

Clinical Strains of *Pseudomonas aeruginosa* Overproducing MexAB-OprM and MexXY Efflux Pumps Simultaneously

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Simultaneous overexpression of the MexAB-OprM and MexXY efflux systems was demonstrated by real-time reverse transcription-PCR and immunoblotting experiments for 12 multiresistant clinical isolates of *Pseudomonas aeruginosa*. DNA sequencing analysis showed that nine of these strains (named *agrZ* mutants) harbored mutations in *mexZ*, the product of which downregulates the expression of the *mexXY* operon. In addition, 8 of the 12 strains exhibited mutations in genes known to control transcription of the *mexAB-oprM* operon. Four of them were *nalB* mutants with alterations in the repressor gene *mexR*, three of them appeared to be *nalC* mutants deficient in gene *PA3721* and overexpressing gene *PA3720*, and one strain was a *nalB nalC* double mutant. For MexAB-OprM as well as for MexXY, no clear correlation could be established between (i) the types of mutations, (ii) the expression level of *mexA* or *mexX*, and (iii) resistance to effluxed antibiotics. Finally, three isolates, named *agrW* mutants, overproduced MexXY and had an intact *mexZ* gene, and four strains overproduced MexAB-OprM and had intact *mexR* and *PA3721* genes (*nalD* mutants). These data show that clinical isolates are able to broaden their drug resistance profiles by coexpressing two Mex efflux pumps and suggest the existence of additional regulators for MexAB-OprM and MexXY.

Polyspecific efflux pumps are essential mechanisms in the defense of *Pseudomonas aeruginosa* against antibiotics, antiseptics, and inhibitors. To date, six of these systems belonging to the RND (resistance nodulation/cell division) family of transporters have been characterized in the pathogen, MexAB-OprM (31), MexCD-OprJ (30), MexEF-OprN (13), MexXY (23, 34), MexJK (3), and MexGHI-OpmD (1). With their partially overlapping substrate specificities, MexAB-OprM and MexXY play a key role in the natural resistance of *P. aeruginosa* to antibiotics. Constitutively expressed in wild-type bacteria, the tripartite system MexAB-OprM confers basal resistance to a wide range of drugs, including β -lactams (except imipenem), fluoroquinolones, tetracyclines, macrolides, chloramphenicol, novobiocin, trimethoprim, and sulfamethoxazole (12, 16, 26, 38). On the other hand, the MexXY proteins, which are produced solely in response to some agents, allow *P. aeruginosa* to adapt rapidly to inhibitory concentrations of aminoglycosides, tetracyclines, and macrolides (7, 20). Several candidate proteins such as OprM (23, 34), OpmB (24), and OmpG and OmpI (11) have been proposed to interact with MexXY in reference strain PAO1 to form a functional tripartite efflux machinery.

Overproduction of MexAB-OprM may lead to significant multidrug resistance in clinical isolates of *P. aeruginosa* (39). In *nalB* mutants, upregulation of the *mexAB-oprM* operon results from various alterations in the adjacent repressor gene *mexR* (32, 35, 36, 39). Other mutants, named *nalC*, harbor intact *mexR* genes (36, 39). Recently, Cao et al. (L. Cao, R. Sriksumar, and K. Poole, Abstr. 42nd Intersci. Conf. Antimicrob. Agents

Chemother., abstr. 430, 2002) reported that *nalC* mutants derived from PAO1 carried mutations in gene *PA3721*. The product of *PA3721* appears to repress a two-gene operon (*PA3720* and *PA3719*) of unknown function whose overexpression in *nalC* mutants may be responsible for MexAB-OprM overproduction.

As for MexAB-OprM, the MexXY proteins may be overproduced constitutively as a result of mutations occurring inside or outside the putative repressor gene named *mexZ* (formerly *amrR*), adjacent to and divergently transcribed from the *mexXY* operon (34, 36a, 37). Mutants of PAO1 are usually two- to eightfold more resistant to aminoglycosides and fluoroquinolones than their wild-type parents (19, 20, 37). However, in vitro screening for spontaneous mutants able to withstand higher concentrations of aminoglycosides often leads to the selection of bacteria with multiple defects in addition to MexXY-mediated efflux (19, 37). The contribution of MexXY to the resistance of clinical isolates such as those recovered from cystic fibrosis patients remains to be explored (37).

While data are accumulating on the occurrence of MexAB-OprM- and MexXY-overproducing strains in the clinical setting (6, 15, 28, 37, 39), little is known about the possible implication of these two systems in the emergence of isolates with reduced susceptibilities to β -lactams, aminoglycosides, and fluoroquinolones, three major classes of antibiotics used for the treatment of *P. aeruginosa* infections. Coexpression of MexCD-OprJ and MexEF-OprN has been described sporadically for fluoroquinolone-resistant cystic fibrosis isolates (8), and one multidrug-resistant strain deficient in the major porin OprF was found to overproduce both MexAB-OprM and MexEF-OprN (33). According to recent observations (14), simultaneous expression of two or three Mex pumps (MexAB-OprM, MexCD-OprJ, and MexEF-OprN) is expected to have

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additive effects on the MICs of common effluxed substrates compared with single-efflux mutants.

In this work, we demonstrate that concomitant overexpression of MexAB and OprM occurs in clinical strains of *P. aeruginosa* and that these two systems may superimpose their drug efflux capabilities, thus contributing to the emergence of multidrug resistance. We also show that overexpression of the two efflux systems may result from mutations affecting multiple regulatory genes.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Twelve clinical isolates of *Pseudomonas aeruginosa* isolated between 1997 and 2000 in the university-affiliated Hospital of Besançon (eastern France) were selected because of their nonenzymatic resistance to both β -lactams and aminoglycosides. These strains, which belonged to serotypes O:1, O:4, O:11, and O:14, were found to be genotypically distinct by random amplified polymorphic DNA analysis (18). They were isolated from urine (WL22, WL24, 1217, 1237, 1562, and 2172), tracheal aspirates (1113, 1727, and 1738), blood catheters (1250 and 2151), or surgical wounds (2085). Bacteria were cultured at 37°C in either Luria-Bertani broth (LB), Mueller-Hinton broth with adjusted concentrations of Ca^{2+} and Mg^{2+} (MHB; BBL, Cockeysville, Md.), or Mueller-Hinton agar plates (MHA; Bio-Rad, Ivry-sur-Seine, France). The wild-type *P. aeruginosa* strain PAO1 (K. Stover) was used as the susceptible reference strain throughout the study, and its MexAB-OprM-overproducing *nalB* mutant strain PT629 (a gift from Thilo Köhler, Gevena, Switzerland) served as a control in gene expression experiments.

Mutants constitutively overproducing MexXY were obtained by incubating strain PAO1 for 2 h in MHB containing 2 μg of gentamicin per ml ($1\times$ MIC) and then plating the bacteria on selective MHA plates supplemented with 2 μg of cefepime per ml ($1\times$ MIC). Western blot and nucleotide sequencing experiments showed that one resistant clone (named Mut-Gr1) overproduced MexY as a result of a single mutation ($\text{C}_{307}\rightarrow\text{T}$) that introduced a stop codon in the coding sequence of the repressor gene *mexZ* (36a). A mutant overproducing both MexAB-OprM and MexXY was obtained by plating an exponential-phase culture of Mut-Gr1 on selective MHA plates supplemented with 10 μg of aztreonam per ml. Reverse transcription-PCR and nucleotide sequencing showed that this double mutant, named ATM4, overproduced MexA as a result of a single-amino-acid substitution ($\text{Ala}_{61}\rightarrow\text{Pro}$) in the repressor MexR.

Bacterial resistance to antibiotics. Clinical strains of *P. aeruginosa* potentially overexpressing the two systems MexAB-OprM and MexXY were retrospectively selected from our laboratory collection on the basis of their drug susceptibility profiles. Typically, MexAB-OprM overproducers exhibit reduced susceptibilities to most β -lactams except imipenem, with MICs of aztreonam at least fourfold higher than that of ticarcillin (39). On the other hand, MexXY-overexpressing mutants show decreased susceptibilities to gentamicin, tobramycin, amikacin, isepamycin, and cefepime, with MICs at least twofold higher than that for wild-type susceptible strains (unpublished observation). None of these isolates displayed typical resistance profiles involving MexCD-OprJ (resistance to cefipime and hypersusceptibility to both ticarcillin and aminoglycosides) or MexEF-OprN (resistance to imipenem and hypersusceptibility to both ticarcillin and aminoglycosides).

Susceptibility testing was performed by the standard microdilution method in MHB with bacterial inocula of about 2.5×10^5 CFU per ml (2). Some of the antibiotics tested were kindly provided by Eli Lilly (tobramycin), Bristol-Meyers Squibb (amikacin, aztreonam, and cefepime), and Glaxo SmithKline (ticarcillin and ceftazidime). Isoelectrofocusing experiments with crude bacterial lysates (6, 22) allowed the selection of 12 strains showing no detectable β -lactamase activity except for faint, wild-type expression of the chromosomally encoded enzyme AmpC (data not shown). The mechanisms of resistance to aminoglycosides in these isolates were deduced from the levels of susceptibility to kanamycin, tobramycin, gentamicin, amikacin, isepamycin, netilmicin, neomycin, 5-epinetilmicin, 2'-netilmicin, 6'-netilmicin, apramycin, and fortimicin, as determined with the aminoglycoside resistance test kit provided by the Schering Plough Research Institute. Apramycin and fortimicin were obtained as titrated powders from Helm (Hamburg, Germany) and Kyowa Hakko Kogyo (Tokyo, Japan), respectively.

Immunodetection of MexY and OprM. Bacterial membranes were isolated and analyzed by Western blotting with MexY- and OprM-specific antisera as previously described (7).

PCR conditions and DNA sequencing. Chromosomal DNA was extracted with the EZNA bacterial DNA kit (Omega, Doraville, Ga.). The coding sequences of

TABLE 1. Primers used for DNA amplification

Primer	5' \rightarrow 3' nucleotide sequence	Reference
nalC1	TCA ACC CTA ACG AGA AAC GCT	This study
nalC2	TCC ACC TCA CCG AAC TGC	This study
mexA-1	CGA CCA GGC CGT GAG CAA GCA GC	Dumas et al. (submitted)
mexA-2	GGA GAC CTT CGC CGC GTT GTC GC	Dumas et al. (submitted)
mexX-1	TGA AGG CGG CCC TGG ACA TCA GC	Dumas et al. (submitted)
mexX-2	GAT CTG CTC GAC GCG GGT CAG CG	Dumas et al. (submitted)
PA3720-1	TCG CCC TGG TCT ATC CGC CGC TC	This study
PA3720-2	CCG CTC AGC AGT GCC TTC GCC AT	This study
rpsL-1	GCA ACT ATC AAC CAG CTG GTG	Dumas et al. (submitted)
rpsL-2	GCT GTG CTC TTG CAG GTT GTG	Dumas et al. (submitted)

mexR and *mexZ* as well as the intergenic regions between *mexA* and *mexR* (*mexA-mexR*) and between *mexX* and *mexZ* (*mexX-mexZ*) were amplified and sequenced as previously described (6). PCR amplification of the putative repressor *nalC* (PA3721) was performed with primers nalC1 and nalC2 (Table 1) in a DNA thermal cycler (Perkin-Elmer, Norwalk, Conn.) for 30 cycles, each cycle consisting of 40 s at 94°C for denaturation, 1 min at 69°C for annealing, and 1 min at 72°C for polymerization. PCR amplicons were sequenced on both strands.

Quantitative real-time reverse transcriptase-PCR. Total RNA were isolated from exponential-phase cultures ($A_{600} = 1$) with the Qiagen RNeasy protocol (Qiagen, Courtaboeuf, France). The RNA samples were further treated with DNase (RQ1 DNase; Promega, Madison, Wis.) and purified by phenol-chloroform extraction and ethanol precipitation (J.-L. Dumas, C. Van Delden, and T. Köhler, submitted for publication). We reverse transcribed 2 μg of total RNA with ImPromII reverse transcriptase (Promega) according to the supplier's instructions. The *mexA* and *mexX* cDNAs were subsequently quantified in a Rotor Gene RG3000 RealTime PCR machine (Corbett Research, Sydney, Australia) with a SybrGreen Quantitect kit (Qiagen) with primers (Dumas et al., submitted for publication) mexA-1/mexA-2 for *mexA* and mexX-1 for *mexX* (Table 1). The expression levels of PA3720 (putative gene *nalC*) were estimated after amplification with primers PA3720-1 and PA3720-2 (Table 1). To correct for differences in the amount of starting materials, the ribosomal *rpsL* gene was chosen as a reference housekeeping gene (primers rpsL-1 and rpsL-2; Table 1). The results are presented as ratios of gene expression between the target gene (*mexA*, *mexX*, or *nalC*) and the reference gene (*rpsL*) (29).

RESULTS AND DISCUSSION

Selection of double-efflux clinical strains of *P. aeruginosa*.

Twelve genotypically distinct strains were selected for their resistance profiles to both β -lactams and aminoglycosides, evoking concomitant overproductions of efflux systems MexAB-OprM and MexXY (see Materials and Methods). None of these isolates were derepressed for the chromosomally encoded AmpC β -lactamase or produced transmissible secondary β -lactamases.

As indicated in Table 2, these strains were two- to eightfold more resistant to ticarcillin, aztreonam, and cefepime than was the wild-type reference strain PAO1. All of them remained susceptible to ceftazidime according to the standard breakpoints (MICs, $\leq 8 \mu\text{g}/\text{ml}$). The 12 strains also displayed reduced susceptibilities to all 12 aminoglycosides tested (Schering Plough kit), including the enzyme-recalcitrant compounds apramycin and fortimicin (Table 2). This decreased susceptibility strongly suggested the expression of one or several non-enzymatic resistance mechanisms to aminoglycosides in the selected bacteria. Except for the well-known chromosomally encoded APH(3')-II enzyme, which naturally provides *P. aeru-*

TABLE 2. Susceptibilities of *P. aeruginosa* strains to antimicrobial agents

Strain	Genotype		Aminoglycoside-modifying enzyme	MIC ^a (μg/ml)								
	<i>nal</i>	<i>agr</i>		Tic	Caz	Fep	Atm	Amk	Tob	Apr	For	Cip
PAO1				16	2	2	4	4	0.5	16	16	0.12
Mut-Gr1		<i>agrZ</i>		16	2	4	4	8	1	64	64	0.5
PT629	<i>nalB</i>			64	4	4	16	4	0.5	16	16	0.25
ATM4	<i>nalB</i>	<i>agrZ</i>		64	4	16	16	8	1	ND	ND	1
WL22	<i>nalB</i>	<i>agrZ</i>	More than 2 ^b	128	8	16	32	128	128	64	128	64
WL24	<i>nalD</i>	<i>agrW</i>	ANT(2'')-I	128	8	16	32	16	64	64	128	64
1113	<i>nalD</i>	<i>agrZ</i>		64	4	8	16	8	1	32	128	0.5
1217	<i>nalD</i>	<i>agrZ</i>	More than 2	64	4	16	16	64	32	128	128	64
1237	<i>nalB</i>	<i>agrZ</i>	AAC(6')-II	64	4	8	16	16	128	64	128	16
1250	<i>nalC</i>	<i>agrZ</i>		64	4	8	16	8	1	16	32	0.5
1562	<i>nalD</i>	<i>agrZ</i>	More than 2	64	4	16	16	128	64	128	128	32
1727	<i>nalB nalC</i>	<i>agrW</i>		128	8	16	32	32	2	64	128	2
1738	<i>nalC</i>	<i>agrW</i>		64	2	8	16	8	1	32	128	0.5
2085	<i>nalB</i>	<i>agrZ</i>	AAC(3)-VI	32	2	8	16	16	4	64	128	8
2151	<i>nalB</i>	<i>agrZ</i>		32	2	8	16	8	1	64	128	32
2172	<i>nalC</i>	<i>agrZ</i>		64	4	8	16	8	1	64	128	1

^a Tic, ticarcillin; Caz, ceftazidime; Fep, cefepime; Atm, aztreonam; Amk, amikacin; Tob, tobramycin; Apr, apramycin; For, fortimicin; Cip, ciprofloxacin. Values in boldface are at least fourfold higher than that for PAO1. ND, not determined.

^b Complex susceptibility profile suggesting the production of two or more different modifying enzymes.

ginosa with high resistance to kanamycin and neomycin (27), no additional enzymatic mechanisms could be detected in six strains (1113, 1250, 1727, 1738, 2151, and 2172), while the aminoglycoside-modifying enzymes ANT(2'')-I, AAC(6')-II, and AAC(3)-VI were phenotypically identified in isolates WL24, 1237, and 2085, respectively. Finally, three isolates (WL22, 1217, and 1562) showed complex susceptibility profiles, suggesting the synthesis of several modifying enzymes. The resistance of the 12 strains to ciprofloxacin varied greatly, with MICs ranging from 0.5 to 64 μg/ml (Table 2).

Overexpression of *mexA* and *mexX*. Upregulation of the MexAB-OprM and MexXY efflux systems in the *P. aeruginosa*

strains was assessed by determining the transcription levels of *mexA* and *mexX* by quantitative real-time reverse transcription-PCR (Table 3). Confirming that the selected isolates are double efflux mutants, *mexA* and *mexX* appeared to be expressed 2.6- to 34.8-fold and 22- to 312-fold, respectively, more than in PAO1. Control experiments performed in parallel showed that the transcription levels of *mexA* and *mexX* in eight randomly chosen, susceptible clinical strains were very similar to that of the reference strain (0.8- ± 0.2-fold and 1- ± 0.3-fold that of PAO1, respectively). These results were in agreement with membrane immunoblots showing increased amounts of MexY and OprM proteins in the mutants (data not shown).

TABLE 3. Genetic analysis of MexAB-OprM- and MexXY-overproducing strains of *P. aeruginosa*

Strain	Nucleotide sequencing data ^a				Mean gene expression ^b		
	<i>mexZ</i> ^c	<i>mexR</i> ^d	<i>PA3721</i> ^e	Vicinity of <i>PA3721</i>	<i>mexX</i>	<i>mexA</i>	<i>PA3720</i>
PAO1					1.0	1.0	1.0
PT629 (<i>nalB</i>)	ND	4 bp deleted at position 363			0.6	3.6	1.71
ATM4 (<i>nalB agrZ</i>)	<u>CAG</u> → <u>TAG</u> (C ₃₀₇ T)	<u>GCA</u> → <u>CCA</u> (A ₆₆ P)			482	6.5	0.11
WL22	48	44, 126	71, 153	G ₋₇₃ →A, A ₊₁₃ →T	168	9.2	2.9
WL24			71, 209		22.0	5.1	0.2
1113	<u>GCC</u> → <u>-CC</u> (A ₃₅ -), <u>ATG</u> → <u>AT</u> -(M ₃₄ -)	126	71, 209	T ₋₂ →C, C inserted (-25)	29.6	4.4	5.5
1217	144, <u>GAG</u> → <u>-AG</u> (E ₁₅₇ -)		71		98.5	6.0	0.5
1237	48, <u>CAG</u> → <u>TAG</u> (Q ₁₀ Z)	44, 126	71, 153, 209, <u>TCA</u> → <u>GCA</u> (S ₄₆ A)	A ₊₁₃ →T	272	7.7	3.7
1250	<u>GGT</u> → <u>G-</u> -(G ₄₆ -), <u>GCG</u> → <u>-CG</u> (A ₄₇ -)		500 bp deleted		312	17.9	147
1562	144		71		129	8.5	1.7
1727		7-bp insertion between 355 and 361	71, <u>GAT</u> → <u>GAA</u> (D ₇₆ E)		300	34.8	9.9
1738			71, 209, <u>CTG</u> → <u>CCG</u> (L ₆₁ P)		82.2	11.7	224
2085	48	44, 126	71, 153	T ₋₂ →C	204	8.7	5.8
2151	<u>GTC</u> → <u>GCC</u> (V ₄₄ A)	44, 126	71, 153, 209	T ₋₂ →C, A ₊₁₃ →T	110	2.6	6.7
2172	<u>GGT</u> → <u>AGT</u> (G ₄₆ S)		71, <u>ATG</u> → <u>ACG</u> (M ₁₅₁ T)		95.4	6.1	194

^a Nucleotide differences compared with the PAO1 genome (www.pseudomonas.com).

^b Relative to PAO1; mean values from two independent experiments.

^c 48, substitution GTC→GCC (V₄₈→A); 144, GCG→GTC (V₁₄₄→A).

^d 44, substitution AAG→ATG (K₄₄→M); 126, GTC→GAG (V₁₂₆→E).

^e 71, substitution GGG→GAG (G₇₁→E); 153, GAG→CAG (E₁₅₃→Q); 209, AGC→CGC (S₂₀₉→R).

Mutations in repressor gene *mexZ*. To gain insight into the mutational events responsible for MexXY upregulation in the 12 double-efflux strains, we amplified and sequenced the intergenic region between *mexX* and *mexZ* as well as *mexZ*, the repressor gene of the *mexXY* operon (C. Vogne, D. Hocquet, J. Ramos Aires, F. El Garch, P. Plésiat, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-434, 2002). Compared with the genome sequence of PAO1 (available at www.pseudomonas.com), nine isolates were found to harbor mutations, resulting in (i) single-amino-acid substitutions in MexZ (strains WL22, 1562, 2085, 2151, and 2172) or (ii) production of aberrant peptides (strains 1113, 1217, 1237, and 1250) (Table 3). Most of these mutations (seven of nine) occurred in the helix-turn-helix N-terminal motif of MexZ (between positions 32 and 53), a domain predicted by the EMBOSS algorithm (available at www.pasteur.fr) to be involved in DNA binding. Two strains (1738 and 2085) exhibited single mutations in the *mexX-mexZ* intergenic region outside the putative promoter sequences of *mexZ* and *mexXY* (data not shown). Finally, strains WL24 and 1727 had nucleotide sequences identical to that of PAO1. No correlation could be established between the various MexZ alterations, the expression level of *mexX*, which varied from 29.6- to 312-fold that of PAO1 (Table 3), and resistance to aminoglycosides (Table 2). Important variations in *mexX* transcription (22- to 300-fold higher than that in PAO1) were also observed in the three strains harboring intact *mexZ* genes (WL24, 1738, and 1727).

In contrast to previous observations made on cystic fibrosis isolates of *P. aeruginosa* (37), stable derepression of *mexXY* in the double-efflux mutants described here was mostly associated with alterations in *mexZ*. We thus propose the names *agrZ* (for aminoglycoside resistance dependent on *mexZ*) and *agrW* (unknown locus) for the genotypes of MexXY-overproducing mutants with altered and intact *mexZ* genes, respectively. In the absence of specific aminoglycoside-modifying enzymes, overexpression of *mexXY* in both types of mutants as well as in the *mexZ*-null mutant Mut-Gr1 was associated with low to moderate resistance to all the aminoglycosides tested (MICs increased two- to eightfold) compared to PAO1. Although relevant in some clinical situations where aminoglycosides diffuse poorly at the site of infection (4), these resistance levels are far below that conferred by aminoglycoside-modifying enzymes for major therapeutic agents such as amikacin (e.g., isolates WL22 and 1562) and tobramycin (e.g., WL22 and 1237) (Table 2). The contribution of the MexXY-OprM efflux system to the overall resistance of strains producing aminoglycoside-modifying enzymes remains to be explored.

Mutations in the *mexABoprM* operon regulatory genes. Sequencing experiments revealed several other mutations in the double-efflux strains (Table 3). One strain (1250) harbored an A→G substitution of unknown significance in the *mexA-mexR* intergenic region at position -5 (position 1 being the A of the *mexR* start codon), which is distant from the MexR binding sites (5). MexR amino acid sequences strictly identical to that published by Poole et al. (32) were found in six putative *nalC* strains (WL24, 1217, 1250, 1562, 1738, and 2172). Five other strains (WL22, 1113, 1237, 2085, and 2151) harbored a Val₁₂₆→Glu substitution in MexR, already observed in susceptible wild-type isolates and considered nonsignificant (28, 39). In this group, four *nalB* mutants (WL22, 1237, 2085, and 2151)

displayed an already known mutation in *mexR* that results in a Lys₄₄→Met substitution (9), affecting the DNA binding domain of MexR (from residues 37 to 97) (17). Finally, a frameshift mutation (7-bp insertion between nucleotides 355 and 361) was discovered in the *mexR* sequence of another *nalB* strain, 1727. Similar genetic events leading to frameshifts in *mexR* have already been reported in MexAB-OprM-overexpressing mutants (36, 39).

In order to further characterize the mutants harboring intact *mexR* genes, we sequenced *PA3721*, the repressor gene of the MexAB-OprM putative activator *PA3720-PA3719* (Cao et al., abstr. 430) and assessed the expression of *PA3720* by real-time reverse transcription-PCR (Table 3). Compared with PAO1, three recurrent substitutions in repressor protein PA3721 were noticed in 11 of 12 isolates: Gly₇₁→Glu, Glu₁₅₃→Gln, and Ser₂₀₉→Arg. These amino acid differences with PAO1 do not seem to have an impact on PA3721 activity, as they were also present in four of four susceptible wild-type environmental strains of *P. aeruginosa* (data not shown). In addition, strain 1237, which overexpressed *PA3720* only 3.7-fold more than PAO1, appeared to have a Ser₄₆→Ala substitution in the PA3721 repressor. More importantly, three isolates were found to strongly overexpress *PA3720* (147- to 224-fold more than in PAO1), suggesting that they are *nalC* mutants. Two of them harbored single-amino-acid substitutions in PA3721: Met₁₅₁→Thr for isolate 2172, and Leu₆₁→Pro for isolate 1738. The third strain (1250) displayed a large 500-bp deletion in *PA3721*. Another strain (1727) already characterized as a *nalB* mutant (see above) and showing moderate overexpression of *PA3720* (9.9-fold more than in PAO1) appeared to carry an Asp₇₆→Glu substitution in PA3721. Finally, various differences in the DNA sequences upstream and downstream of the *PA3721* gene were observed between five isolates with low levels of *PA3720* expression (WL22, 1113, 1237, 2085, and 2151) and PAO1 (Table 3). The effect of these nucleotide changes on *PA3720* transcription is unclear.

As for MexXY, the transcription levels of *mexA* in the isolates were not clearly correlated with particular types of mutations in *mexR* or *PA3721*. The MICs of β-lactams such as ticarcillin and aztreonam, which are known to be good substrates for MexAB-OprM (21), also did not vary with the degree of *mexA* overexpression (Table 2). This observation is not surprising by itself, as multiple factors are involved in determining the susceptibility of a given isolate to antibiotics (e.g., outer membrane permeability, affinity of drug targets, degree of inducibility of AmpC enzyme, etc.) (25). In contrast to previous results obtained with reference strain PAO1 (36), the clinical *nalC* mutant isolates reported here and elsewhere (39) did not appear to be less resistant than the *nalB* mutants to β-lactams.

In addition to well known *nalB* strains (32, 36, 39), this work identified three *nalC* mutants and one *nalB nalC* mutant among the 12 MexAB-OprM/MexXY-overproducing strains selected. This indicates that *nalC* mutants with alterations in *PA3721*, like those initially characterized in vitro from PAO1 (Cao et al., abstr. 430), are rather prevalent among resistant clinical isolates. As indicated in Table 3, mutations in both *mexR* and *PA3721* could have additive effects on *mexA* expression, as *nalB nalC* isolate 1727 displayed the highest *mexA* transcription levels (about twofold higher than that of *nalB* or

nalC mutants). A similar observation has been made for a Δ *acrR* Δ *mar* double mutant of *Escherichia coli* which expressed greater amounts of *acrB* mRNA than single *mar* mutants (10). Interestingly, interplay between *mexR* and *nalC* has already been suspected in PAO1 (36). Finally, no less than four *P. aeruginosa* strains (WL24, 1113, 1217, and 1562), which we propose to call *nalD* type mutants, appeared to contain no mutation in the known regulatory genes for MexAB and OprM.

Lee et al. reported that concomitant overproduction of two Mex pumps in PAO1 produces additive effects on the resistance to shared antibiotic substrates (14). This tends to suggest that clinical strains of *P. aeruginosa* may increase their resistance to a given compound by coexpressing two Mex efflux systems. In support of this, the clinical double-efflux mutants studied here appeared to be more resistant to cefepime (Table 2) than mutants overproducing MexAB-OprM (PT629) or MexXY (Mut-Gr1) alone (MIC, 8 to 16 versus 4 μ g/ml). Convincing evidence of cooperation between MexXY and MexAB-OprM for the extrusion of common substrates is also provided by the double mutant ATM4, which was two- to fourfold more resistant to cefepime and ciprofloxacin than the single mutants Mut-Gr1 and PT629, respectively (Table 2). Besides this, our results show that superimposition of the resistance profiles conferred by two efflux systems, such as MexAB-OprM (β -lactams) and MexXY (aminoglycosides), may be an efficient way for infectious strains to become less susceptible to numerous antibiotics. In a therapeutic perspective, efflux inhibitors of broad specificity would be potentially interesting to reverse the resistance or prevent the emergence of such double-efflux mutants.

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