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Contribution of the MexXY Multidrug Transporter to Aminoglycoside Resistance in *Pseudomonas aeruginosa* Clinical Isolates

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MexXY is an aminoglycoside-inducible multidrug transporter shown to contribute to intrinsic and acquired aminoglycoside resistance in laboratory isolates of *Pseudomonas aeruginosa*. To assess its contribution to aminoglycoside resistance in 14 clinical isolates demonstrating a panaminoglycoside resistance phenotype unlikely to be explained solely by aminoglycoside modification, expression of *mexXY* by these isolates was examined by reverse transcription-PCR. Elevated levels of *mexXY* expression were evident for most strains compared with those detected for an aminoglycoside-susceptible control strain, although there was no correlation between *mexXY* levels and the aminoglycoside MICs for the resistant strains, indicating that if MexXY was playing a role, other factors were also contributing. Deletion of *mexXY* from 9 of the 14 isolates resulted in enhanced susceptibilities to multiple aminoglycosides, confirming the contribution of this efflux system to the aminoglycoside resistance of these clinical isolates. Still, the impact of MexXY loss varied, with some strains clearly more or less dependent on MexXY for aminoglycoside resistance. Expression of *mexXY* also varied in these strains, with some showing high-level expression of the efflux genes independent of aminoglycoside exposure (aminoglycoside-independent hyperexpression) and others showing hyperexpression of the efflux genes that was to a greater or lesser degree aminoglycoside dependent. None of these strains carried mutations in *mexZ*, which encodes a negative regulator of *mexXY* expression, or in the *mexZ-mexXY* intergenic region. Thus, *mexXY* hyperexpression in aminoglycoside-resistant clinical isolates occurs via mutation in one or more as yet unidentified genes.

Pseudomonas aeruginosa is a major cause of opportunistic infections in immunocompromised patients, and the organism demonstrates a high level of intrinsic and acquired resistance to a variety of structurally unrelated antibiotics. While this organism causes a panoply of infections, one of the most frequent sites of *P. aeruginosa* infection remains the lungs of cystic fibrosis (CF) patients (10, 26). Long-term care of CF patients involves oral and parenteral administration of various groups of antibiotics, including the aminoglycosides, which are a family of related cationic antibiotics that find frequent use in the treatment of pseudomonal lung infections (7, 24). The recurrent use of aminoglycosides has unfortunately led to recalcitrant subpopulations that demonstrate elevated levels of resistance to this family of antimicrobials (10, 26). While in nonpseudomonal infections the development of aminoglycoside resistance is predominantly mediated through aminoglycoside-modifying enzymes (13, 14), the aminoglycoside resistance observed in *P. aeruginosa* is not due solely to inactivation of these antibiotics by the aminoglycoside-modifying enzymes. Although such enzymatic resistance mechanisms have been described in *P. aeruginosa* (4, 23, 28), they do not appear to be the predominant mechanism responsible for panaminoglycoside resistance in this organism (10, 26). Rather, aminoglycoside resistance, especially in clinical isolates from CF patients, is often caused by a poorly understood mechanism termed "impermeability resistance" (10, 26) that is defined by a general lack of aminoglycoside susceptibility (7, 24) as a result of reduced drug uptake and/or accumulation (2, 9, 17, 18). Transient, so-called adaptive resistance to aminoglycosides has also been reported in *P. aeruginosa* following exposure of susceptible strains to drugs (2, 6, 9, 17, 18). Although a mechanism was not invoked, the reversible nature of this resistance suggested that it was regulatory rather than mutational in nature.

Increasingly, resistance to multiple antimicrobials in *P. aeruginosa* is explained by the operation of multidrug efflux systems, of which several have been identified in this organism to date (19, 22). One of these, encoded by *mexXY* (also known as *amrAB*), exports aminoglycosides and thus provides resistance to multiple aminoglycosides (1, 15, 29). This system has been implicated in both impermeability resistance (29) (in which efflux and not membrane impermeability explain the reduced levels of accumulation) and adaptive aminoglycoside resistance (6). The MexXY efflux pump is homologous to several previously studied pseudomonal multidrug efflux systems of the resistance-nodulation-division (RND) family, the family increasingly recognized as the most significant vis-à-vis the contribution to resistance to clinically relevant agents in gram-negative organisms (20). These pumps consist of an RND inner membrane drug-proton antiporter, a channel-forming outer membrane factor (OMF), and a periplasmic so-called membrane fusion protein (MFP) that is believed to facilitate assembly of the RND and OMF components into a functional efflux pump (21). The MexXY MFP-RND components are believed to associate with the previously identified OprM outer membrane component of the MexAB-OprM efflux system (1, 15); and this association is capable of extruding a variety of antibiotics in addition to aminoglycosides, including macrolides, tetracyclines, β-lactams, and quinolones (1, 12, 15). Still, a recent study (8) highlighting the involvement of outer membrane proteins

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TABLE 1. Bacterial strains and plasmids used in the study

Strain or plasmid	Genotype	Source or reference
P. aeruginosa		
K767	PAO1 wild type	R. Hancock (University of British Columbia)
K258	Clinical isolate	D. Speert (University of British Columbia)
K260	Clinical isolate	D. Speert (University of British Columbia)
K2152 to K2156	Clinical isolates	L. Tomalty (Kingston General Hospital)
K2157 to K2163	Clinical isolates	D. Livermore (Antibiotic Resistance Monitoring and Reference Laboratory)
K1525	K767 Δ*mexXY*	3
K2164	K2152 Δ*mexXY*	Present study
K2165	K2153 Δ*mexXY*	Present study
K2166	K2155 Δ*mexXY*	Present study
K2167	K2156 Δ*mexXY*	Present study
K2168	K2160 Δ*mexXY*	Present study
K2169	K2161 Δ*mexXY*	Present study
K2170	K2162 Δ*mexXY*	Present study
K2171	K258 Δ*mexXY*	Present study
K2172	K2163 Δ*mexXY*	Present study
E. coli S17-1	Donor strain used to promote transfer of pEX18Tc derivatives into *P. aeruginosa*; *thi pro hsdR recA* Tra^+	27
Plasmids		
pEX18Tc	Gene replacement vector; *sacB* Tc^r	5
pCSV05-01	pEX18Tc::Δ*mexXY*	3

OpmG, OpmI, and, to a lesser extent, OpmH in intrinsic aminoglycoside resistance in *P. aeruginosa* suggests that one or more of these may also function as the OMF for the MexXY pump. Expression of *mexXY* is negatively regulated by the product of the divergently transcribed *mexZ* (*amrR*) gene (1, 15, 29) and is inducible by substrate antibiotics, including aminoglycosides (11). The latter observation, in fact, explains the contribution of MexXY to adaptive aminoglycoside resistance (6, 10), although the role of MexZ in adaptive or mutational resistance that results from MexXY expression (29) remains undefined. In the study described in the present report, we examined the expression of this efflux system in clinical aminoglycoside-resistant isolates of *P. aeruginosa* and assessed its contribution to this broad-spectrum panaminoglycoside resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. All bacterial strains were grown in Luria-Bertani (LB) broth supplemented with 0.2% (wt/vol) sodium chloride and were incubated at 37°C for 18 h with shaking (90 rpm). For induction of *mexXY*, 100 μl of an overnight culture was inoculated into 10 ml of LB broth supplemented with kanamycin at one-quarter the MIC. Cultures of *Escherichia coli* S17-1 (27) carrying pCSV05-01 required the addition of tetracycline (10 μg/ml) to maintain the plasmid. In conjugations involving *E. coli* S17-1 (donor) and clinical strains of *P. aeruginosa* (recipients), the latter were incubated at 42°C without shaking. Solid media were prepared by addition of 1.5% (wt/vol) Bacto Agar and contained tetracycline (50 μg/ml), chloramphenicol (5 μg/ml), and sucrose (10% [wt/vol]), as required.

Quantification of *mexXY* by RT-PCR. Total bacterial RNA was isolated from 1.5 ml of late-log-phase *P. aeruginosa* cultures (with and without exposure to subinhibitory concentrations of kanamycin) by using the Qiagen RNeasy Mini kit (Qiagen Inc., Mississauga, Ontario, Canada) and treated with RNase-free DNase (Promega, Madison, Wis.) (1 U of enzyme/μg of RNA for 60 min at 37°C, followed by 15 min at 65°C). Reverse transcription (RT)-PCR was performed with the Qiagen OneStep RT-PCR kit (Qiagen Inc.) according to the instructions of the manufacturer. Primer pair *rpsLF* (5′-GCA ACT ATC AAC CAG CTG-3′) and *rpsLR* (5′-GCTGTG CTC TTG CAG GTT GTG-3′) and primer pair *mexXF* (5′-CAT CAG CGA ACG CGA GTA CAC-3′) and *mexXR* (5′-CAA TTC GCG ATG CGG ATT G-3′) were used to detect the *rpsL* and *mexX* messages, respectively. The reaction mixtures were incubated for 30 min at 50°C, followed by 15 min at 95°C and 18 or 20 cycles (for *rpsL*) or 27 or 29 cycles (for *mexXY*) of 1 min at 95°C, 1 min at 60°C and 1.5 min at 72°C, before finishing with a final 7 min of elongation at 72°C. The amount of product was assessed by gel electrophoresis in conjunction with densitometry measurements with gel analysis software (ONE-DSCAN; Scanalytics).

Amplification of *mexZ* by PCR. Total chromosomal DNA was prepared from overnight cultures as described by Sambrook and Russel (25), and *mexZ* was amplified by PCR with primers *mexZF* (5′-ATT GGA TGT GCA TGG GTG-3′) and *mexZR* (5′-TGG AGA TCG AAG GCA GC-3′). A 50-μl PCR mixture included 10 ng of chromosomal DNA, 0.6 μM each primer, 5% (vol/vol) dimethyl sulfoxide, 1× ThermoPol buffer (New England Biolabs), 0.2 mM deoxynucleoside triphosphate, and 2 U of Vent DNA polymerase (New England Biolabs). The mixture was incubated for 1 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, with a final 5 min of elongation at 72°C. The PCR products were purified with the Qiagen PCR Purification kit and sequenced by Cortec DNA Service Laboratories Inc. (Kingston, Ontario, Canada) with the PCR primers.

Construction of *mexXY* deletion mutants. Chromosomal deletions of *mexXY* were engineered into clinical isolates of *P. aeruginosa* by using *mexXY* gene replacement vector pCSV05-01 (3) following conjugal transfer of the plasmid from *E. coli* S17-1 (3). Briefly, 100 μl of a log-phase *E. coli* S17-1 LB broth culture was transferred to LB agar plates and immediately overlaid with an equal volume of a stationary-phase culture of a *P. aeruginosa* clinical isolate. Following incubation at 37°C for 18 h, the bacterial cells were resuspended in 1 ml of LB broth and diluted 10-fold before being plated onto LB agar plates containing 5 μg of chloramphenicol per ml (to counterselect *E. coli* S17-1) and 70 μg of tetracycline per ml. *P. aeruginosa* transconjugants harboring chromosomal inserts of pCSV05-01 were recovered from these plates and streaked onto LB agar containing sucrose (10% [wt/vol]). Sucrose-resistant colonies were then screened for deletion of *mexXY* by PCR with primers *mexzbI* (5′-AAG CTT AGG CTT GCG TTC GCA CTT GAG GTA GAG-3′) and *mexhbI* (5′-A CCG GAA TTC CAC CAG GAA GAA CAG CGG TAC-3′) as described before (3). The reaction mixtures were formulated as described above for the *mexZ* PCR and were incubated for 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 58°C, and 4 min at 72°C, with a final 5 min of elongation at 72°C.

Antimicrobial susceptibility testing. The antimicrobial susceptibilities of the clinical *P. aeruginosa* strains and their Δ*mexXY* derivatives were assessed in microtiter trays by a twofold serial dilution technique (8). Briefly, 50-μl aliquots of log-phase cells grown in LB broth were added to an equal volume of LB broth containing serial twofold dilutions of antibiotic to yield a final cell concentration of 2.75×10^5 cells/ml. Following incubation at 37°C for 18 h, growth was assessed

TABLE 2. Aminoglycoside susceptibilities of clinical *P. aeruginosa* strains

Strain[a]	MIC (μg/ml)[b]								
	AMI	GEN	KAN	LIV	NEO	PAR	SPC	STR	TOB
K767	2	4	128	16	64	512	1,024	16	1
K2152 (699)	8	8	128	64	128	512	2,048	32	4
K2153 (524)	16	16	128	64	128	512	2,048	64	4
K2154 (0006-3)	16	512	512	256	256	1,024	>2048	256	256
K2155 (4420)	1	4	16	16	8	32	512	16	1
K2156 (8812-2)	32	32	512	128	128	512	2,048	256	8
K2157 (R68)	64	64/128	1,024	512	512	>2,048	>2,048	256	128
K2158 (R69)	64	64	1,024	512	512	>2,048	>2,048	128	32
K2159 (R92)	8	8	128	16	64	64	64	4	8
K2160 (R103)	64	32	512	512	128	512	512	128	16
K2161 (R109)	64	64	1,024	256	512	>2,048	256	64	16
K2162 (R115)	256	256	>2,048	>2,048	1,024	>2,048	>2,048	256	64
K258 (C517M)	32	16	512	128	256	>2,048	2,048	64	16
K260 (C510M)	16	16	128	128	64	256	512	64	4
K2163 (PS380)	16	8	128	64	32	256	1,024	32	8

[a] The designation of the laboratory of K. Poole is given, with the original hospital designation given in parentheses.
[b] Abbreviations; AMI, amikacin; GEN, gentamicin; KAN, kanamycin; LIV, lividomycin; NEO, neomycin; PAR, paromomycin; SPC, spectinomycin; STR, streptomycin; TOB, tobramycin.

visually and the MIC was reported as the lowest concentration of antibiotic inhibiting visible growth.

RESULTS AND DISCUSSION

Aminoglycoside resistance in clinical strains of *P. aeruginosa*. Fourteen clinical isolates and the designated PAO1 laboratory strain of *P. aeruginosa* (strain K767) were assessed for their susceptibilities to a range of aminoglycoside antibiotics. With few exceptions, the strains demonstrated panaminoglycoside resistance (Table 2), with MICs generally being above the accepted NCCLS breakpoint (16) for parenterally administered aminoglycosides used in the treatment of *P. aeruginosa* infections. Wild-type PAO1 reference strain K767 and two clinical isolates (isolates K2155 and K2159) were, however, substantially more susceptible to the aminoglycosides than the other strains (Table 2). Clinical isolates K2155 and K2159, particularly K2155, would nonetheless prove to be useful comparators in later assessments of *mexXY* expression and its contribution to aminoglycoside resistance in the other isolates. While most strains showed a range of susceptibilities to the various aminoglycosides, the MICs of spectinomycin, lividomycin, paromomycin, neomycin, and kanamycin were found to be universally elevated for all strains examined (Table 2). Given that *P. aeruginosa* is known to harbor a number of aminoglycoside-modifying enzymes, including APH(3′) (28), AAC(3) (4), AAC(6′) (4), and ANT(4′) (23), it is possible that enzymatic modification may contribute to the high level of resistance to these antibiotics that was observed. It is extremely unlikely, however, that aminoglycoside modification would satisfactorily explain the panaminoglycoside resistance observed in most of the clinical isolates, as shown in Table 2 (2, 6, 9). Indeed, this broad-range aminoglycoside resistance is reminiscent of the impermeability resistance that has previously been described in *P. aeruginosa* (7, 9, 10, 24).

Expression of *mexXY* in aminoglycoside-resistant *P. aeruginosa*. In light of suggestions that MexXY may contribute to aminoglycoside impermeability resistance, attempts were made initially to assess *mexXY* expression by RT-PCR and to correlate this with the resistance levels of the clinical isolates. RNA samples from the isolates grown in LB broth in the absence of aminoglycoside antibiotics were standardized by using *rpsL* gene expression (Fig. 1A), and although RNA was clearly added to the RT-PCR mixtures in equivalent amounts, a high degree of variation in the level of *mexXY* gene expression was observed among the aminoglycoside-resistant strains examined (Fig. 1C). Several isolates, including K2153, K2156, K2160, and K2161, showed high levels of *mexXY* expression relative to that for "susceptible" strain K2155 (Fig. 1C, top panel, lanes 4, 6, 10, and 11, respectively); and this was made more evident when the PCR was increased to 29 cycles (Fig. 1C, bottom panel, lanes 4, 6, 10, and 11, respectively). Westbrock-Wadman and colleagues (29) have reported similar increases in *amrAB* (*mexXY*) expression in strains from which *amrR* (*mexZ*) was deleted, although such mutants were not aminoglycoside resistant. PCR amplification and subsequent sequencing of *mexZ* and the *mexZ-mexXY* intergenic region failed to reveal any mutations in the *mexZ* genes of the resistant clinical strains described here. Similarly, in vitro-selected *P. aeruginosa* mutants that are resistant to tobramycin as a result of elevated levels of MexXY expression and that lack *mexZ* mutations have also been described (29). Two isolates, K2157 and K2158, did, however, carry the same mutation in the *mexZ-mexXY* intragenic region, a C-to-T transition at position −23 relative to the ATG start codon of *mexX*, although whether this would affect MexZ repression of *mexXY* is unclear. Interestingly, "susceptible" strain K2155 was the only strain that carried a mutation in *mexZ*, producing a L41Q substitution in the MexZ protein which is unlikely to affect its repressor activity, given the barely detectable level of *mexX* expression seen in this strain prior to antibiotic induction (Fig. 1C, top panel). Thus, despite the known role of MexZ as a negative regulator of *mexXY* expression, hyperexpression of this system in aminoglycoside-resistant clinical isolates is achieved by mutations in a gene(s) other than *mexZ*. Interestingly, K2159 failed to express detectable *mexXY* (Fig. 1C and D, lanes 9), and a *mexZ* gene could not be amplified from its

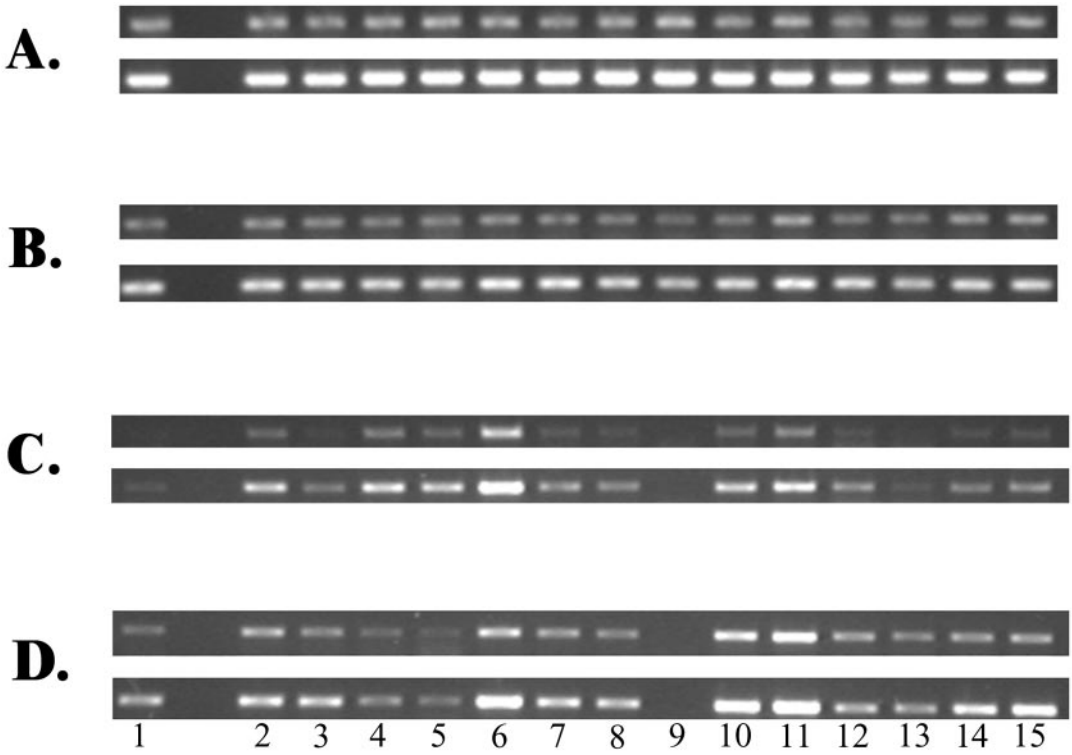

FIG. 1. Expression of *rpsL* (A and B) and *mexX* (C and D) in *P. aeruginosa* clinical isolates grown without (A and C) and with (B and D) kanamycin (at one-quarter the MIC) as determined by semiquantitative RT-PCR. Lanes 1, K2155; lanes 2, PAO1 strain K767; lanes 3, K2152; lanes 4, K2153; lanes 5, K2154; lanes 6, K2156; lanes 7, K2157; lanes 8, K2158; lanes 9, K2159; lanes 10, K2160; lanes 11, K2161; lanes 12, K2162; lanes 13, K258; lanes 14, K260; lanes 15, K2163. The *rpsL* reaction served as an internal control that ensured that equal amounts of RNA were used in all of the RT-PCRs shown. The PCR portion of the *rpsL* reactions was carried out for 18 cycles (upper panels) or 20 cycles (lower panels). The PCR portion of the *mexX* reactions was carried out for 27 cycles (upper panels) or 29 cycles (lower panels).

genome, suggesting that a *mexXY* locus is absent or defective in this isolate, possibly explaining its general aminoglycoside susceptibility (Table 2).

Despite the variability of *mexXY* expression observed in the clinical strains and although many of the aminoglycoside-resistant strains expressed *mexXY* at levels above the level of expression by K2155, there was no clear correlation between *mexXY* expression and aminoglycoside resistance. Indeed, strain K2162 (Fig. 1C, lane 12) expressed substantially less *mexXY* than several isolates (e.g., K2153 and K2156; Fig. 1C lanes 4 and 6, respectively), yet the MICs of a range of aminoglycosides were 4- to 16-fold higher for K2162 than for these isolates (Table 2). Still, *mexXY* expression is typically aminoglycoside inducible (6, 11), and as MIC determinations clearly involve drug exposure, it was possible that aminoglycoside MICs would correlate better with aminoglycoside-induced *mexXY* levels. Thus, *mexXY* expression by the clinical isolates following aminoglycoside (i.e., kanamycin) exposure was examined (Fig. 1D). Not unexpectedly, many of the isolates, including generally susceptible strain K2155, showed increased levels of *mexXY* expression in the presence of kanamycin compared to the levels of expression by their counterparts not exposed to kanamycin (compare Fig. 1C and D). Again, too, most of the aminoglycoside-resistant clinical isolates showed higher levels of *mexXY* expression than K2155 (Fig. 1D). Still, no good correlation between *mexXY* levels and aminoglycoside resistance was observed, with many highly resistant isolates (e.g., K2154, K2157, K2158, and K2162; Fig. 1D lanes 5, 7, 8, and 12, respectively) expressing *mexXY* at levels comparable to the levels expressed by K2155 (Fig. 1D, lane 1) and much less than the levels expressed by more susceptible isolates (e.g., K2156, K2160, K2161, and K2163; Fig. 1D lanes 6, 10, 11, and 15, respectively). Thus, while *mexXY* is clearly expressed at high levels in a number of clinical aminoglycoside-resistant *P. aeruginosa* isolates, at this point we are uncertain about its contribution to resistance.

Contribution of MexXY to aminoglycoside resistance. To better assess the contribution of MexXY to aminoglycoside resistance in clinical isolates, the genes were deleted from these isolates and the impact on aminoglycoside resistance was assessed. Despite numerous attempts, deletion of *mexXY* was achieved in only nine strains (Table 3), although for each of these strains the aminoglycoside MICs decreased a minimum of twofold, indicating that MexXY does, in fact, contribute to aminoglycoside resistance in clinical isolates. Interestingly, the biggest decline in MICs upon deletion of *mexXY* (8- to 64-fold) was found for strain K2156, which generally showed the highest levels of *mexXY* expression (see K2167 in Table 3), indicating that MexXY is a major determinant of aminoglycoside resistance in this isolate. In two other isolates that also showed substantial levels of *mexXY* expression (i.e., K2160 and K2161), the loss of *mexXY* had a substantial impact (2- to 32-fold declines in MICs; see K2168 and K2169 in Table 3) on the panaminoglycoside resistance of these strains. Still, K2163 also

TABLE 3. Aminoglycoside susceptibilities of Δ*mexXY* derivatives of clinical *P. aeruginosa* strains

Strain	MIC (μg/ml)[a]											
	AMI	GEN	KAN	LIV	NEO	PAR	SPC	STR	TOB	ERY	TET	NOR
K767	2	4	128	16	64	512	1,024	16	1	512	16	0.5
K1525	1	2	64	4	16	32	128	2	1	128	16	0.5
ΔMIC[b]	2	2	2	4	4	16	8	8	1	4	1	1
K2152	8	8	128	64	128	512	2,048	32	4	512	32	1
K2164	4	4	64	8	32	32	128	4	2	128	32	1
ΔMIC	2	2	2	8	4	16	16	8	2	4	1	1
K2153	16	16	128	64	128	512	2,048	64	4	512	32	1
K2165	2	4	64	16	16	16	128	8	4	128	128	1
ΔMIC	8	4	2	4	8	32	16	8	1	4	0.25	1
K2155	1	4	16	16	8	32	512	16	1	256	32	4
K2166	0.5	2	16	4	8	8	128	4	1	32	32	4
ΔMIC	2	2	1	4	1	4	4	4	1	8	1	1
K2156	32	32	512	128	128	512	2,048	256	8	512	16	8
K2167	1	4	64	4	16	32	64	4	1	256	16	4
ΔMIC	32	8	8	32	8	16	32	64	8	2	1	2
K2160	64	32	512	512	128	512	512	128	16	256	16	1
K2168	16	8	256	32	64	64	64	16	8	32	16	1
ΔMIC	4	4	2	16	2	8	8	8	2	8	1	1
K2161	64	64	1,024	256	512	>2,048	256	64	16	512	16	0.5
K2169	8	8	256	32	64	64	64	8	8	256	16	0.5
ΔMIC	8	8	4	8	8	32	4	8	2	2	1	1
K2162	256	256	2,048	2,048	1,024	>2,048	2,048	256	64	512	32	2
K2170	32	16	512	64	128	256	128	16	16	512	32	2
ΔMIC	8	16	4	32	8	8	16	16	4	1	1	1
K258	32	16	512	128	256	2,048	2,048	64	16	1,024	64	1
K2171	8	4	256	32	64	64	512	16	8	1,024	64	1
ΔMIC	4	4	2	4	4	32	4	4	2	1	1	1
K2163	16	8	128	64	32	256	1,024	32	8	256	32	1
K2172	8	2	64	16	16	32	256	8	4	128	32	1
ΔMIC	2	4	2	4	2	8	4	4	2	2	1	1

[a] Abbreviations: AMI, amikacin; GEN, gentamicin; KAN, kanamycin; LIV, lividomycin; NEO, neomycin; PAR, paromomycin; SPC, spectinomycin; STR, streptomycin; TOB, tobramycin; ERY, erythromycin; TET, tetracycline; NOR, norfloxacin.
[b] ΔMIC, ratio of MIC for the parent strain and the MIC for the corresponding Δ*mexXY* derivative.

showed substantial levels of *mexXY* expression, and the loss of *mexXY* had only a modest impact on susceptibility (two- to eightfold declines in MICs; see K2172 in Table 3). In this instance, another resistance determinant(s) is likely playing an important role and, for some aminoglycosides, at least can compensate for the loss of this efflux system, although the presence of mutations affecting MexXY expression or activity cannot be ruled out. In contrast, some strains expressing modest levels of *mexXY* (e.g., K2162) still showed substantial increases in aminoglycoside susceptibilities upon the loss of *mexXY* (up to 32-fold decreases in MICs; see K2170 in Table 3). In this instance, *mexXY* is clearly a major determinant of aminoglycoside resistance in this isolate. It is interesting, however, that even in strains in which MexXY is clearly having a major impact (e.g., K2160, K2161, and K2162), the loss of *mexXY* yields strains that are still substantially aminoglycoside resistant (the MICs for K2168, K2169, and K2170 are much greater than those for the other Δ*mexXY* derivatives). Clearly, then, another factor(s) is also contributing to the high-level aminoglycoside resistance in these isolates.

While MexXY was initially identified as an aminoglycoside efflux system, this pump is also capable of extruding unrelated antibiotics, including erythromycin, tetracycline, and norfloxacin (1, 15, 29). We therefore assessed the impact of *mexXY* deletion on the susceptibilities of the clinical strains to these nonaminoglycoside antimicrobials. While the loss of MexXY had a modest impact on erythromycin resistance, with MICs declining less than or equal to eightfold for all strains, the tetracycline and norfloxacin MICs did not change at all (Table 3). This is reminiscent of earlier studies in which the loss of *mexXY* in otherwise wild-type laboratory strains had a negligible impact on susceptibilities to these agents, apparently because they could be exported by other efflux systems (e.g., MexAB-OprM), which could thus compensate for the loss of MexXY (15, 29).

***mexXY* expression patterns in aminoglycoside-resistant clinical isolates.** While no correlation between aminoglycoside resistance and *mexXY* expression in the presence or absence of an added aminoglycoside antibiotic was found to exist, clear patterns of *mexXY* expression were evident among those ami-

noglycoside-resistant isolates in which MexXY was a contributing factor. In some instances, the best example being K2156, *mexXY* was expressed at levels markedly higher than the level of expression seen by K2155 in the absence of antibiotic (Fig. 1C, lane 6); and no increase in *mexXY* expression was observed following drug (i.e., kanamycin) exposure (Fig. 1D, lane 6). A similar pattern of drug-independent hyperexpression was observable for clinical strains K2153 (Fig. 1C and D, lanes 4) and K2154 (Fig. 1C and D, lanes 5), although a contribution of MexXY to aminoglycoside resistance was not confirmed for K2154. In most instances, however, maximal *mexXY* expression by the clinical isolates was achieved upon aminoglycoside exposure, although two distinct patterns of drug-dependent *mexXY* hyperexpression were evident. These two patterns were differentiated by the levels of *mexXY* expressed prior to aminoglycoside exposure. One group produced very modest levels of *mexXY* in the absence of aminoglycoside (e.g., K260 and K2163; Fig. 1C, lanes 14 and 15, respectively), while the other group expressed substantial *mexXY* even in the absence of an aminoglycoside (e.g., K2160 and K2161; Fig. 1C, lanes 10 and 11, respectively). These different patterns of expression seen in the clinical aminoglycoside-resistant isolates suggest that different mutations are responsible for the enhanced *mexXY* expression observed and that MexXY-mediated aminoglycoside resistance can arise in *P. aeruginosa* in several ways. While the drug-independent *mexXY* hyperexpression seen in, for example, K2156 is somewhat reminiscent of hyperexpression of the *mexAB-oprM* and *mexCD-oprJ* multidrug efflux systems by *nalB* and *nfxB* mutants, respectively, as a result of mutations in linked repressor genes and is consistent with a mutation in *mexZ*, such mutations, as mentioned previously, were absent from all aminoglycoside-resistant isolates examined. Thus, not only is *mexXY* hyperexpression alone insufficient for aminoglycoside resistance, but mutations in *mexZ* are not associated with the *mexXY* hyperexpression that clearly occurs in and that contributes to the aminoglycoside resistance of at least some clinical isolates. Thus, additional genes contribute both to MexXY-dependent aminoglycoside resistance in *P. aeruginosa* and to *mexXY* hyperexpression in aminoglycoside-resistant strains.

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