystal structures, shows that this area in AcrD is in line with many more acidic residues that may attract polycationic substrates (Yu et al., 2003). Treatment with sub-inhibitory concentrations of kanamycin induced adaptive resistance to aminoglycosides, which was dependent on acrD (Sidhu et al., 2012). Aminoglycosides are very hydrophilic and polycationic and assumingly permeate through the porin channel in E. coli, unlike P. aeruginosa (Nikaido and Pages, 2012) in addition to so called “self-promoted” aminoglycoside uptake across the outer membrane of both of E. coli and P. aeruginosa (Hancock et al., 1991; Hancock, 1998). The MICs of aminoglycosides on E. coli, unlike P. aeruginosa, might be poor indicators of aminoglycoside efflux. There are numerous AcrD homologs in other Enterobacteriaceae (Poole, 2004). Although the AcrD of S. enterica serovar Typhimurium ATCC 14028s was studied comprehensively, no significant difference between the AcrDs of E. coli and S. enterica has been observed so far (Nishino et al., 2009; Horiyama et al., 2011; Yamazaki et al., 2011). Interestingly, AcrD pumps mediate resistance to the substrates of MexAB (e.g., carbenicillin, aztreonam, and novobiocin). However, AcrD pumps did not mediate resistance to the substrates of MexXY (e.g., cefpirome, erythromycin, and tetrathenlyphosphonium) or shared substrates of both MexAB and MexXY (e.g., fluoroquinolone and tetracycline) when differences of the MICs were compared between a parent strain and its transformant overproducing the pump (Srikumar et al., 1997; Mine et al., 1999; Morita et al., 2003a; Nishino et al., 2009; Horiyama et al., 2011; Yamazaki et al., 2011). MexAB-OprM was also shown to contribute to aminoglycoside resistance, presumably via active efflux in the
low-ionic-strength medium used in this particular study (Li et al., 2003). AcrD and the MdrABC pump were iron-regulated, induced in low-iron conditions, and export the siderophore enterobactin (Bochner et al., 2008). We also report that MexAB-OprM was inducible under conditions of iron limitation and compensated for a growth defect in an iron-deficient medium in the presence of the non-metabolizable iron chelator 2,2′-dipyridyl (Dvide et al., 1993a,b). AcrD seems to be a functional homolog of MexB rather than MexA, as determined from substrate specificity and physiological function, consistent with the fact that phylogenetic analysis showed that AcrD is closer to MexB than to MexY (Figure 1).

**AmrAB-OprA IS A MULTIDRUG EFFLUX SYSTEM THAT MEDIATES AMINOGLYCOSIDE RESISTANCE IN *B. pseudomallei***

*Burkholderia pseudomallei* is the etiologic agent of melioidosis, a rare but serious disease endemic to South Asia, Northern Australia, and other parts of the tropics (Mima and Schweizer, 2010). Melioidosis is very difficult to treat because of the intrinsic resistance to many antimicrobial agents including aminoglycosides, macrolides, polymyxins, and some β-lactams (Mima and Schweizer, 2010). AmrAB-OprA was identified as an efflux determinant of resistance to aminoglycosides and macrolides in the *B. pseudomallei* 1026b clinical isolate (Moore et al., 1999). This pump was actually the first to be demonstrated responsible for the aminoglycoside resistance of RND pumps in Gram-negative bacteria. The gene product of amrB, which is located immediately upstream and divergently transcribed from *amrA* in *B. pseudomallei* 1026b (Moore et al., 1999), showed strong homology (60% (73%) identity (positive)) to MexZ, which acts as a transcriptional repressor of the mcrXY operon of *P. aeruginosa* PA01 (Matsuo et al., 2004; Alguel et al., 2010).

While the majority of *B. pseudomallei* clinical isolates exhibit high levels of aminoglycoside and macrolide resistance, rare isolates are susceptible to these antibiotics (Simpson et al., 1999; Trunck et al., 2009). While it is noted that the resistance profile of those isolates matches that of the *amrAB-oprA* mutants (Simpson et al., 1999), it was shown experimentally that *amrAB-oprA* was missing in *B. pseudomallei* 708a, an aminoglycoside- and macrolide-susceptible clinical isolate, and this loss was associated with the deletion of >130 kb of genetic material (Trunck et al., 2009). The expression of *amrAB-oprA* increased resistance to not only aminoglycosides and macrolides but also fluoroquinolones and tetracyclines in a BpeA-B oprA pump-deficient mutant of 1026b (Mima and Schweizer, 2010). Judging from the substrate specificity and sequence similarity (Mima and Schweizer, 2010), we have no doubt that AmrAB is a functional ortholog of MexXY in *B. pseudomallei*. BpeA-B oprA of *B. pseudomallei* also reportedly mediates aminoglycoside resistance in strain KHW (Chan et al., 2004), while this pump did not confer aminoglycoside resistance in 1026b (Mima and Schweizer, 2010). In addition, the BpeB RND transporter was also shown to be closely related to MexB of *P. aeruginosa*, both functionally and phylogenetically (Mima and Schweizer, 2010), consistent with their phylogenetic analysis (Figure 1).

**Table 1 | Genetic organization of aminoglycoside efflux operons of clinical significance and their regulators in non-fermentative Gram-negative pathogens.**

| Organism | Efflux operon | Product Function | Regulator |
|----------|---------------|-----------------|----------|
| *P. aeruginosa* | mexXY (MexA) | Amino glycoside, fluoroquinolones, tetracyclines, erythromycin, cefotaxime, trimethoprim, and chloramphenicol | ParRS |
| | MexX (MexB) | | |
| | MexY (MexZ) | | |
| | MexW | | |
| | OprM | | |
| A. xylosoxidans | aexX | Aminoglycoside, fluoroquinolones, tetracyclines, erythromycin, cefotaxime, trimethoprim, and chloramphenicol | AexR |
| | OprZ | | |
| B. pseudomallei | amrAB | Aminoglycoside, fluoroquinolones, tetracyclines, erythromycin, cefotaxime, trimethoprim, and chloramphenicol | AmrR |
| | oprA | | |
| A. baumannii | adeAB (adeC) | Aminoglycoside, fluoroquinolones, tetracyclines, erythromycin, cefotaxime, trimethoprim, and chloramphenicol | AdeRS |
| | AcrD | | |
| A. xylosoxidans | aexX | Aminoglycoside, fluoroquinolones, tetracyclines, erythromycin, cefotaxime, trimethoprim, and chloramphenicol | AexR |
| | OprZ | | |
| B. pseudomallei | amrAB | Aminoglycoside, fluoroquinolones, tetracyclines, erythromycin, cefotaxime, trimethoprim, and chloramphenicol | AmrR |
| | oprA | | |
| A. baumannii | adeAB (adeC) | Aminoglycoside, fluoroquinolones, tetracyclines, erythromycin, cefotaxime, trimethoprim, and chloramphenicol | AdeRS |
| | AcrD | | |

As described above, AmrB orthologs are conserved among various human pathogens belonging to *Burkholderia* species, but not *B. gladioli*. Actually, an AmrAB-OprA ortholog was shown to be a major aminoglycoside resistance contributor in *B. cenocepacia*, a member of the *B. cepacia* complex (Hamad et al., 2010).

**AdeABC IS A MULTIDRUG EFFLUX SYSTEM THAT MEDIATES AMINOGLYCOSIDE RESISTANCE IN *A. baumannii***

*Acinetobacter baumannii* is the most frequently implicated species in nosocomial infections among *Acinetobacter* spp. (Cogne et al., 2011). AdeABC was identified as an RND-type efflux pump involved in resistance to multiple antimicrobials including aminoglycosides, fluoroquinolones, tetracyclines, erythromycin, cefotaxime, trimethoprim, and chloramphenicol in *A. baumannii* BM4454, a low-level pan-aminoglycoside resistant clinical isolate...
AdeSGly30Asp) in AdeRS have been shown to be responsible for the AdeC, as observed for the MexXY pump with its linked outer-membrane channel OpxA, which was not essential in P. aeruginosa PA7 (Morita et al., 2012). The adeC gene was usually present, but the adeS gene was not found in ~40% of clinical isolates (Nemec et al., 2007). Our phylogenetic analysis showed that AdeC is more closely related to OpxM and Opy than to OpxA in the OpxM outer membrane family of P. aeruginosa (Figure 2).

The AdeABC operon is expressed at low levels in natural isolates of A. baumannii due to stringent control by the AdeRS two-component system, which is encoded adjacent to adeABC, but transcribed in the opposite direction (Marchand et al., 2004, Table 1). Mutations (e.g., AdeP+RIs, AdeS+RIs, or AdeC+RIs) in AdeS have been shown to be responsible for the constitutive expression of AdeABC (Marchand et al., 2004), which reminds us that mutations (e.g., ParPMet59Ile) in the ParRS two-component system are responsible for the constitutive expression of MexXY in P. aeruginosa (Miller et al., 2011). Overexpression of the AdeABC system in a tigecycline-non-susceptible clinical isolate was due to the transposition of a copy of IsaS/Isd into adeS (Buzin et al., 2007). Very recently, a truncated AdeS kinase protein generated by an IsaS/Isd insertion was shown to be correlated with enhanced adeABC expression in A. baumannii (Sun et al., 2012). Other regulatory mechanism(s) were shown to be involved in adeABC overexpression without any previously known mutation (Sun et al., 2010). Recently the AdeABC ortholog was shown to be a contributor to multiple antimicrobials, including aminoglycosides, in Acinetobacter genomospecies 1F3TU, a non-A. baumannii species (Boca et al., 2011).

**FUTURE PERSPECTIVES**

MexXY is one of the potential targets for novel anti-pseudomonas agents. Its inhibitor is able to not only potentiate previously used ineffective antimicrobial agents (e.g., aminoglycosides against aminoglycoside-resistant P. aeruginosa and B. cepacia complex), but also to speed up the development of novel anti-pseudomonas agents. Because there are a significant number of potential drug targets encoded by the genome of P. aeruginosa (e.g., products of essential genes, Morita et al., 2010), it is the most promising therapeutic strategy to conquer the impermeability barriers of these bacteria. The efflux inhibitor MP 601384, which has specificity toward aminoglycoside-accommodating RND efflux systems and is not toxic to bacteria, is the only MexXY inhibitor reported so far (Jassem et al., 2011). Uncultured bacteria and plants are predicted to be a significant reservoir of novel antimicrobial agents (Stavri et al., 2007; Piel, 2011). Screening novel antibacterial agents, including a MexXY inhibitor, is currently in progress in our laboratory (e.g., Shota et al., 2004).

**ACKNOWLEDGMENTS**

This work was supported in part by a Grant-in-Aid for Young Scientists (B) (Kakenhi 23790106) from the Japan Society for the Promotion of Science and a research grant from the Institute of Pharmaceutical Life Sciences, Aichi Gakuin University.
Morita et al. Efflux-mediated aminoglycoside resistance

Fehlberg, L. C., Xavier, D. E., Peraro, Damier-Piolle, L., Magnet, S., Brede, Deplano, A., Denis, O., Poirel, L., Hocquet, D., Noirhomme, C., and Poletti, P. (2011). The Pseudomonas aeruginosa oppor- tunistic pathogen and human infections. Environ Microbiol 13, 1655–1665.

Duan, C., Xu, Y., Prentice, S. J., Sun, F. E., and Bradford, P. A. (2003). Efflux-mediated resistance to tig- cycline (GAR96) in Pseudomonas aeruginosa F5O1. Antimicrob. Agents Chemother. 47, 872–879.

Deplano, A., Denis, O., Poirel, L., Hoc- quiet, D., Noirhomme, C., and Poletti, P. (2005). Molecular characterization of an epidemic clone of panantibiotic- resistant Pseudomonas aeruginosa. J. Clin Microbiol. 43, 1198–1204.

Ela, S., Manola, H., and Nakae, T. (2003). An elegant means of self-protection in gram-negative bacteria by recognizing and extruding xenobiotics from the periplasmic space. J. Biol. Chem. 278, 2085–2088.

Edgar, B., and Bé, E. (1997). MdrA, an Escherichia coli mutless resistance protein with an extraordinarily broad substrate range. Cell 89, 1137–1145.

González, E., Hansen, W., Penney, J., and Sawarrowsky, E. (1988). In vitro susceptibility of Alcaligenes denitrificans subsp. peregrinus to 26 antimicrobi- al agents. Antimicrob. Agents Chemother. 32, 276–278.

Goldsberg, J. R., Hancis, R. E., Pars- akian, R. E., Lopez, J., and Cornelis, P. (2000). Pseudomonas aeruginosa J. Bacteriol. 182, 2469–2662.

Hampe, N., Lisan, C. B., Bahmanyar, A., and Balaban, T. J. (2011). The MtbR protein of Mycobacterium tuberculosis is a member of the resistance/nodulation/division protein family constituting part of an efflux system. Microbiology 157, 2117–2125.

Hampe, N., Skolmon, A. M., and Valamont, M. A. (2010). Con- struction of antimicrobial-resistant Burkholderia cenocepacia strains for use in studies of two bacterial bacte- ria with the gentamicin protection assay. Appl. Environ. Microbiol. 76, 3170–3176.

Hancock, R. E., Farm, S. W., Li, Z. S., and Poole, K. (1991). Interaction of aminoglycosides with the outer membranes and purified lipopolysaccharide and OmpF porins of Escherichia coli. Antimicrob. Agents Chemother. 35, 1309–1314.

Hancock, R. E. (1990). Resistance mechanisms in Pseudomonas aerug- inosa. Antimicrob. Agents Chemother. 37, 2117–2125.

Harnois, F., Wiegand, L., Pièle, W., and Wiedemann, B. (2005). Resistance mechanisms of multidrug-resistant Pseudomonas aeruginosa strains from Germany and correlation with hypermutation. Antimicrob. Agents Chemother. 51, 402–407.

Hirakawa, H., Nishino, K., Hirata, T., and Yamaguchi, A. (2003). Comparative study of drug resis- tance mediated by overexpression of two-component signal transduction sys- tems in Escherichia coli. J. Bacteriol. 185, 3051–3056.

Houpt, D., Voge, C., El Garch, F., Vezon, A., Goeh, N., Lee, A., et al. (2003). MexXY-OprM efflux pump is necessary for a adaptive resis- tance of Pseudomonas aeruginosa to aminoglycosides. Antimicrob. Agents Chemother. 47, 1757–1759.

Houpt, D., Nordmann, P., El Garch, F., Cabanne, L., and Poletti, P. (2006). Involvement of the MexXY- OprM efflux system in emergence of aminoglycoside resistance in clinical strains of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 50, 1459–1511.

Houpt, D., Berthelet, P., Houssin- Delbret, M., Favre, R., Iannone, K., Bajot, O., et al. (2007). Pseu- domonas aeruginosa may accumulate drug resistance mechanism without losing its ability to cause bloodstream infections. Antimicrob. Agents Chemother. 51, 3511–3516.

Houpt, D., MILLER, A., Bane, K., FLA- pat, F., Talon, D., Moran, D. L., et al. (2005). Relationship between antibi- otic use and resistance of MexXY- OprM overexpressors among clinical isolates of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 52, 1173–1175.

Horttyna, T, Yamaguchi, A., and Nakae, T. (2011). toll-like re- sponse of multidrug efflux systems in Salmonella enterica serovar Typhimurium. J. Antimicrob. Chemother. 65, 1732–1736.

Ishimura, K., and Y ourassowsky, E. (1988). An elegant means of self-protection in gram-negative bacteria by recognizing and extruding xenobiotics from the periplasmic space. J. Biol. Chem. 278, 2085–2088.

Kobayashi, N., Agraobawa, B., Budhram, S., Chetwetmanee, S., Cials, S., Foddb, P. A., et al. (2009). The National Center for Biotechnology Information’s Protein Cluster Database. Nucleic Acids Res. 37, D218–D222.

Klopp, T., Michae-Hampshurst, M., Hanse, U., Goebel, N., Curry, L. K., and Fouché, J. C. (2007). Characterization of Mex-Med-Ofp1, a positively regulated efflux family system of Pseudomonas aeruginosa. Mol. Microbiol. 23, 345–354.

Kushima, N., Iwamoto, K., Kunin, D., Kuss, J. L., and Nislan, D. P. (2008). Expression of the MexXY-Ofp1 efflux system in Pseu- domonas aeruginosa with discord- ant cefalotin/cefadoline suscepti- bility profiles. Infl. Drug Resistant 1, 51–55.

Lau, C. H., Fraud, S., Jones, M., Peterson, S. N., and Poletti, K. (2012). Reduced expression of the pfl-opm oprp12525c9-12777e12777 is required for resistance to aminoglycoside-resistant mutants of Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 56, 5717–5719.

Lee, S., Hinz, A., Baueh, A., Anger- meyer, A., and Sonntag, C. (2009). Targeting a bacterial stress response to enhance antibiotic action. Proc. Natl. Acad. Sci. U.S.A. 106, 14570–14575.

frontiersin.org
Morita et al. 2012. “Efflux-mediated aminoglycoside resistance.” Antimicrob. Agents Chemother. 56, 4493–4498.
Nikaido, H. (1994). Prevention of drug resistance barriers and active efflux. Science 264, 385-388.

Nikaido, H. (1996). Multidrug efflux pumps of Gram-negative bacteria. J. Bacteriol. 178, 5835-5850.

Nikaido, H. (2011). Structure and mechanism of RND-type multidrug efflux pumps. Adv. Enzymol. Relat. Areas Mol. Biol. 77, 1-68.

Nikaido, H., and Pagos, J. M. (2012). Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS Microbiol. Rev. 36, 360-363.

Nishino, K., and Yamaguchi, A. (2001). Analysis of a complete library of multidrug efflux pump sequences. Trends Microbiol. 9, 2381-2387.

Nishino, K., Yamasaki, T., and Hirakawa, H. (2002). Characterization of outer membrane efflux systems of Pseudomonas aeruginosa. Microbiol. Immunol. 46, 1001-1006.

Nishino, M., and Yamaguchi, A. (2009). Regulation and characterization of an AdeABC efflux operon. Antonie van Leeuwenhoek 95, 1001-1006.

Nishino, M., and Yamaguchi, A. (2010). Differen- tial selection of single-step AmpC or PEN- 

Nishino, N., and Yamaguchi, A. (2001). Waves of multidrug resistance in Pseudomonas aeruginosa. J. Bacteriol. 183, 5303-5305.

Nishino, K., Kubler, T., and Groisman, E. A. (2000). Virulence and drug resistance roles of multidrug efflux systems of Salmonella enterica serovar Typhimurium. Mol. Microbiol. 36, 126-141.

Nitobi, Y., Teru, S., and Kishimoto, K. (2003). Inner membrane efflux pump AcrB reveals a proximal mul-

drug resistance efflux pumps in Pseudomonas aeruginosa. J. Bacteriol. 185, 2215-2224.

Nobata, T., Gotoh, N., Tsujimoto, H., and Flamm, R. K. (2010). Differen-
tial selection of single-step AmpC or PEN- 

Nobata, T., Yamasaki, Y., and Hirata, T. (2003). Overexpression of the mexC-

Nobata, T., Yamasaki, Y., and Hirata, T. (2005). Mutations in PA2491 (mexS) cause reduced susceptibility to tigecycline in Pseudomonas aeruginosa. Antimicrob. Agents Chemother. 50, 259-264.

Nomura, S., Nakahama, B., Yamamoto, E., and Yamasaki, A. (2002). Crystal structure of bacterial multidrug efflux transporter AcrB. Nature 419, 387-393.

Norata, T., Gotchi, N., and Nishino, T. (2001). Characterization of outer membrane efflux pumps OppN, OppM and OppN of Pseudomonas aeruginosa: molecular cloning and development of specific antiserum. FEMS Microbiol. Lett. 207, 37-43.

Noguchi, K., and Nikaido, H. (2009). Kinetic behavior of the multidrug efflux pump AcrB of Escherichia coli. Proc. Natl. Acad. Sci. USA 106, 7031-7036.

Nakahima, S., Sakakura, K., Yamasaki, S., Nishino, K., and Yamasaki, A. (2011). Structure of the multidrug exporter AcrB reveals a proximal multi-
drug resistance efflux pumps in Pseudomonas aeruginosa. J. Bacteriol. 193, 5854-5859.

Nakahira, H., Tatsuta, A., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochem. Biophys. Acta 1795, 514-523.

Nakagawa, N., Sakanishi, H., Hishida, K., and Yamaguchi, A. (2000). Effect of NlpE on expression of multidrug resistance in Escherichia coli. Antonie van Leeuwenhoek 77, 1-60.

Nakano, H., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1795, 514-523.

Nakano, K., Tatsuta, A., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1795, 514-523.

Nakano, K., and Yamaguchi, A. (2000). Expression of multidrug resistance in Escherichia coli. J. Bacteriol. 182, 1754-1756.

Nakano, K., Terui, S., and Yamasaki, K. (2010). Multiple anti-

Nakano, K., Terui, S., and Yamasaki, K. (2010). Multiple anti-

Nakano, K., and Yamaguchi, A. (2007). Multidrug resistance mediated by efflux pumps in Pseudomonas aeruginosa. Mol. Microbiol. 61, 126-141.

Nagase, N., and Yamagishi, T. (2012). Multidrug efflux pump is associated with decreased susceptibility to tigecycline in Acinetobacter baumannii. J. Antimicrob. Chemother. 67, 1211-1221.

Nakamura, S., Nakahama, B., Yamamoto, E., and Yamasaki, A. (2002). Crystal structure of bacterial multidrug efflux transporter AcrB. Nature 419, 387-393.

Muraoka, H., and Piddock, L. J. (2006). Clinically rele-
ted multidrug efflux transporter AcrB. J. Biol. Chem. 281, 31152-31156.

Morita, T., Gotoh, N., and Nishino, T. (2001). Characterization of outer membrane efflux pumps OppN, OppM and OppN of Pseudomonas aeruginosa: molecular cloning and development of specific antiserum. FEMS Microbiol. Lett. 207, 37-43.

Noguchi, K., and Nikaido, H. (2009). Kinetic behavior of the multidrug efflux pump AcrB of Escherichia coli. Proc. Natl. Acad. Sci. USA 106, 7031-7036.

Nakahira, H., Tatsuta, A., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1795, 514-523.

Nakano, K., and Yamaguchi, A. (2000). Effect of NlpE on expression of multidrug resistance in Escherichia coli. Antonie van Leeuwenhoek 77, 1-60.

Nakano, K., Tatsuta, A., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1795, 514-523.

Nakano, K., and Yamaguchi, A. (2000). Effect of NlpE on expression of multidrug resistance in Escherichia coli. Antonie van Leeuwenhoek 77, 1-60.

Nakano, K., Tatsuta, A., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1795, 514-523.

Nakano, K., and Yamaguchi, A. (2000). Effect of NlpE on expression of multidrug resistance in Escherichia coli. Antonie van Leeuwenhoek 77, 1-60.

Nakano, K., Tatsuta, A., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1795, 514-523.

Nakano, K., and Yamaguchi, A. (2000). Effect of NlpE on expression of multidrug resistance in Escherichia coli. Antonie van Leeuwenhoek 77, 1-60.

Nakano, K., Tatsuta, A., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1795, 514-523.

Nakano, K., Tatsuta, A., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1795, 514-523.

Nakano, K., Tatsuta, A., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1795, 514-523.

Nakano, K., Tatsuta, A., and Yamasaki, A. (2009). Regulation and physiological function of multidrug efflux pumps in Escherichia coli and Salmonella. Biochim. Biophys. Acta 1795, 514-523.
beta-lactam specificity of multidrug efflux pumps in Pseudomonas aeruginosa Journal of Bacteriology 179, 7875–7881.
Stavrakas, M., Pichlik, I., and Gibernov, S. (2007). Bacterial efflux pump inhibitors from natural sources. Antimicrob. Agents Chemother. 53, 1274–1280.
Steen, C. K., Flamm, X., Enz, A., Miguel, S., Weigelt, K., Hickey, M., et al. (2008). Complete genome sequence of Pseudomonas aeruginosa PAO1, an opportunist pathogen. Nature 456, 592–596.
Sun, J. K., Chan, M. C., Chang, T. Y., Wang, W. Y., and Chu, S. H. (2010). Overexpression of the adult gene in clinical isolates of tigecycline-nonresponsive Acinetobacter baumannii without insertion mutations in aadA. Antimicrob. Agents Chemother. 54, 4834–4838.
Sun, J. R., Peng, C. L., Chan, M. C., Minna, Y., Lin, J. C., Su, C. M., et al. (2012). A truncated AdeI kinase protein generated by ISHAl insertion correlates with tigecycline resistance in Acinetobacter baumannii. PLoS ONE 7(10), e43771. doi: 10.1371/journal.pone.0043771
Takahisa, Y., and Nishikawa, H. (2006). Threonine-978 in the transmembrane segment of the multidrug efflux pump AcrB of Escherichia coli is crucial for drug transport as a probable component of the proton relay network. J. Bacteriol. 188, 7284–7289.
Tanaka, N., and Shuman, S. (2011). BbbP is the RNA ligase component of a Shigella oib RNA repair system. J. Biol. Chem. 286, 7727–7731.
Thibault, F. M., Hernandez, E., Vidal, D., Gravert, M., and Cavalli, J. D. (2011). Characterization of 65 isolates of Burkholderia pseudomallei and Burkholderia mallei to 35 antimicrobial agents. Antimicrob. Agents Chemother. 57, 1134–1138.
Treich, L. A., Propst, K. L., Wuthrich-Kamen, V., Teisseyre, A., Beckstrom-Sternberg, S. M., Beckstrom-Sternberg, J. S., et al. (2009). Molecular basis of rare aminoglycoside susceptibility and pathogenesis of Burkholderia pseudomallei clinical isolates from Thailand. PLoS Negl. Trop. Dis. 3, e529. doi: 10.1371/journal.pntd.0000529
Vermis, K., Vanadunme, P. A., and Naka, H. J. (2003). Burkholderia cepacia complex genotypes: utilization of carbon sources, susceptibility to antimicrobial agents and growth on selective media. J. Appl. Microbiol. 95, 1191–1199.
Vettoretti, L., Florio, N., Hocquet, D., Deba, R., Fleurant, P., Talon, D., et al. (2009b). Emergence of clinical drug-resistant Pseudomonas aeruginosa in a French university hospital. J. Clin. Microbiol. Infect. 29, 1217–1222.
Vettoretti, L., Fleurant, P., Mullier, C., El Garch, E., Phan, G., Attias, L., et al. (2009b). Efflux substrate in Pseudomonas aeruginosa isolates from cystic fibrosis patients. Antimicrob. Agents Chemother. 55, 1987–1997.
Vogoe, C., Aires, J. R., Bailey, C., Hocquet, D., and Fleurant, P. (2004). Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among Pseudomonas aeruginosa isolates from patients with cystic fibrosis. Antimicrob. Agents Chemother. 48, 1076–1080.
Wohlbach-Walman, S., Sherman, D. M., Hickey, M. J., Couder, S. N., Zhu, Y. Q., Warren, P., et al. (1999). Characterization of a Pseudomonas aeruginosa efflux pump contributing to aminoglycoside impermeability. Antimicrob. Agents Chemother. 43, 2875–2883.
Wong, G. L., Lam, D. K., Fleming, L., Le, R., Whiteside, M. D., Yu, N. Y., et al. (2011). Pseudomonas Genome Database: improved comparative analysis and population genomes capability for Pseudomonas genomes. Nucleic Acids Res. 39, 7206–7209.
Wolter, D. J., Smith-Moland, E., Goering, R. V., Hamon, N. D., and Lister, P. D. (2004). Multidrug resistance associated with mexXY expression in clinical isolates of Pseudomonas aeruginosa from a Texas hospital. Diagn. Microbiol. Infect. Dis. 50, 45–50.
Woka, M. A., and St John, A. C. (1998). Effects of production of abnormal proteins on the rate of killing of Escherichia coli by streptomycin. Antimicrob. Agents Chemother. 42, 534–538.
Xavier, D. E., Picas, R. C., Girardello, R., Feldberg, I. L., and Gales, A. C. (2010). Efflux pump expression and its association with poor down-regulation and beta-lactamase production among Pseudomonas aeruginosa causing bloodstream infections in Brazil. BMC Microbiol. 10, 217. doi: 10.1186/1471-2180-10-217.
Yamamoto, M., Uda, A., Kado, M., Matsumoto, Y., Fujishima, J., Nakae, T., et al. (2009). Role of MexD and PmrA571 in transcriptional regulation of mexXY in Pseudomonas aeruginosa. BMC Microbiology 9, 312–321.
Yamauchi, S., Nagano, S., Hayashi, Nishino, K. (2011). AcrA dependence of the AcrAB efflux pump in Salmonella enterica serovar Typhimurium J. Bacteriol. 193, 453–457.
Yaraiah, H., Lebednik, M., and Schmid, S. (1995). Emergence of an Escherichia coli 12-kDa multidrug transporter, exchanges toxic cations and H+ and is soluble in organic solvents. J. Biol. Chem. 270, 6856–6865.
Yu, E. W., Aires, J. R., and Nishikawa, H. (2003). AcrB multidrug efflux pump of Escherichia coli: composite substrate-binding cavity of exceptional flexibility generates its extremely wide substrate specificity. J. Bacteriol. 185, 5677–5684.