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Oxidative Stress Induction of the MexXY Multidrug Efflux Genes and Promotion of Aminoglycoside Resistance Development in *Pseudomonas aeruginosa*[▽]

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Exposure to reactive oxygen species (ROS) (e.g., peroxide) was shown to induce expression of the PA5471 gene, which was previously shown to be required for antimicrobial induction of the MexXY components of the MexXY-OprM multidrug efflux system and aminoglycoside resistance determinant in *Pseudomonas aeruginosa*. *mexXY* was also induced by peroxide exposure, and this too was PA5471 dependent. The prospect of ROS promoting *mexXY* expression and aminoglycoside resistance recalls *P. aeruginosa* infection of the chronically inflamed lungs of cystic fibrosis (CF) patients, where the organism is exposed to ROS and where MexXY-OprM predominates as the mechanism of aminoglycoside resistance. While ROS did not enhance aminoglycoside resistance *in vitro*, long-term (8-day) exposure of *P. aeruginosa* to peroxide (mimicking chronic *in vivo* ROS exposure) increased aminoglycoside resistance frequency, dependent upon PA5471 and *mexXY*. This enhanced resistance frequency was also seen in a mutant strain overexpressing PA5471, in the absence of peroxide, suggesting that induction of PA5471 by peroxide was key to peroxide enhancement of aminoglycoside resistance frequency. Resistant mutants selected following peroxide exposure were typically pan-aminoglycoside-resistant, with *mexXY* generally required for this resistance. Moreover, PA5471 was required for *mexXY* expression and aminoglycoside resistance in these as well as several CF isolates examined.

Multidrug efflux systems of the three-component resistance-nodulation-division (RND) family contribute significantly to intrinsic and acquired resistance to antimicrobials in a number of Gram-negative bacteria (39, 41). *Pseudomonas aeruginosa*, an opportunistic human pathogen (28), expresses several RND-type multidrug efflux systems, of which four, MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM, are reported to be significant determinants of multidrug resistance in lab and clinical isolates (38). MexXY-OprM is somewhat unique in *P. aeruginosa* in that the *mexXY* operon is induced upon exposure to many of the antibiotics that this efflux system exports (31). Still, only those agents known to target the ribosome promote *mexXY* expression (26, 31, 34), and this is compromised by so-called ribosome protection mechanisms (26), suggesting that the MexXY-OprM efflux system is recruited in response to ribosome disruption or defects in translation and not antibiotics *per se*. Consistent with this, mutations in *fmt* (encoding a methionyl-tRNA-formyltransferase) and *folD* (involved in folate biosynthesis and production of the formyl group added to initiator methionine), which are expected to negatively affect protein synthesis, increase expression of *mexXY* (6). Upregulation of *mexXY* by antimicrobials or *fmt*/*folD* mutations is dependent upon a gene, PA5471, encoding a conserved hypothetical protein whose expression is also promoted by ribosome-disrupting antimicrobials (34) and by *fmt*/*folD* mutations (6). Despite this primary link to translation disruption, the MexXY-OprM efflux system is a significant determinant of resistance to antimicrobials in clinical isolates, particularly aminoglycosides (19, 40, 52) but also β-lactams (3, 19, 23, 37, 51). Indeed, while it is uncommon as a mechanism of aminoglycoside resistance in most clinical strains of *P. aeruginosa*, MexXY-OprM is the predominant mechanism of resistance to these agents in cystic fibrosis (CF) isolates (19, 40, 52). Consistent with *mexXY* expression being commonplace in CF lung isolates. *mexX* is induced *in vitro* upon exposure of *P. aeruginosa* to human airway epithelial cells (17) and *mexY* shows enhanced expression in this organism in the CF lung (DNA array performed on RNA isolated from sputum) (48).

Recent transcriptome studies revealed that PA5471 is substantially upregulated in *P. aeruginosa* cells subjected to oxidative stress imposed by disinfectants such as peroxide (H_2O_2) (7) and peracetic acid (8), although *mexXY* expression was not reported (only highly up-/downregulated genes were reported). Intriguingly, the CF lung is rich in reactive oxygen species (ROS) (11) owing to the chronic inflammation that is apparently the result of the CF transmembrane conductance regulator (CFTR) defect that characterizes this disease and of chronic *P. aeruginosa* infection (27, 45). Given that MexXY-mediated efflux is the most common mechanism of aminoglycoside resistance in *P. aeruginosa* CF isolates (43), the implication is that ROS may be promoting the development of aminoglycoside resistance in CF lung isolates, mediated by PA5471 and MexXY. Thus, the impact of ROS (H_2O_2) on *mexXY* expression and development of MexXY-OprM-dependent aminoglycoside resistance *in vitro* was examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial cells were cultured in Luria broth (L broth)

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Reference
E. coli		
DH5α	ϕ80d *lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* F^- Δ(*lacZYA-argF*)*U169*	1
S17-1	*thi pro hsdR recA* Tra^+	46
P. aeruginosa		
K767	PAO1 prototroph	30
K1525	K767 Δ*mexXY*	14
K2413	K767 ΔPA5471	34
K2415	K767 Δ*mexZ*	34
K2416	K2415 ΔPA5471	34
K2817	K767 PA5471.1$_{Q3Am}$ (PA5471^{++})	33
K2965	Amikacin-resistant derivative of K767 selected following an 8-day exposure to H_2O_2	This study
K2966	Amikacin-resistant derivative of K767 selected following an 8-day exposure to H_2O_2	This study
K2967	Amikacin-resistant derivative of K767 selected following an 8-day exposure to H_2O_2	This study
K2968	Amikacin-resistant derivative of K767 selected following an 8-day exposure to H_2O_2	This study
K2969	K2965 Δ*mexXY*	This study
K2970	K2966 Δ*mexXY*	This study
K2971	K2867 Δ*mexXY*	This study
K2972	K2968 Δ*mexXY*	This study
K2973	K2966 ΔPA5471	This study
K2974	K2967 ΔPA5471	This study
K2975	K2968 ΔPA5471	This study
K2152	CF isolate	47
K2427	K2152 ΔPA5471	This study
K2164	K2152 Δ*mexXY*	47
K2153	CF isolate	47
K2428	K2153 ΔPA5471	This study
K2165	K2153 Δ*mexXY*	47
K2160	CF isolate	47
K2430	K2160 ΔPA5471	This study
K2168	K2160 Δ*mexXY*	47
K2161	CF isolate	47
K2431	K2161 ΔPA5471	This study
K2169	K2161 Δ*mexXY*	47
K2158	CF isolate	47
K2432	K2158 ΔPA5471	This study
K2171	K2158 Δ*mexXY*	47
K2163	CF isolate	47
K2433	K2163 ΔPA5471	This study
K2172	K2163 Δ*mexXY*	47
Plasmids		
pEX18Tc	Gene-replacement vector; *sacB* Tc^r	21
pYM008	pEX18Tc::ΔPA5471	34
pCSV05	pEX18Tc::Δ*mexXY*	14

[a] Tc^r, tetracycline resistance.

and on Luria agar with antibiotics, as necessary, at 37°C. Plasmid pEX18Tc and its derivatives were maintained in *Escherichia coli* with 10 μg/ml of tetracycline. Δ*mexXY* and ΔPA5471 derivatives of *P. aeruginosa* were constructed by mobilizing pEX18Tc::Δ*mexXY* (pCSV05-01) and pEX18Tc::ΔPA5471 (pYM008), respectively, into *P. aeruginosa* from *E. coli* S17-1 as described previously (5) with modifications. Briefly, 700 μl of plasmid-carrying *E. coli* S17-1 (log phase, cultured at 37°C) was mixed with 300 μl of *P. aeruginosa* (stationary phase, cultured at 42°C) in a microcentrifuge tube and centrifuged, and the pellet was resuspended in 100 μl of L broth and spotted onto the center of an L-agar plate. Following incubation at 37°C for 6 h, bacteria were recovered from the L-agar plate in 100 μl L broth, and *P. aeruginosa* transconjugants harboring chromosomal inserts of the deletion vectors were selected on L-agar plates containing tetracycline (75 μg/ml) and chloramphenicol (5 μg/ml; to counterselect *E. coli* S17-1). These were subsequently streaked onto L agar containing sucrose (10% [wt/vol]) as before (5), with sucrose-resistant colonies screened for the appropriate deletion using colony PCR.

Colony PCR. To identify Δ*mexXY* and ΔPA5471 derivatives of *P. aeruginosa*, single colonies were recovered from sucrose plates and resuspended in 30 μl sterile distilled H_2O, which was then heated for 5 min at 95°C and centrifuged for 1 min at 13,000 rpm. The *mexXY* operon and PA5471 were amplified with primer pairs mexXY-KO-Scr-F (5′-CACCAGGAAGAACAGCGGTA-3′) and mexXY-KO-Scr-R (5′-CAGA-TCATAAGGATATGTTA-3′), and PA5471-KO-Scr-F (5′-CCTGGGAAGGCTATACCAACG-3′) and PA5471-KO-Scr-R (5′-GCTTC ATCGGCACCATCAT-3′), respectively, in a 10-μl PCR mixture containing 2 μl of colony lysate, 0.6 μM each primer, 0.2 mM each deoxynucleoside triphosphate, 0.5 U of *Taq* Polymerase (New England BioLabs, Ltd., Pickering, Ontario, Canada), 1× ThermoPol buffer and 5% (vol/vol) dimethyl sulfoxide (DMSO). The mixture was heated for 5 min at 95°C, followed by 30 cycles of 0.5 min at 95°C, 0.5 min at 51°C (*mexXY*) or 60°C (PA5471), and 5 min (*mexXY*) or 3.5 min (PA5471) at 72°C, finishing with 7 min at 72°C. Products were then visualized on agarose gels.

Selection of aminoglycoside-resistant mutants following exposure to H_2O_2. Overnight cultures (in L broth) of various *P. aeruginosa* strains were diluted 1:49 in fresh L broth and grown for 2 h. H_2O_2 (1 mM final concentration) was then added three times at 2-hour intervals, after which cultures were allowed to recover overnight. This was repeated daily over 8 days, at which time serial dilutions were plated on L agar (to enumerate total cell numbers) and L agar supplemented with amikacin (at 2.5× MIC) or tobramycin (at 1× MIC) (to enumerate numbers of amikacin- or tobramycin-resistant bacteria and to calculate the resistance frequency). Eight-day unexposed *P. aeruginosa* controls were processed in parallel. Randomly selected amikacin-resistant colonies were subsequently picked, passaged eight times on L-agar plates, and then assessed for resistance to amikacin and to additional aminoglycosides. Stable pan-aminoglycoside-resistant mutants were saved for further study. In some experiments, aminoglycoside-resistant mutants were recovered and resistance frequency determined following overnight growth (16 h) only.

DNA methods. Standard protocols were generally used for restriction endonuclease digestion, ligation, transformation, plasmid isolation, and agarose gel electrophoresis, as described by Sambrook and Russell (44). Plasmid DNAs were also prepared from *E. coli* or *P. aeruginosa* using a GeneJET Plasmid Miniprep kit (Fermentas Canada Inc., Burlington, Ontario, Canada) or QIAfilter Plasmid Midikit (Qiagen Inc., Mississauga, Ontario, Canada) according to the protocols provided by the manufacturer. Chromosomal DNA of *P. aeruginosa* was extracted using a DNeasy Blood & Tissue kit (Qiagen) according to the protocol provided by the manufacturer. PCR products were purified using a Wizard SV gel and PCR clean-up system (Fisher Scientific, Ltd., Nepean, Ontario, Canada) and, when cloned, sequenced to verify that no mutations were introduced during PCR. Competent *P. aeruginosa* (9) and *E. coli* (24) cells were prepared as described previously. Oligonucleotide synthesis was carried out by Integrated DNA Technologies (Coralville, IA), and nucleotide sequencing was carried out by ACGT Corp. (Toronto, Ontario, Canada) using universal and custom primers.

Susceptibility testing. The antimicrobial susceptibilities of various *P. aeruginosa* strains were assessed in 96-well microtiter plates using 2-fold serial dilutions as described previously (27).

RT-PCR. Total bacterial RNA was isolated from log-phase *P. aeruginosa* L-broth cultures (with and without 1 mM H_2O_2), using a High Pure RNA isolation kit (Roche Canada, Mississauga, Ontario, Canada), Turbo DNA-free DNase (Applied Biosystems Canada, Streetsville, Ontario, Canada), and a protocol provided by the manufacturer (Roche). The reverse transcription-PCR (RT-PCR) was performed with ca. 50 ng RNA using the Qiagen one-step RT-PCR kit according to a protocol provided by the manufacturer. Primers and reaction conditions for amplification of *rpoD*, PA5471, and *mexX* have been described previously (34). RT-free control reactions were carried out to ensure that there was no DNA contamination of RNA preparations.

PCR amplification of *mexZ*, *mexXY*, and PA5471.1. To screen various pan-aminoglycoside-resistant *P. aeruginosa* strains for mutations in *mexZ* (including its promoter region), *mexXY*, and PA5471.1, the genes were amplified from the chromosome prior to sequencing. The *mexZ* gene, including the entirety of the *mexZ-mexXY* intergenic region, was amplified with primers mexZ-295-F (5′-AC GATCACGCCGACCTCG-3′) and mexZ-80-R (5′-GAGG-AAGACGCCCAG

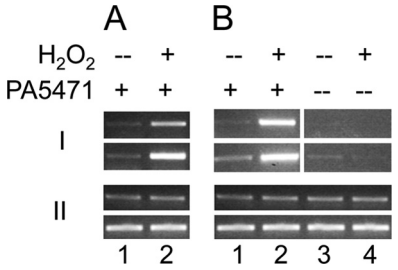

FIG. 1. (I) Influence of H_2O_2 on expression of PA5471 (A) and *mexXY* (B) in *P. aeruginosa*. Wild-type *P. aeruginosa* strain K767 (lanes 1 and 2) and its ΔPA5471 derivative K2413 (lanes 3 and 4) were grown to mid-log phase and cultured without or with 1 mM H_2O_2 for 30 min, and PA5471 or *mexXY* expression was assessed using RT-PCR. (II) Expression of *rpoD* was also assessed and served as an internal control that ensured that equal amounts of RNA were employed in all of the RT-PCRs shown. The PCR portion of the reactions was carried out for 28 (PA5471), 32 (*mexX*), or 20 (*rpoD*) cycles (upper panels of I or II) and for 30, (PA5471), 34 (*mexX*), or 22 (*rpoD*) cycles (lower panels of I or II).

CGGCT-3′) in a 50-μl PCR mixture formulated as before for PA5471 (34), except that Phusion high-fidelity DNA polymerase (New England BioLabs, Ltd., Pickering, Ontario, Canada) was used (1 U) in 1× Phusion GC buffer and $MgCl_2$ was omitted. The mixture was heated for 0.5 min at 98°C, followed by 30 cycles of 0.5 min at 98°C, 0.5 min at 65°C, and 0.3 min at 72°C, before finishing with 7 min at 72°C. The *mexXY* operon was amplified in two overlapping PCR products using primers mexXY-1-F (5′-TGAGTTGCGGTGCCCTTT-3′) and mexXY-1-R (5′-CGAACGCCGAGGTGTCA-TA-3′) for fragment 1 and primers mexXY-2-F (5′-AGCGAGTACGGCTTCGTCT-3′) and mexXY-2-R (5′-GGT CGGTGAACTGCTGTTG-3′) for fragment 2. Both fragments were amplified in a 50-μl PCR mixture formulated as described above for *mexZ* with the exception that 5% (vol/vol) DMSO was included and the first 72°C incubation was for 1 min. Amplification of PA5471.1 was achieved as described previously (33).

RESULTS

Peroxide induction of *mexXY* dependent on PA5471. Transcriptome analysis has revealed that exposure of *P. aeruginosa* to oxidative stress-promoting agents such as peroxide (7) and peracetic acid (8) induces expression of the PA5471 gene, which is required for induction of the *mexXY* multidrug efflux operon in response to ribosome-targeting antimicrobials (34). Using RT-PCR, peroxide induction of PA5471 was confirmed (Fig. 1A lane 2 [cf. lane 1]). Given that ribosome-targeting-drug inducibility of *mexXY* follows from induction of PA5471 by the same drugs (34), it was of interest to examine whether *mexXY* was also peroxide inducible. As seen in Fig. 1B, lane 2 (cf. lane 1), this efflux operon was indeed induced by peroxide. Moreover, this induction was dependent on PA5471, being lost in the ΔPA5471 mutant K2413 (Fig. 1B, lane 4 [cf. lane 3]). Despite the induction of *mexXY* by peroxide, however, the MexXY-OprM efflux system did not contribute to peroxide resistance (the peroxide MIC for the Δ*mexXY* strain K1525 remained the same as that for wild-type parent strain K767, 2 mM). The mechanism by which peroxide induces PA5471 (and subsequently *mexXY*) expression is as yet unresolved but may involve the same transcriptional attenuation mechanism that explains antibiotic inducibility of PA5471 (i.e., peroxide may interfere with ribosome function, leading to loss of attenuation and thus read-through transcription of PA5471) (33).

Peroxide treatment enhances recovery of aminoglycoside-resistant mutants of PAO1 dependent on PA5471 and MexXY. Given the observation that oxidative stress enhances expression of the MexXY components of a multidrug efflux system, the possibility existed that it might positively influence MexXY-OprM-mediated antimicrobial resistance. The presence of half the MIC of peroxide did not, however, influence the resistance of wild-type *P. aeruginosa* PAO1 strain K767 to any MexXY-OprM antimicrobial substrate tested (e.g., aminoglycosides, erythromycin, tetracycline, and chloramphenicol), presumably because these antimicrobials themselves induce *mexXY* expression (31) (i.e., peroxide induction of *mexXY* and its subsequent effect on antimicrobial resistance are "masked" by the positive impact that the antimicrobials have on their own resistance by virtue of their induction of the efflux genes in the MIC assay). Still, ROS induction of *mexXY* suggested that the MexXY-OprM efflux system likely plays a positive role in an oxidative stress response such that peroxide exposure over time might provide a selective pressure for *mexXY*-expressing antimicrobial-resistant mutants. It is, for example, interesting to note that MexXY-OprM-mediated efflux is the predominant mechanism of aminoglycoside resistance in *P. aeruginosa* isolates recovered from the lungs of cystic fibrosis (CF) patients (40) an environment noted for its richness in ROS (25, 43). The implication here is that ROS may be promoting the development of aminoglycoside resistance in CF lung isolates mediated by PA5471 and MexXY-OprM. In a modest attempt to mimic chronic exposure of *P. aeruginosa* to ROS *in vitro* and assess the impact on *mexXY* and aminoglycoside resistance, various strains of *P. aeruginosa* were exposed to three doses of half the MIC of peroxide daily over 8 days and the impact on aminoglycoside resistance frequency assessed, using amikacin and tobramycin as representative aminoglycosides that are commonly used to treat *P. aeruginosa* CF lung infections (4, 35, 49). Chronic *in vitro* exposure of wild-type *P. aeruginosa* (K767) to peroxide produced a 4-fold increase in amikacin resistance frequency relative to that of an ROS-free control (at 2.5× MIC) (Table 2). Peroxide exposure for 1 day did not promote any increase in amikacin resistance frequency in *P. aeruginosa* K767 (data not shown), indicating that longer-term exposure was necessary for this effect. This peroxide-promoted enhancement of aminoglycoside resistance frequency was dependent on both *mexXY* (amikacin-resistant mutants were not selectable in the Δ*mexXY* strain K1525) and PA5471 (peroxide had no effect on amikacin resistance frequency in the ΔPA5471 strain K2413) (Table 2). This indicated that MexXY was absolutely required for resistance to amikacin at 2.5× MIC and that peroxide-inducible PA5471 was required for peroxide enhancement of the amikacin resistance frequency. Intriguingly, overexpression of PA5471 alone (owing to a chromosomal mutation in the PA5471.1 open reading frame [ORF] upstream of PA5471 [33]) was able to enhance amikacin resistance frequency 8-fold in the absence of peroxide (see K2817 in Table 2). This enhancement of resistance frequency was seen following as little as 16 h of cultivation of K2817 (data not shown). These data suggested that the positive effect of peroxide on amikacin resistance frequency resulted from peroxide promotion of PA5471 expression and not some other impact of peroxide. Similar results were obtained for tobramycin (data not shown).

MexXY-dependent pan-aminoglycoside resistance in peroxide-exposed wild-type *P. aeruginosa*. Ten randomly selected amikacin-resistant peroxide-exposed mutants derived from

TABLE 2. PA5471-dependent peroxide (H_2O_2) enhancement of amikacin resistance frequency in *P. aeruginosa*[a]

Strain	Relevant phenotype	Peroxide	Amikacin resistance frequency	Fold change[d]
K767	Wild type	−	7.8E−6	
		+	2.6E−5	3.3 (4.6 ± 1.5)
K2413	PA547$^-$	−	1.0E−6	
		+	1.0E−6	1.0 (1.2 ± 0.1)
K1525	MexXY$^-$	−	—[c]	
		+	—	
K2415	MexZ$^-$ (MexXY^{++})	−	3.2E−5	
		+	2.7E−4	8.4 (6.7 ± 1.6)
K2416	MexZ$^-$ (MexXY^{++}) PA547$^-$	−	4.3E−5	
		+	5.8E−5	1.3 (1.5 ± 0.1)
K767	PA5471.1$_{WT}$ (PA5471$^+$)[b]	−	6.4E−6	
K2817	PA5471.1$_{Q3Am}$ (PA5471^{++})[b]	−	5.4E−5	8.4 (8.0 ± 0.6)

[a] The indicated *P. aeruginosa* strains were exposed (+) or not (−) to H_2O_2 (1 mM) over 8 days, mutants resistant to amikacin (2.5× MIC for each strain) were selected and enumerated, and the resistance frequency was determined. Results of a representative experiment is shown.

[b] The relative PA5471 level is in parentheses (+, expressed at wild-type levels; ++, hyperexpressed). PA5471 hyperexpression was achieved via introduction of a nonsense mutation (Q3Am) into the PA5471.1 ORF in generating strain K2817. PA5471.1$_{WT}$, wild-type PA5471.1.

[c] —, no mutants capable of growth at 2.5× MIC were recovered.

[d] Except for strains K767 (second entry only) and K2817, the fold change in amikacin resistance frequency in peroxide-treated versus untreated *P. aeruginosa* is shown. For strains K767 (second entry) and K2817, the fold change in amikacin resistance frequency in *P. aeruginosa* hyperexpressing versus not hyperexpressing PA5471 is shown. Numbers in parentheses represent the mean ± standard deviation from three independent experiments.

wild-type *P. aeruginosa* K767 were screened for resistance to additional aminoglycosides. All showed enhanced resistance to the four aminoglycosides tested (Table 3), indicating that amikacin readily selected pan-aminoglycoside-resistant mutants. To assess the involvement of MexXY in this resistance, four mutants were examined for *mexXY* expression using RT-PCR. Two mutants (K2966 and K2968) (Fig. 2, lanes 2 and 4)

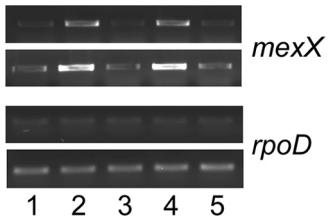

FIG. 2. *mexXY* expression in pan-aminoglycoside-resistant *P. aeruginosa* selected on amikacin following exposure to H_2O_2. Expression was assessed in K767 (lane 1) and four randomly selected amikacin (and pan-aminoglycoside)-resistant mutants (lane 2, K2966; lane 3, K2965; lane 4, K2968; lane 5, K2967) using RT-PCR. Expression of *rpoD* was also assessed and served as an internal control that ensured that equal amounts of RNA were employed in all of the RT-PCRs shown. The PCR portion the RT-PCRs was carried out for 32 (*mexX*) or 20 (*rpoD*) cycles (upper panels) and for 34 (*mexX*) or 22 (*rpoD*) cycles (lower panels).

showed elevated *mexXY* expression relative to that in K767, while two (K2965 and K2967) (Fig. 2, lanes 3 and 5) did not. Neither of the *mexXY*-expressing mutants harbored a mutation in *mexZ*, encoding the repressor of the *mexXY* operon (32), or the *mexZ-mexXY* intergenic region. Consistent with MexXY being responsible for the pan-aminoglycoside resistance of K2966 and K2968, deletion of *mexXY* from these mutants fully reversed resistance, to levels seen for a Δ*mexXY* derivative of K767, K1525 (Table 4). Interestingly, deletion of *mexXY* from K2967 also fully reversed resistance, to levels seen for K1525 (Table 4). Thus, despite no overt increase in *mexXY* expression in this mutant, MexXY-OprM was required for its pan-aminoglycoside-resistant phenotype. No mutations, however, were observed in the *mexXY* genes of K2967 (a missense mutation in *mexY* has previously been linked to a modest [2-fold] increase in aminoglycoside resistance [50]).

PA5471-dependent MexXY-mediated aminoglycoside resistance in peroxide-exposed *P. aeruginosa* and in CF isolates. PA5471 is required for antimicrobial (38) and peroxide (see

TABLE 3. Pan-aminoglycoside resistance of amikacin-resistant mutants of *P. aeruginosa* selected following an 8-day peroxide exposure[a]

Strain[b]	MIC (μg/ml)[c]			
	AMI	TOB	GEN	PAR
K767 (wild type)	4	1	4	256
AMI^r-T1 (K2965)	16	4	8	512
AMI^r-T2	8	2	4	≥2,048
AMI^r-T3	8	2	8	1,024
AMI^r-T4	8	4	8	≥2,048
AMI^r-T5 (K2966)	16	2	8	≥2,048
AMI^r-T6	8	4	8	1,024
AMI^r-T7 (K2967)	8	4	8	1,024
AMI^r-T8	8	2	8	≥2,048
AMI^r-T9	≥16	2	8	≥2,048
AMI^r-T10 (K2968)	8	2	8	2,048

[a] Wild-type *P. aeruginosa* K767 was exposed to peroxide (half the MIC; 1 mM) for 8 days and mutants resistant to 2.5× MIC for amikacin selected and screened for resistance to additional aminoglycosides. Results for 10 randomly selected mutants are shown.

[b] Four mutants that were studied in greater detail are noted with strain designations in parentheses.

[c] AMI, amikacin; TOB, tobramycin; GEN, gentamicin; PAR, paromomycin.

TABLE 4. MexXY-dependent pan-aminoglycoside resistance of amikacin-resistant mutants derived from peroxide-exposed *P. aeruginosa* PAO1 strain K767[a]

Strain	MexXY[b]	MIC (μg/ml)[c]			
		AMI	TOB	GEN	PAR
K767	+	4	1	4	256
K1525	−	1–2	0.5–1	1–2	32
K2965[d]	+	16	4	8	512
K2969	−	4	4	4	32
K2967[d]	+	8	4	8	1,024
K2971	−	2	1	1	32
K2966[d]	+	16	2	8	≥2,048
K2970	−	2	1	1	64
K2968[d]	+	8	2	8	2,048
K2972	−	2	1	1–2	32

[a] The *mexXY* genes were deleted from four representative pan-aminoglycoside-resistant mutants (Table 3) and the impact on aminoglycoside susceptibility assessed. Data for wild-type strain K767 and its ΔPA5471 derivative K2413 are shown for comparison purposes.

[b] MexXY status of the indicated strains. +, present; −, absent owing to deletion.

[c] AMI, amikacin; TOB, tobramycin; GEN, gentamicin; PAR, paromomycin.

[d] Mutant strain selected on amikacin.

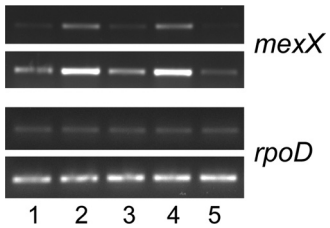

FIG. 3. PA5471-dependent MexXY expression in pan-aminoglycoside-resistant mutants selected following peroxide exposure. Expression of *mexXY* was assessed in K767 (wild type; lane 1), K2966 (pan-aminoglycoside-resistant mutant; lane 2), K2973 (K2966 ΔPA5471; lane 3), K2967 (pan-aminoglycoside-resistant mutant; lane 4), and K2974 (K2967 ΔPA5471; lane 5) using RT-PCR. Expression of *rpoD* was also assessed and served as an internal control that ensured that equal amounts of RNA were employed in all of the RT-PCRs shown. The PCR portion of the RT-PCRs was carried out for 32 (*mexX*) or 20 (*rpoD*) cycles (upper panels) and for 34 (*mexX*) or 22 (*rpoD*) cycles (lower panels).

above) induction of *mexXY* expression. It is unclear, however, whether it is required for *mexXY* expression in pan-aminoglycoside-resistant mutants recovered following peroxide exposure *in vitro* or in isolates recovered from the CF lung. To assess this, PA5471 was deleted from the *mexXY*-expressing pan-aminoglycoside-resistant mutants K2966 and K2968 described above and from several *mexXY*-expressing pan-aminoglycoside-resistant CF isolates described previously (47), and the impact on *mexXY* expression (Fig. 3 and 4) and aminoglycoside resistance (Table 5) was determined. Deletion of PA5471 from K2966 and K2967 abrogated the increased *mexXY* expression of these mutants (Fig. 3, lanes 3 and 5 [cf. lanes 2 and 4]) and concomitantly increased susceptibility to aminoglycosides, though not to the same extent as seen for the Δ*mexXY* derivatives of these strains or for a PA5471 knockout of K767 (K2413) (Table 4). Elimination of PA5471 from the CF isolates reduced *mexXY* expression to some extent in every instance but one (CF isolate K2153) (Fig. 4, compare lanes 3 and 4). Interestingly, elimination of PA5471 in K2153 also had a minimal impact on aminoglycoside susceptibility and much less than was seen when *mexXY* was eliminated from this isolate, in contrast to the case for the other isolates, where loss of PA5471 or *mexXY* had a similar impact (Table 5). Thus,

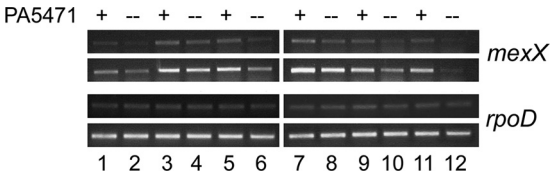

FIG. 4. PA5471-dependent *mexXY* expression in pan-aminoglycoside-resistant CF isolates. Expression of *mexXY* was assessed in CF isolates and their PA5471 deletion derivatives using RT-PCR. Lane 1, K2152; lane 2, K2427 (K2152 ΔPA5471); lane 3, K2153; lane 4, K2428 (K2153ΔPA5471); lane 5, K2160; lane 6, K2430 (K2160 ΔPA5471); lane 7, K2161; lane 8, K2431 (K2161 ΔPA5471); lane 9, K2158; lane 10, K2432 (K2158 ΔPA5471); lane 11, K2163; lane 12, K2433 (K2163 ΔPA5471). Expression of *rpoD* was also assessed and served as an internal control that ensured that equal amounts of RNA were employed in all of the RT-PCRs shown. The PCR portion of the RT-PCRs was carried out for 32 (*mexX*) or 20 (*rpoD*) cycles (upper panels and for 34 (*mexX*) or 22 (*rpoD*) cycles (lower panels).

TABLE 5. PA5471-dependent MexXY-mediated pan-aminoglycoside resistance in peroxide-exposed and CF isolates of *P. aeruginosa*[a]

Strain	PA5471[b]	MIC (μg/ml)[c]			
		AMI	TOB	GEN	PAR
K767	+	4	1	4	256
K2413	−	1–2	1	1–2	16
K2966[d]	+	16	2	8	>2,048
K2973	−	4	1	2	128
K2968[d]	+	8	2	8	2,048
K2975	−	2	1	2	128
K2967[d]	+	8	4	8	1,024
K2974	−	2	1	2	32
K2152[e]	+	16	4	16	512
K2427	−	16 (8)	4 (4)	8 (4)	64 (32)
K2153[e]	+	16	8	16	512
K2428	−	8 (2)	4 (4)	8 (2)	256 (32)
K2160[e]	+	64	32	64	2,048
K2430	−	32 (16)	16 (16)	16 (16)	256 (64)
K216[e]	+	32	16	64	2,048
K2431	−	16 (16)	16 (8)	16 (8)	128 (64)
K2158[e]	+	32	16	32	1,024
K2432	−	8 (8)	8 (8)	8 (8)	128 (64)
K2163[e]	+	16	8	16	128
K2433	−	8 (8)	8 (8)	8 (4)	64 (32)

[a] The PA5471 gene was deleted from *mexXY*-expressing pan-aminoglycoside-resistant (i) mutants selected on amikacin following peroxide exposure and (ii) CF isolates, and the impact on aminoglycoside susceptibility was assessed. Data for wild-type strain K767 and its ΔPA5471 derivative K2413 are shown for comparison purposes.

[b] PA5471 status of the indicated strains. +, present; −, absent owing to deletion.

[c] AMI, amikacin; TOB, tobramycin; GEN, gentamicin; PAR, paromomycin. Numbers in parentheses represent MICs for Δ*mexXY* derivatives of the various CF isolates and have been published previously (47). They are provided to permit comparison with the ΔPA5471 derivatives of those same CF isolates.

[d] Mutant strain selected on amikacin.

[e] Clinical CF isolate.

PA5471 seems to be generally necessary for *mexXY* expression/MexXY-OprM-mediated pan-aminoglycoside resistance in lab-selected and CF isolates. Despite this, none of the aforementioned pan-aminoglycoside-resistant lab or CF isolates showed any increase in PA5471 expression or carried a mutation in the PA5471.1 ORF. This observation that PA5471 is necessary for *mexXY* expression and pan-aminoglycoside resistance is consistent with the observation that none of the lab/CF isolates harbored mutations in *mexZ* or the *mexZ-mexXY* promoter region; such mutations have been shown to yield *mexXY* expression and pan-aminoglycoside resistance independent of PA5471 (34). Indeed, in selecting *mexXY*-expressing pan-aminoglycoside-resistant mutants following peroxide exposure of *P. aeruginosa in vitro*, the only instance where *mexZ* mutants were recovered was when a PA5471 deletion mutant, K2413, was employed (data not shown). This argues that most mutations that yield *mexXY* expression and the attendant pan-aminoglycoside resistance "operate" through PA5471.

DISCUSSION

In vitro exposure to ROS increases the frequency with which aminoglycoside resistant mutants of *P. aeruginosa* are recovered, dependent upon MexXY-OprM and PA5471. It is interesting to note, however, that MexXY-OprM-dependent aminoglycoside resistance does not necessarily follow from increased

mexXY expression and indeed, enhanced *mexXY* expression alone, as seen, for example, in *mexZ* deletion strain K2145, appears to be insufficient for resistance. Thus, additional genes/mutations must operate with/through MexXY-OprM to promote aminoglycoside resistance. In the lab and clinical isolates studied here, this gene(s)/mutation(s) appears to act "through" PA5471, with *mexXY* expression and aminoglycoside resistance being compromised in the absence of this gene. This perhaps is not surprising, given that PA5471 acts naturally to promote *mexXY* expression (34). While *mexXY* expression can occur independently of PA5471 in the case of *mexZ* mutants (34) and indeed *mexXY*-expressing aminoglycoside-resistant *mexZ* mutants were readily selected in this study using the PA5471-deficient mutant strain K2413, such mutants were not recovered in this study from otherwise wild-type cells, and the clinical strains studied here similarly lacked mutations in *mexZ* (47). Presumably, the frequency of mutations that affect *mexXY* expression via PA5471 is substantially higher than the *mexZ* mutation frequency (perhaps owing to the existence of multiple genes whose disruption affects PA5471 and *mexXY* expression). In this regard, and recognizing that PA5471 and *mexXY* are induced in response to ribosome disruption with antimicrobials, it may be that mutation of various genes linked to translation/protein synthesis can upregulate *mexXY* via PA5471. It has been shown, for example, that spontaneous mutations in the *fmt* gene, encoding a methionyl-tRNA-formyltransferase, yield increased PA5471 and *mexXY* expression (6), as does transposon disruption of the *rplY* gene, encoding a probable ribosomal protein, L25 (16). Neither of these genes, however, was mutated in the *in vitro*-selected *mexXY*-expressing pan-aminoglycoside-resistant mutants described in the current study.

While ROS are known to damage DNA and so have the potential to be mutagenic (10), the increased resistance frequency seen for peroxide-treated *P. aeruginosa* is not explainable by ROS-promoted mutagenesis inasmuch as its effect is lost in strains lacking PA5471. The observation, too, that PA5471 hyperexpression in the absence of peroxide provides a similar increase in aminoglycoside resistance frequency argues that ROS increase resistance frequency as a consequence of their positive impact on PA5471 expression. Their enhancement of aminoglycoside resistance frequency is not, however, explainable solely by their positive influence on *mexXY* expression, since this enhancement was also seen in a *mexZ* deletion mutant already hyperexpressing *mexXY*, enhancement which was also PA5471 dependent. Presumably, PA5471 expression provides a selective pressure for mutations that ultimately affect aminoglycoside susceptibility, possibly via its influences on expression of additional genes in *P. aeruginosa*. DNA array studies have, for example, revealed that many genes are influenced, both positively and negatively, by the PA5471 status of the cell (C. Dean, unpublished data).

Aminoglycosides have been and continue to be widely used in treating *P. aeruginosa* lung infections in CF (4, 42) and so undoubtedly provide some selective pressure for the development of MexXY-mediated aminoglycoside resistance. Certainly, *mexXY*-expressing pan-aminoglycoside-resistant mutants could be recovered in the current study from *P. aeruginosa* not exposed to peroxide (data not shown), in agreement with earlier studies (22). Still, this does not explain the general lack of other aminoglycoside resistance mechanisms in CF isolates (19, 45), which should be as readily selected by aminoglycosides. At the very least, ROS in the CF lung may enrich for *mexXY*-expressing mutants that can be selected by aminoglycosides during therapy and may provide selective pressure for maintaining such mutants during periods where antibiotics are not being used.

The positive influence of ROS on *mexXY* expression and MexXY-OprM-dependent aminoglycoside resistance notwithstanding, why both ROS and ribosome-targeting antimicrobials induce *mexXY* expression in *P. aeruginosa* and do so via PA5471 is uncertain. A possible explanation lies in the link between translational (in)fidelity and protein oxidation. It is known, for example, that translational fidelity is reduced in nongrowing senescent bacteria, which thus accumulate abnormal polypeptides that are prone to cell-mediated oxidation/oxidative damage, with oxidation somehow identifying these as candidates for destruction and/or removal (2, 11, 12, 13, 29). Ribosome disruption with antibiotics also leads to accumulation of abnormal polypeptides in bacteria (18, 20, 50), which may similarly be subjected to natural oxidative processes in the cell that target them for destruction or removal (possibly by MexXY-OprM). Indeed, using antibiotics or mutations to compromise ribosome function, the production of aberrant proteins that are subsequently prone to oxidation has been seen in *E. coli* (15). Application of an exogenous oxidative stress (e.g., with peroxide *in vitro* or ROS in the CF lung) will also lead to oxidation of normal polypeptides in bacteria (11, 13, 36), possibly targeting them for destruction and removal via the same mechanism (hence the common recruitment of PA5471 and, possibly, MexXY by ribosome-targeting antibiotics and ROS). It is also possible that ROS, like ribosome-targeting antimicrobials, directly disrupt ribosomes, leading to accumulation of the aberrant polypeptides that may be substrates for PA5471/MexXY. Either way, PA5471/MexXY may contribute to a natural process for removal of abnormal proteins that accumulate in response to aging and environmental stresses (including antibiotics).

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