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Antibiotic Inducibility of the MexXY Multidrug Efflux System of *Pseudomonas aeruginosa*: Involvement of the Antibiotic-Inducible PA5471 Gene Product

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The MexXY components of the MexXY-OprM multidrug efflux system of *Pseudomonas aeruginosa* are encoded by a MexZ repressor-regulated operon that is inducible by antibiotics that target the ribosome. Mutant strains disrupted in a gene, PA5471, were shown to be compromised for drug-inducible *mexXY* expression and, therefore, MexXY-OprM-mediated antimicrobial resistance. The PA5471 gene was inducible by the same ribosome-targeting agents that induce *mexXY* expression. Moreover, vector-driven expression of cloned PA5471 was sufficient to promote *mexXY* expression and MexXY-mediated resistance in the absence of antibiotic exposure, consistent with PA5471 directly or indirectly activating *mexXY* expression following its own upregulation in response to antibiotics. The requirement for PA5471 for *mexXY* expression and antimicrobial resistance was, however, obviated in mutants lacking the MexZ repressor of *mexXY* expression, suggesting that PA5471 directly or indirectly modulates MexZ activity in effecting *mexXY* expression. While the recruitment of PA5471 and MexXY in response to ribosome disruption by antimicrobials is consistent with their genes playing a role in protecting cells from the adverse consequences of disrupting the translation process, reminiscent of *trans*-translation, these genes appear to operate independently in their contribution to resistance: mutants defective in *trans*-translation showed a much more modest (twofold) decrease in resistance to ribosome-targeting agents than those lacking PA5471 or MexXY, and this decrease was observed whether functional PA5471/MexXY was present or not.

Multidrug efflux systems of the resistance-nodulation-division (RND) family contribute significantly to intrinsic and acquired resistance to antimicrobials in a number of gram-negative bacteria (43, 45). Despite their significance as determinants of antibiotic resistance, however, RND-type multidrug exporters also, in many instances, accommodate biocides (42, 45), organic solvents (48), detergents (43), including bile salts (9, 18, 46, 60), toxic fatty acids/lipids (54), and in some instances, plant-derived antimicrobials (phytoalexins and isoflavonoids) (7, 39), metabolic inhibitors (52), organometallic compounds (tributyltin) (25), quorum-sensing effector molecules (13, 26, 40), and, possibly, virulence factors (21) in addition to antibiotics. Clearly, RND pumps can and do function as other than antibiotic exporters.

Pseudomonas aeruginosa expresses several three-component RND-type multidrug efflux systems, among 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 (41, 44). A clear indication, however, that antimicrobial export may not be the intended function of many of these systems comes from the observation that while these pumps accommodate many of the same antimicrobials, each appears to be independently regulated by linked regulatory genes (44), but not (with the exception of MexXY [30]) in response to antibiotics.

The MexXY-OprM system is unique in *P. aeruginosa* in that the *mexXY* operon is induced by exposure to many of the antibiotics that this efflux system exports (30). While this is consistent with efflux of these agents being the intended function of the MexXY-OprM system, it is interesting that not all antibiotic substrates, but only those agents known to target the ribosome, induce *mexXY* expression (23). Moreover, in contrast to other drug-inducible multidrug efflux systems (e.g., QacA, an MF family exporter in *Staphylococcus aureus*), where drug binding to the cognate regulator (i.e., QacR) alleviates repression of the efflux gene (i.e., *qacA*) (16), providing some support for these systems as intended determinants of drug efflux, MexXY antimicrobial substrates that induce *mexXY* expression do not interact with or directly modulate the activity of the *mexXY* repressor, MexZ (32). Also, the observation that ribosome protection mechanisms compromise drug-inducible *mexXY* expression (23) supports this efflux system being recruited in response to ribosome disruption and not to antibiotics per se. One possibility is that the action of these agents on their ribosomal targets induces the expression of MexXY-OprM in order to counter/alleviate some stress or adverse effect resultant from ribosome disruption. Certainly, transcriptomic and proteomic studies confirm that agents that interfere with prokaryotic translation impact the expression of a myriad of genes (1, 5, 14, 27, 38, 47, 50, 55), in some instances including genes associated with stress responses (27, 38, 47, 50). In an effort to define MexXY's role in *P. aeruginosa*'s response to translation inhibition, attempts were made to identify additional genes involved in MexXY-dependent antibiotic resistance by screening a transposon insertion mutant library for mutants compromised for resistance to MexXY substrate antibiotics. We report here the identification of a gene, PA5471,

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TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Relevant characteristics[a]	Reference
E. coli strains		
DH5α	ϕ80d *lacZΔM15 endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* F^- Δ(*lacZYA-argF*)*U169*	4
S17-1	*thi pro hsdR recA* Tra^+	56
KAM3	Δ*acrB* Δ(*lac-pro*) *supE thi hsd*Δ*5*/F′ *traD36 proA*$^+$ *B*$^+$ *lacI*^{q} *lacZ* Δ*M15*	36
P. aeruginosa strains		
K767	PAO1, wild type	29
K1525	K767 Δ*mexXY*	58
K2439	K1525 Δ*ssrA*	This study
K2440	K1525 Δ*smpB*	This study
K2413	K767 ΔPA5471	This study
K2414	K767 Δ*mexXY* ΔPA5471	This study
K2415	K767 Δ*mexZ*	This study
K2416	K767 Δ*mexZ* ΔPA5471	This study
K2417	K767 ΔPA5471-PA5470	This study
K2437	K767 Δ*ssrA*	This study
K2438	K767 Δ*smpB*	This study
K2162	Clinical isolate displaying MexXY-dependent pan-aminoglycoside resistance	58
K2418	K2162 ΔPA5471	This study
K2435	K2162 PA5471::mini-Tn*5*–*tet*	This study
K2436	K2162 PA5471::mini-Tn*5*–*tet*	This study
YM34	PAO1 Δ*mexAB* Δ*mexCD-oprJ* Δ*mexEF-oprN*	37
YM44	YM34 Δ*mexXY*	37
Plasmids		
pEX18Tc	Broad-host-range gene replacement vector; *sacB* Tc^r	22
pYM008	pEX18Tc::ΔPA5471	This study
pYM015	pEX18Tc::ΔPA5471-PA5470	This study
pYM021	pEX18Tc::Δ*mexZ*	This study
pYM022	pEX18Tc::Δ*ssrA*	This study
pYM023	pEX18Tc::Δ*smpB*	This study
pUCP20T	Broad-host-range cloning vector; Mob^+ Ap^r Cb^r	53
pYM010	pUCP20T::PA5471	This study
pYM013	pUCP20T::PA5471-PA5470	This study
pYM017	pUCP20T::*mexZ*	This study
pBC KS(+)	Phagemid cloning vector; Cm^r	Stratagene
pTEM4	pBR322::*mexXY*; Ap^r	35
pMMB190	Broad-host-range, low-copy-number cloning vector; *lacI*^{q} Ap^r Cb^r	34
pYM004	pMMB190::*mexXY*	This study

[a] Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance; Cm^r, chloramphenicol resistance.

which, like *mexXY*, is drug inducible and is required for drug-inducible *mexXY* expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used for this study are listed in Table 1. Bacterial cells were cultured in Luria broth (L broth) and on Luria agar (8) with antibiotics, as necessary, at 37°C. Plasmid pEX18Tc and its derivatives were maintained in *Escherichia coli* with 10 μg/ml of tetracycline. Plasmids pUCP20T and pMMB190 and their derivatives were maintained in *E. coli* with 100 μg/ml ampicillin and in *P. aeruginosa* PAO1 strain K767 and its derivatives with 200 μg/ml carbenicillin.

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 (51). Plasmid DNAs were also prepared from *E. coli* or *P. aeruginosa* using a QIAprep Spin miniprep kit or QIAfilter Plasmid Midi kit (QIAGEN Inc., Mississauga, Ontario, Canada) according to the protocols provided by the manufacturer. Genomic DNA of *P. aeruginosa* was extracted following the protocol of Barcak et al. (6). DNA fragments used for cloning were extracted from agarose gels using a QIAquick gel extraction kit (QIAGEN). PCR products were purified using a QIAquick PCR purification kit (QIAGEN) and, when cloned, sequenced to verify that no mutations were introduced during PCR. Competent *P. aeruginosa* (10) and *E. coli* (51) cells were prepared as described previously. Chromosomal DNA flanking the mini-Tn*5*–*tet* element in aminoglycoside-susceptible K2162 insertion mutants was sequenced using the primer mini-Tn*5*-Right (8). Oligonucleotide synthesis was carried out by Cortec DNA Services (Kingston, Ontario, Canada), and nucleotide sequencing was carried out by ACGT Corp. (Toronto, Ontario, Canada). Once the flanking DNA sequences were obtained, disrupted genes were identified by BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) searches of the available *P. aeruginosa* genome sequence (59; http://www.pseudomonas.com).

Transposon insertion mutagenesis. *P. aeruginosa* strain K2162, a MexXY-expressing, pan-aminoglycoside-resistant clinical isolate of *P. aeruginosa* (Table 1), was mutagenized with mini-Tn*5*–*tet* (12) as described previously (8), with mini-Tn*5*–*tet*-carrying K2162 mutants selected on L agar containing tetracycline (64 μg/ml) and imipenem (0.5 μg/ml; to counterselect donor *E. coli* used to mobilize plasmid-borne mini-Tn*5*–*tet* into K2162). Mutants showing increased aminoglycoside susceptibilities were screened initially for lack of growth on L agar containing either paromomycin (1,024 μg/ml) or spectinomycin (256 μg/ml) and later for increased susceptibilities to multiple aminoglycosides using a broth assay (see below).

RT-PCR. Total bacterial RNAs were isolated from log-phase *P. aeruginosa* L broth cultures (with and without subinhibitory concentrations of antibiotics, as follows: kanamycin, cefotaxime, and norfloxacin at one-fourth the MIC and erythromycin, tetracycline, and chloramphenicol at one-eighth the MIC), using a QIAGEN RNeasy mini kit, RNase-free DNase (QIAGEN), and a protocol provided by the manufacturer. Reverse transcriptase PCR (RT-PCR) was performed with ca. 500 ng RNA and primer pairs internal to *rpoD*, *mexX*, PA5471, PA5470, and *mexZ* (Table 2), using a QIAGEN One Step RT-PCR kit according to a protocol provided by the manufacturer. To assess whether PA5470 and

TABLE 2. Oligonucleotides used for this study

Primer	Sequence (5′→3′)[a]	Purpose
RT-rpoD-F	GATCCGGAACAGGTGGAAGAC	RT-PCR of *rpoD*
RT-rpoD-R	TCAGCAGTTCCACGGTACCC	RT-PCR of *rpoD*
RT-mexX-F	CATCAGCGAACGCGAGTACAC	RT-PCR of *mexX*
RT-mexX-R	CAATTCGCGATGCGGATTG	RT-PCR of *mexX*
RT-PA5471-F	ACAGCACCTGGATCGAAGGC	RT-PCR of PA5471
RT-PA5471-R	TTCGATGCAGTCGCTCCAG	RT-PCR of PA5471
RT-PA5470-F	GATCCTGCTGCAACTCTCCG	RT-PCR of PA5470
RT-PA5470-R	ACCAGTTCTTGCGCGCAT	RT-PCR of PA5470
RT-mexZ-F	AAACCCGCGACGGCATACT	RT-PCR of *mexZ*
RT-mexZ-R	ACTGGCGGAGAAAGCCCAT	RT-PCR of *mexZ*
E-PA5471-F	GCTAGAATTCGATCTACCGTTTCAATCACATGGAT; EcoRI	PCR cloning of PA5471
X-PA5471-R	GATCTCTAGAGGCCACCTCCTCGATTACCT; XbaI	PCR cloning of PA5471
EH-mexX-F	GAATTCAAGCTTCAAGCTCGCGAGTTCACGA; EcoRI, HindIII	PCR cloning of *mexXY*
N-mexX-R	ACGTTGGACGAGGCGATCTC	PCR cloning of *mexXY*
mexZ-F	TCGTGAACTCGCGAGCTTG	PCR cloning of *mexZ*
mexZ-R	CACATCAGCGAGGAAGACGC	PCR cloning of *mexZ*
PA5471DU-F	GATCAAGCTTCCTGGGAAGGCTATACCAACG[b]	Deletion of PA5471; upstream fragment
PA5471DU-R	GCTAGGTACCGCCCATAATCCAATCCATGTG[b]; KpnI	Deletion of PA5471; upstream fragment
PA5471DD-F	GCTAGGTACCCGGAAGCCGGTGCTGACCTACA; KpnI	Deletion of PA5471; downstream fragment
PA5471DD-R	GCTAGAATTCGCTTCATCGGCACCATCAT; EcoRI	Deletion of PA5471; downstream fragment
XPA5470-F	GATCTCTAGATGCTCGACATCAACCACAACC	Deletion of PA5471-PA5470
PA5470-DDR	GCTAGAATTCCCAACGACGCCTTCTACTAC; EcoRI	Deletion of PA5471-PA5470
mexZDU-F	GATCAAGCTTAATTCGCGATGCGGATTG; HindIII	Deletion of *mexZ*; upstream fragment
mexZDU-R	GATCTCTAGACACTGAACGTCCTCACAAGGG; XbaI	Deletion of *mexZ*; upstream fragment
mexZDD-F	GATCTCTAGACGCAGTTCTCCCTCCTGTTG; XbaI	Deletion of *mexZ*; downstream fragment
mexZDD-R	GCTAGAATTCGAAGGAAATCTTGGTGGCGA; EcoRI	Deletion of *mexZ*; downstream fragment
PA0826.2DU-F	GATCAAGCTTTCGAATACCGCCTGCAAGC; HindIII	Deletion of *ssrA*; upstream fragment
PA0826.2DU-R	CTAGTCTAGACCGGCGTCGAATCCTAATC; XbaI	Deletion of *ssrA*; upstream fragment
PA0826.2DD-F	CTAGTCTAGAGCATGTAGAACCGATAGCGGA; XbaI	Deletion of *ssrA*; downstream fragment
PA0826.2DD-R	GCTAGGTACCCAGTCCTTCCTGGCGGCTAT; KpnI	Deletion of *ssrA*; downstream fragment
PA4768DU-F	GCTAGAATTCGCGGTAGATCTCCACCCGTT; EcoRI	Deletion of *smpB*; upstream fragment
PA4768DU-R	GATCTCTAGAGACCATAGGCGGCGCATTATAG; XbaI	Deletion of *smpB*; upstream fragment
PA4768DD-F	GATCTCTAGAGACTTCGACAAGCGCCACAC; XbaI	Deletion of *smpB*; downstream fragment
PA4768DD-R	GATCAAGCTTAGAGGCTTTGCGACGAAACTT; HindIII	Deletion of *smpB*; downstream fragment

[a] In some instances, restriction sites were introduced into oligonucleotides to be used for PCR, and these are underlined in the sequences, with the corresponding restriction endonucleases indicated.

[b] The PCR product amplified with these primers and cloned into pCR-BluntII-TOPO was excised following PstI-KpnI digestion (a PstI site is present within the pCR-BluntII-TOPO multicloning site) prior to cloning into pEX18Tc.

PA5471 were expressed from a polycistronic message, the primer pair PA5471-F and PA5470-R (Table 2) was used. RT-free (i.e., PCR) controls were carried out to ensure that there was no DNA contamination of RNA preparations.

Cloning of PA5471 and PA5470. The PA5471 gene was amplified using primers EPA5471-F and XPA5471-R (Table 2) in a 50-μl PCR mixture containing 10 ng of chromosomal DNA, a 0.6 μM concentration of each primer, a 0.2 mM concentration of each deoxynucleoside triphosphate, 1 mM $MgSO_4$, 1 U of KOD Hot Start DNA polymerase (EMD Biosciences, Inc., Madison, WI), 1× KOD Hot Start polymerase buffer, and 4.0% (vol/vol) dimethyl sulfoxide. The mixture was heated for 2 min at 94°C, followed by 35 cycles of 0.25 min at 94°C, 0.5 min at 60°C, and 2 min at 68°C and a final step of 10 min at 68°C. The PA5471-containing PCR product was cloned into pSportI (Invitrogen, Carlsbad, CA), released by digestion with EcoRI and BamHI, and cloned into pUCP20T to yield pYM010. To clone the PA5471-PA5470 operon into pUCP20T, PA5470 was excised from pCR-PA5470+DD (see below) following digestion with XbaI and BamHI, cloned into pSportI, and subsequently released from this vector by BsiWI-BamHI digestion. This fragment and a PA5471-containing fragment released from pSportI (see above) following digestion with EcoRI and BsiWI were then jointly cloned into pUCP20T to yield pYM013.

Cloning of *mexXY*. The *mexX* gene was amplified with primers EHmexX-F and NmexX-R (Table 2) from plasmid pTEM4 (1 ng). The reaction mixture was formulated as described above for the amplification of PA5471, using the same parameters. The blunt-ended PCR fragment was first cloned into plasmid pCR-BluntII-TOPO (Invitrogen, Carlsbad, CA) before being released as an EcoRI-NotI fragment and cloned into pSportI. The *mexY* gene was subsequently excised from pTEM4 via digestion with NotI and BamHI and cloned into *mexX*-carrying pSportI, after which the *mexXY* gene pair was released via digestion with EcoRI and cloned into pMMB190 to yield pYM004.

Cloning of *mexZ*. The *mexZ* gene was amplified with primers mexZ-F and mexZ-R (Table 2) in a reaction mixture formulated as described above for PA5471, with the exception that dimethyl sulfoxide was included at 4.0% (vol/vol) and Vent DNA polymerase (2 U; New England Biolabs, Ltd., Pickering, Ontario, Canada) in 1× ThermoPol buffer (New England Biolabs, Ltd., Pickering, Ontario, Canada) replaced the KOD enzyme. Reaction mixtures were heated for 3 min at 94°C, followed by 35 cycles of 0.5 min at 94°C, 0.75 min at 65°C, and 1 min at 72°C and a final 10-min elongation at 72°C. The *mexZ*-containing PCR product was first cloned into pCR-BluntII-TOPO as described above, excised from this vector using HindIII and XbaI, and then cloned into pUCP20T, yielding pYM017.

Construction of gene deletions in *P. aeruginosa*. To introduce in-frame gene deletions into strains of *P. aeruginosa*, deletion constructs were first prepared in plasmid pEX18Tc by cloning PCR-amplified 1-kb DNA fragments corresponding to the regions upstream and downstream of the gene sequences to be deleted. Typically, these were amplified from the chromosome of *P. aeruginosa* PAO1 strain K767 and first cloned individually into pCR-BluntII-TOPO, from which they were sequenced to verify the absence of PCR-introduced mutations before being excised following restriction digestion (PCR primers were tagged with restriction sites [Table 2]) and sequentially cloned into pEX18Tc. While the upstream fragment used for construction of the PA5471 deletion served for construction of a ΔPA5471-PA5470 double deletion, attempts to amplify sequences 3′ of PA5470 failed to yield a correct product. Thus, the PA5470 gene together with sequences ca. 1 kb downstream of it were amplified using primers XPA5470-F and PA5470DD-R (Table 2) and cloned into pCR-BluntII-TOPO, and the PA5470 downstream fragment was then excised from this vector (pCR-PA5470+DD) via digestion with KpnI and EcoRI for cloning into pEX18Tc. PCR mixtures were formulated as described above for the amplification of *mexZ*

TABLE 3. Involvement of PA5471 in antimicrobial resistance in *P. aeruginosa*

Strain	Relevant phenotype	MIC (μg/ml)[a]													
		Ami	Gen	Kan	Neo	Par	Spc	Str	Tob	Cef	Cam	Car	Ery	Nor	Tet
K2162	WT	128	256	1,024	512	>2,048	1,024	256	128	64	256	512	512	1	16
K2435	PA5471$^-$	—	32	—	—	256	128	32	—	—	—	512	—	—	—
K2436	PA5471$^-$	—	32	—	—	256	128	32	—	—	—	512	—	—	—
K2418	PA5471$^-$	32	64	512	256	512	128	64	64	64	256	512	512	1	16
K767	WT	4	8	128	64	256	512	32	4	16	32	32	512	0.5	16
K2413	PA5471$^-$	2	4	64	64	64	128	8	4	16	32	32	128	0.5	8
K1525	MexXY$^-$	2	4	64	32	32	64	4	4	16	32	32	64	0.5	8
K2414	PA5471$^-$ MexXY$^-$	2	4	64	32	32	64	4	4	16	32	32	64	0.5	8
K2413(pUCP20T)	PA5471$^-$	2	4	64	64	64	128	8	4	8	32	—	128	0.5	8
K2413(pYM010)	PA5471$^+$	8	8	128	128	512	512	64	4	8	32	—	512	1	8
K2413(pMMB190)	PA5471$^-$	2	4	64	64	64	128	8	4	8	32	—	128	0.5	8
K2413(pYM004)	PA5471$^-$ MexXY^{++}	8	8	128	128	512	512	64	4	8	32	—	512	1	8
K2417(pUCP20T)	PA5471$^-$ PA5470$^-$	2	2	64	64	64	128	8	2	16	32	—	128	0.5	8
K2417(pYM010)	PA5471$^+$ PA5470$^-$	8	8	256	128	512	1,024	64	4	16	32	—	512	1	16

[a] Ami, amikacin; Gen, gentamicin; Kan, kanamycin; Neo, neomycin; Par, paromomycin; Spc, spectinomycin; Str, streptomycin; Tob, tobramycin; Cef, cefotaxime; Cam, chloramphenicol; Car, carbenicillin; Ery, erythromycin; Nor, norfloxacin; Tet, tetracycline. —, not determined.

and heated for 3 min at 95°C, followed by 35 (ΔPA5471, ΔPA547-PA5470, and Δ*mexZ*) or 40 (Δ*ssrA* and Δ*smpB*) cycles of 0.5 min at 95°C, 0.75 min at 60°C, and 2 min at 72°C and a final step of 10 min at 72°C. The resulting deletions lacked all but the first 2 (including the ATG start) and last 14 codons (ΔPA5471), all but the GTG start codon and the last 11 codons (Δ*mexZ*), all but the first 25 and last 61 bp (tmRNA gene *ssrA*), and all but the start codon and the last 27 codons (Δ*smpB*). The ΔPA547-PA5470 construct lacked all but the first two codons of PA5471 and all of the PA5470 codons.

The deletion-carrying pEX18Tc derivatives were mobilized into *P. aeruginosa* from *E. coli* S17-1 (8). Briefly, 100 μl of log-phase *E. coli* S17-1 cultured in tetracycline-containing (10 μg/ml) L broth was transferred to L agar plates and immediately overlaid with an equal volume of a log-phase L broth culture of *P. aeruginosa*. Following incubation at 37°C for 18 h, the bacterial cells were resuspended in 1 ml of 0.85% NaCl and diluted 10-fold before being plated onto L agar plates containing tetracycline (75 μg/ml) and chloramphenicol (5 μg/ml; to counterselect *E. coli* S17-1). *P. aeruginosa* transconjugants harboring chromosomal inserts of the plasmid were recovered from these plates and streaked onto L agar containing sucrose (10% [wt/vol]). Sucrose-resistant colonies were then screened for the appropriate deletion using colony PCR (49).

Antimicrobial susceptibility testing. The antimicrobial susceptibilities of the various *P. aeruginosa* strains were assessed in microtiter plates by a twofold serial dilution technique (24). In some experiments, $MgCl_2$ was included in the growth medium (5 mM) since this appears to enhance MexXY-mediated antimicrobial resistance (28).

RESULTS

Involvement of PA5471 in MexXY-mediated antimicrobial resistance. Inducible (by antibiotics) (23, 30) and mutational (23, 58, 64) up-regulation of MexXY is associated with resistance to multiple antimicrobials in *P. aeruginosa*, although the details of MexXY expression in each instance remain obscure. A recent paper, however, highlights the significance of drug-ribosome interactions in ultimately stimulating *mexXY* expression (23), a finding consistent with earlier observations that while MexXY accommodates and thus provides resistance to a variety of antimicrobials (30, 31), only those targeting the ribosome (e.g., aminoglycosides) actually induce *mexXY* expression (23). To gain some insights into the details of drug-inducible *mexXY* expression, including the identity of any additional gene(s) needed for this, a *P. aeruginosa* clinical strain in which MexXY is expressed and implicated in antimicrobial resistance (i.e., K2162) (58) was subjected to random transposon insertion mutagenesis (with mini-Tn*5*–*tet*) and screened for a loss of resistance to representative MexXY antimicrobial substrates. A library of mini-Tn*5*–*tet* mutants of K2162 was thus constructed, and mutants initially showing enhanced susceptibility to the aminoglycosides paromomycin and streptomycin (good MexXY substrates [58]) were selected. Subsequent screening for increased susceptibilities to the aminoglycosides spectinomycin and gentamicin, but not to antimicrobials known not to be MexXY substrates (e.g., carbenicillin and imipenem), identified seven mutants with generalized increased susceptibilities to aminoglycosides. Of these, only two (K2435 and K2436; see Table 3, for susceptibility data) lacked mini-Tn*5*–*tet* insertions in *mexXY*. Cloning and sequencing of the disrupted genes in each instance revealed that the mini-Tn*5*–*tet* element had inserted in the putative promoter region of (K2435) or within (K2436) an opening reading frame dubbed PA5471 by the Pseudomonas Genome Project (http://www.pseudomonas.com). PA5471 encodes a predicted product of 43,508 Da identified as a conserved hypothetical protein and a member of the UPF0027 uncharacterized protein family (Protein Families Database of Alignments and HMMS accession number pfam01139 [http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF01139]), which has numerous members broadly distributed among bacteria (gram-positive and gram-negative) and archaea.

The creation of an in-frame deletion of PA5471 in K2162 also compromised resistance to a variety of aminoglycosides, but not to MexXY antimicrobial substrates known not to induce this efflux system (e.g., cefotaxime and carbenicillin; see strain K2418 in Table 3), confirming the contribution of this gene to pan-aminoglycoside resistance. The elimination of PA5471 in wild-type PAO1 strain K767 also increased the susceptibilities to aminoglycosides as well as other ribosome-targeting agents, such as erythromycin and tetracycline (see strain K2413 in Table 3), reminiscent of the impact of a *mexXY* deletion on resistance in this strain (see strain K1525 in Table 3). These data suggested that PA5471 plays a role in MexXY-mediated antimicrobial resistance, and consistent with this, deletion of PA5471 in strains already lacking MexXY had no impact on antimicrobial resistance (Table 3, compare K2414 with K1525). As expected, the cloned PA5471 gene (on plas-

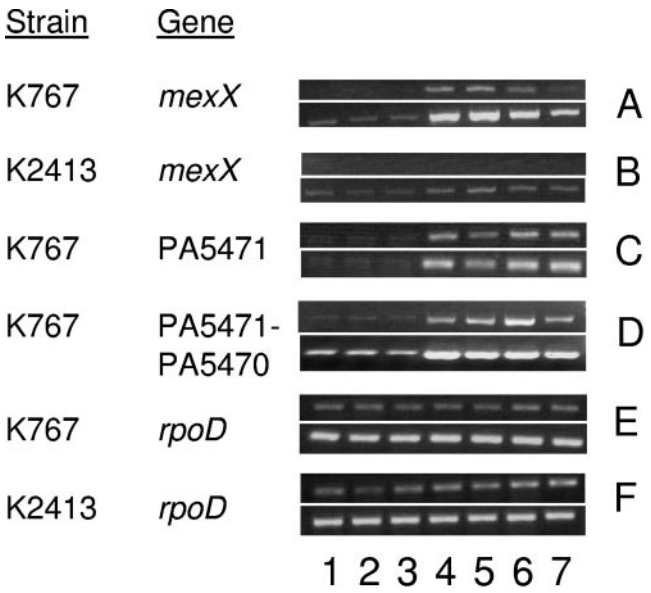

FIG. 1. Requirement for PA5471 for antibiotic-inducible *mexXY* expression. The expression of *mexX*, PA5471, and *rpoD* was assessed in *P. aeruginosa* strains K767 and K2413 (K767 ΔPA5471) grown without antibiotics (lane 1) or with norfloxacin (lane 2), cefotaxime (lane 3), chloramphenicol (lane 4), erythromycin (lane 5), tetracycline (lane 6), and kanamycin (lane 7) by semiquantitative RT-PCR. The *rpoD* reaction 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 (top panel) and 30 (bottom panel) cycles for *mexX*, 19 (top panel) and 21 (bottom panel) cycles for PA5471 and *rpoD*, and 31 (top panel) and 33 (bottom panel) cycles for PA5471-PA5470. Data are representative of two or three replicates.

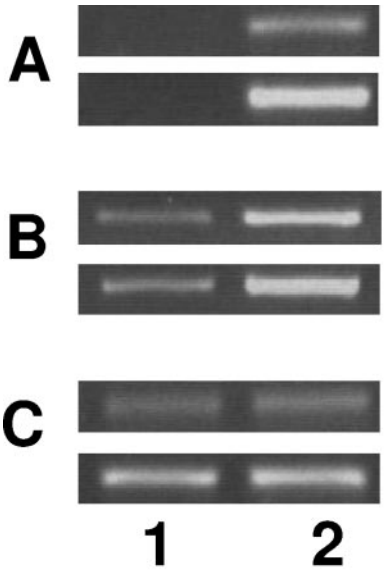

FIG. 2. PA5471 stimulates *mexXY* expression in the absence of antibiotics. The expression of PA5471 (A), *mexX* (B), and *rpoD* (C) was assessed in *P. aeruginosa* strain K767 carrying pUCP20T (lane 1) or pYM010 (pUCP20T::PA5471), using semiquantitative RT-PCR. The *rpoD* reaction served as an internal 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 15 (top panel) and 17 (bottom panel) cycles for PA5471, 19 (top panel) and 21 (bottom panel) cycles for *rpoD*, and 30 (top panel) and 32 (bottom panel) cycles for *mexX*. Data are representative of two or three replicates.

mid pYM010) restored antimicrobial resistance in the PA5471 deletion strain K2413 (Table 3).

Requirement for PA5471 for drug-inducible *mexXY* expression. One way in which PA5471 could contribute to MexXY-mediated antimicrobial resistance is via involvement in the process of drug induction of *mexXY* expression. Indeed, the observation that expression of the cloned *mexXY* genes from a vector-borne promoter on plasmid pYM004 was sufficient to reverse the drug susceptibility of the PA5471 deletion in strain K2413 (Table 3) is consistent with PA5471 being required only for the expression of *mexXY*. To assess a contribution of PA5471 to drug-inducible expression of *mexXY*, the impact of PA5471 loss on *mexX* (as a measure of *mexXY*) gene expression was examined. As expected, agents that target the ribosome, including chloramphenicol, tetracycline, erythromycin, and kanamycin, induced the expression of *mexXY* in log-phase cells of *P. aeruginosa* strain K767 (Fig. 1A, cf. lanes 4 to 7 and lane 1), while those that do not (e.g., norfloxacin and cefotaxime, a fluoroquinolone and a β-lactam, respectively) did not (Fig. 1A, lanes 2 and 3). Elimination of PA5471 in K767, however, severely compromised drug-inducible *mexXY* expression (Fig. 1B, lanes 4 to 7; compare with Fig. 1A, lanes 4 to 7). The *mexXY* message was still detectable in these mutants (Fig. 1B, lower panel), at levels comparable to or minimally above that seen in cells not exposed to antibiotics (Fig. 1A and B, lanes 1), consistent with PA5471 having a specific involvement in drug induction of *mexXY* expression. The fact that the PA5471 mutant K2413 still expressed some *mexXY* was also consistent with observations that it was more resistant to some MexXY antimicrobials than was the Δ*mexXY* knockout K1525 (Table 3). As expected, given its involvement in drug induction of *mexXY* expression, the expression of PA5471 was stimulated in log-phase cells of K767 (Fig. 1C, cf. lanes 4 to 7 and lane 1) by the same ribosome-targeting antimicrobials that induce *mexXY*, but not by agents that do not induce *mexXY* expression (Fig. 1C, lanes 2 and 3).

PA5471, but not PA5470, is required for *mexXY* expression. Examination of the *P. aeruginosa* genome reveals that PA5471 occurs upstream of and in a possible operon with an open reading frame dubbed PA5470. PA5470 is predicted to encode a peptide chain release factor of 22,282 Da (http://www.pseudomonas.com). RT-PCR confirmed both the drug inducibility of PA5470 and its expression from a polycistronic message that also contains PA5471 (Fig. 1D, lanes 4 to 7). Still, the observation that resistance to MexXY antimicrobial substrates was restored with the cloned PA5471 gene alone (on plasmid pYM010) in a PA5471-PA5470 double deletion mutant (K2417) (Table 3) suggested that PA5471 alone was needed for drug-inducible *mexXY* expression. Moreover, expression of the cloned PA5471 gene from a vector-borne promoter on plasmid pYM010 (Fig. 2A, lane 2) stimulated *mexXY* expression in strain K767 in the absence of antibiotic (Fig. 2B, cf. lane 2 and lane 1), indicating that drug induction of *mexXY* is a consequence, directly or indirectly, of PA5471 upregulation in response to antibiotic exposure. The cloned PA5471 gene also promoted resistance to norfloxacin, a noninducing MexXY

TABLE 4. Influence of plasmid-expressed PA5471 on norfloxacin resistance in *P. aeruginosa*

Strain	Relevant property	MIC (μg/ml) of norfloxacin[a]	
		$-Mg^{2+}$	$+Mg^{2+}$
YM34(pUCP20T)[b]	MexXY$^+$	0.25	2
YM34(pYM010)	MexXY$^+$ PA5471^{++c}	0.5	8
YM44(pUCP20T)	MexXY$^-$	0.12	0.25
YM44(pYM010)	MexXY$^-$ PA5471^{++c}	0.12	0.25

[a] Norfloxacin MICs were determined in the presence and absence of 5 mM $MgCl_2$. Mg^{2+} enhances MexXY-mediated antimicrobial resistance, possibly by enhancing the activity of this efflux system (28).
[b] Lacks MexAB-OprM, MexCD-OprJ, and MexEF-OprN.
[c] PA5471 was overexpressed from plasmid pYM010.

substrate (Fig. 1A, lane 2), in MexXY$^+$ (i.e., YM34) but not MexXY$^-$ (i.e., YM44) *P. aeruginosa* (Table 4), consistent with PA5471 positively affecting *mexXY* expression.

PA5471 acts via the MexZ repressor in mediating drug-inducible *mexXY* expression. The gene *mexZ* occurs upstream of *mexXY* and encodes a repressor of *mexXY* expression (3, 32). While MexZ does not directly mediate the drug inducibility of *mexXY* (i.e., antibiotics do not bind to MexZ and modulate its repressor activity [32]), it may respond to PA5471 or the activity of this protein in ultimately effecting drug-inducible *mexXY* expression. To address this possibility, the impact of PA5471 loss on antimicrobial resistance and *mexXY* expression in a strain carrying a *mexZ* deletion was assessed. If PA5471 should act, directly or indirectly, to modulate MexZ repressor activity, such that PA5471 expression in response to antimicrobials leads to derepression of *mexXY*, then the loss of PA5471 in a mutant already lacking *mexZ* should have no adverse impact on *mexXY* expression or resistance. Conversely, and in light of previous observations that *mexZ* knockouts do not demonstrate maximal *mexXY* expression (23), if drug-inducible/PA5471-dependent *mexXY* expression is independent of MexZ, then the loss of PA5471 would compromise drug-inducible *mexXY* expression and thus resistance, even in a *mexZ* mutant expected to already demonstrate an increase in *mexXY* expression and resistance relative to its MexZ$^+$ counterpart (i.e., increased *mexXY* expression in a *mexZ* knockout would not mask an additional contribution of PA5471 and thus a negative impact of PA5471 loss on *mexXY* expression and resistance). As expected, the loss of *mexZ* (in K767 derivative K2415) increased the resistance to multiple antimicrobials, though only modestly (Table 5), consistent with the increase in *mexXY* expression seen in this mutant even without antibiotic

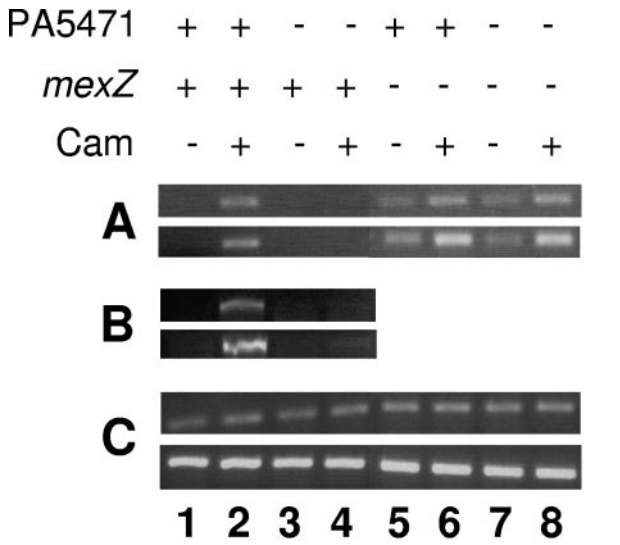

FIG. 3. Requirement for PA5471 for drug-inducible *mexXY* expression in MexZ$^+$ but not MexZ$^-$ *P. aeruginosa*. The expression of *mexX* (A), *mexZ* (B), and *rpoD* (C) was assessed in strain K767 (PA5471$^+$ MexZ$^+$) and its derivatives K2413 (PA5471$^-$ MexZ$^+$), K2415 (PA5471$^+$ MexZ$^-$), and K2416 (PA5471$^-$ MexZ$^-$) grown without (−) and with (+) chloramphenicol (Cam; 8 μg/ml), using semiquantitative RT-PCR. The *rpoD* reaction 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 (*mexX*), 40 (*mexZ*), or 20 (*rpoD*) cycles (top) and 30 (*mexX*), 42 (*mexZ*), or 22 (*rpoD*) cycles (bottom). Data are representative of two or three replicates.

(e.g., chloramphenicol) induction (Fig. 3A, cf. lane 5 and lane 1). Indeed, without drug exposure, the *mexZ* deletion mutant K2415 expressed this efflux system at levels comparable to that seen for the drug-exposed MexZ$^+$ parental strain K767 (Fig. 3A, compare lanes 2 and 5). In contrast to the adverse impact of a PA5471 deletion on the resistance of otherwise wild-type cells (Table 3), however, the loss of PA5471 in the *mexZ* deletion strain K2415 had no effect on resistance (Table 5, compare strains K2416 and K2415). Consistent with this, *mexXY* expression in the *mexZ* mutant strain was not adversely impacted by the loss of PA5471 (Fig. 3A, cf. lane 7 and lane 5).

Intriguingly, drug (chloramphenicol)-exposed K2415 (K767 Δ*mexZ*) still showed some increase in *mexXY* expression (Fig. 3A, cf. lane 6 and lane 5), and this drug inducibility of *mexXY* in the absence of *mexZ* was also not compromised by a subsequent loss of PA5471 (Fig. 3A, cf. lane 8 and lane 7). Clearly, then, PA5471 is required for drug-inducible *mexXY* expression

TABLE 5. Influence of *mexZ* on PA5471-dependent antimicrobial resistance

Strain	Relevant genotype	MIC (μg/ml)[a]										
		Ami	Gen	Kan	Neo	Par	Spc	Str	Tob	Ery	Nor	Tet
K767		4	8	128	64	256	512	32	—	512	0.5	16
K2415	Δ*mexZ*	4	8	128	**128**	**512**	**1,024**	**64**	—	512	**1**	16
K2416	Δ*mexZ* ΔPA5471[b]	4	8	128	128	512	1,024	64	—	512	1	16

[a] Antibiotic abbreviations are defined in Table 3. —, not determined. The loss of PA5471 did not impact the MICs of these agents in K767 (Table 3), and thus the impact of PA5471 loss on the MICs of these agents was not studied here. Agents for which resistance was increased upon loss of *mexZ* are indicated in bold.
[b] In contrast to the lack of any impact of PA5471 loss on resistance of the MexZ$^-$ strain K2415, the loss of PA5471 in the MexZ$^+$ strain K767 compromised resistance to all agents listed here, except for neomycin and norfloxacin (Table 3, compare K2418 and K767).

only in strains expressing MexZ, consistent with it functioning to directly or indirectly modulate the activity of this repressor. In agreement with this, too, antibiotics or PA5471 did not adversely impact *mexZ* expression (data not shown), i.e., did not increase *mexXY* expression via a negative influence on *mexZ* expression, and thus must act at the level of MexZ activity. *mexZ* expression was, in fact, antibiotic (e.g., chloramphenicol) inducible (Fig. 3B, cf. lane 2 and lane 1), and this was dependent on PA5471 (Fig. 3B, lane 4), exactly mirroring the antibiotic and PA5471 dependence of *mexXY* expression. This is consistent with *mexZ* being subject to autoregulation, as for repressors of other multidrug efflux systems (41, 44), and given the low levels of *mexZ* mRNA detected (it took a minimum of 40 cycles to detect *mexZ* using RT-PCR), it is not inconsistent with antibiotics and/or PA5471 positively impacting *mexXY* expression via modulation of MexZ repressor activity.

PA5471-dependent MexXY operates independently of *trans*-translation. Aminoglycosides and other ribosome-targeting agents promote mistranslation and stop codon readthrough, the latter of which results in ribosome stalling at the 3′ ends of mRNAs and thus in the depletion of free tRNAs and ribosomes needed for translation (2, 19, 61, 62). Stalled ribosomes are rescued in bacteria by a process known as *trans*-translation that requires a specialized RNA species termed tmRNA (which functions as both a tRNA and an mRNA) and a small accessory protein, SmpB (17, 65). To assess, then, whether MexXY functions as part of a *trans*-translation process in *P. aeruginosa* that serves to counter the adverse effects of ribosome-targeting antimicrobials, homologues of the tmRNA (i.e., *ssrA*, or PA0826.2) and *smpB* (PA4768) genes were disrupted in MexXY$^+$ (K767) and MexXY$^-$ (K1525) strains, and the impact on antimicrobial resistance was assessed. The loss of *ssrA* or *smpB* had a modest (twofold decrease) but reproducible impact on resistance to aminoglycosides (amikacin, gentamicin, kanamycin, neomycin, paromomycin, and spectinomycin) and chloramphenicol in the MexXY$^+$ K767 derivatives K2437 and K2438, respectively, and this was seen even in the absence of MexXY (in strains K2439 and K2440) (data not shown), consistent with tmRNA/SmpB and PA5471/MexXY operating independently of one another in promoting resistance to these agents. The observation, too, that the loss of *ssrA* or *smpB* in K767 did not adversely impact resistance to erythromycin (data not shown), while the loss of *mexXY* clearly did (Table 3, K1525), further supports these systems operating independently in *P. aeruginosa*, with PA5471/MexXY apparently playing no role in the process of *trans*-translation.

DISCUSSION

PA5471 is a member of a family of proteins (UPF0027) that are broadly conserved in bacteria and archaea, consistent with it playing a basic, housekeeping function in *P. aeruginosa*. Interestingly, however, a linkage of PA5471-like genes to a putative release factor gene is seen in a more limited number of organisms that include a variety of enterobacteria (*Erwinia carotovora* subsp. *atroseptica* SCRI1043, *Salmonella enterica* serovar Typhi, *Salmonella enterica* serovar Paratyphi, *Salmonella enterica* serovar Choleraesuis, *Escherichia coli* CFT073, *Escherichia coli* K-12, and *Shigella flexneri* 2a) and only three pseudomonads (*Pseudomonas fluorescens* Pf-5, *Ralstonia solanacearum*, and *Burkholderia cepacia* R18154). Whether this reflects a specific and unique function of PA5471 in these organisms or simply a lack of linkage of PA5470/PA5471 homologues in most bacteria harboring PA5471-like genes is unclear. Interestingly, a homologue of PA5471 from *E. coli*, *ykfJ* (b0235; GenBank accession numbers NP_414770 and CAH19161), was also shown to be inducible by an agent, 4-azaleucine, known to interfere with translation (50), and it too is linked to a putative peptide release factor gene (GenBank accession numbers NP_414771 and CAH19162).

Ribosome-targeting antibiotics, including those shown here to induce PA5471-PA5470 and *mexXY* expression, typically cause mistranslation and/or stop codon readthrough, leading to an accumulation of aberrant polypeptides or stalling of ribosomes at the 3′ ends of mRNAs (e.g., aminoglycosides and chloramphenicol [2, 19]), dissociation of incomplete peptidyl-tRNAs from the ribosome (e.g., macrolides [20]), or an accumulation of truncated peptidyl-tRNAs and ribosome stalling (e.g., tetracycline and chloramphenicol [61]). Stalled ribosomes pose a serious problem in that they deplete pools of free tRNAs and ribosomes, and the accumulation of peptidyl-tRNAs is toxic to cells (33). While genes for a *trans*-translation system implicated in the alleviation of drug-induced ribosome stalling were identified in *P. aeruginosa* and were shown here to contribute, albeit modestly, to aminoglycoside and chloramphenicol resistance, as for other organisms (e.g., *E. coli* [2] and *Synechocystis* sp. [11]), MexXY clearly does not participate in this process.

The PA5470 gene is present on a polycistronic message that also encodes PA5471, which is annotated as a peptide chain release factor and carries signature sequences of peptidyl-tRNA hydrolases (http://www.tigr.org/tigr-scripts/CMR2/GenePage.spl?db=ntpa03&locus=PA5470). Intriguingly, a peptidyl-tRNA hydrolase (Pth) in *E. coli* is responsible for recycling of peptidyl-tRNAs formed, for example, as a result of antibiotic action (57). One possibility, then, is that PA5470 participates in the release of aberrant peptides from peptidyl-tRNAs that accumulate in response to drug treatment. Still, this gene is dispensable with regards to MexXY recruitment and MexXY-mediated antibiotic resistance, arguing that while PA5470 and PA5471 (and thus MexXY) may function in a common process that is initiated by ribosome disruption, antibiotic resistance promoted by PA5471/MexXY is independent of this common function. Should PA5470 function in the release of aberrant peptides, PA5471 and MexXY (and possibly others) may play a role in downstream processing of these peptides or the recruitment of components responsible for this. In such a scenario, MexXY may play an intended role in export of these anomalous peptides or processed products thereof and, given the anticipated variation in amino acid sequence and composition of these components (drugs will be targeting ribosomes translating a myriad of different mRNAs and disrupting them at various stages of translation, producing a very heterogeneous mixture of aberrant peptidyl-tRNAs), may need to be flexible with regards to substrate recognition. Such flexibility might then explain the ability of MexXY-OprM to accommodate a diverse array of unintended antimicrobial substrates. Certainly, the observations that substantial *mexXY* expression has only a modest positive impact on antimicrobial resistance (Tables 4 and 5) and that the loss of this efflux mechanism only modestly increases susceptibilities to many anti-

microbials (Table 3) suggest that antimicrobials are not the intended or preferred substrates. Consistent with this, too, a recent DNA array study demonstrated that both PA5471 and PA5470 are inducible (two- to threefold) under anaerobic conditions, in parallel with several ribosome-related genes, possibly due to some adverse impact of anaerobiosis on ribosome function (15; unpublished data).

While there are as yet no definitive clues to the function of PA5471 in *P. aeruginosa* or how it effects *mexXY* upregulation, directly or indirectly, it does not impact *mexZ* expression and thus clearly works to modulate the activity of the MexZ repressor of *mexXY* expression—the loss of PA5471 only compromises drug-inducible *mexXY* expression in MexZ$^+$ and not MexZ$^-$ strains. The observation that antibiotics still enhance *mexXY* expression in a *mexZ* knockout mirrors previous results (23) and is consistent with the presence of additional pathways in *P. aeruginosa* by which *mexXY* can be unregulated in response to antibiotics. Mutations in a gene(s) other than *mexZ* (as yet unidentified) are, in fact, also associated with *mexXY* upregulation (63), although whether these play a role in drug-inducible *mexXY* expression independent of MexZ is unknown. In any case, these additional pathways must be masked by MexZ repressor activity, inasmuch as drug-inducible *mexXY* expression is not observed in MexZ$^+$ PA5471 deletion mutants.

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