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Cumulative Effects of Several Nonenzymatic Mechanisms on the Resistance of *Pseudomonas aeruginosa* to Aminoglycosides[▽]

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Screening of a Tn*5*-Hg insertional library (12,000 clones) constructed in wild-type *Pseudomonas aeruginosa* strain PAO1 identified four genes (namely, *galU*, *nuoG*, *mexZ*, and *rplY*) whose disruption individually led to increased resistance to aminoglycosides (means of twofold). Inactivation of these genes was associated with (i) impaired outer membrane uptake, (ii) reduced active transport, (iii) increased MexXY-OprM-mediated active efflux, and (iv) alteration of target of aminoglycosides, respectively. In addition, suppression of the gene *rplY*, which codes for ribosomal protein L25, was found to result in both moderate upregulation of the efflux system MexXY-OprM and hypersusceptibility to β-lactam antibiotics. Construction of double, triple, and quadruple mutants demonstrated cumulative effects of the different mechanisms on aminoglycoside resistance, with MICs increasing from 16- to 64-fold in the quadruple mutant compared to the wild-type strain PAO1. Altogether, these results illustrate how *P. aeruginosa* may gradually develop high resistance to these antibiotics via intrinsic (i.e., nonenzymatic) mechanisms, as in cystic fibrosis patients.

Aminoglycosides are widely used in the treatment of cystic fibrosis (CF) patients with *Pseudomonas aeruginosa* chronic lung infection. However, repeated intravenous and/or aerosolized administration of these antibiotics over years generally leads to a gradual increase in resistance of the pulmonary populations of *P. aeruginosa*. This evolution toward greater resistance does not usually rely on the acquisition of mobile genes encoding aminoglycoside-modifying enzymes (17, 25, 35). Actually, most CF isolates exhibiting significant resistance to aminoglycosides overproduce the chromosomally encoded multidrug efflux system MexXY-OprM as a result of mutations occurring in repressor gene *mexZ*, the product of which downregulates the expression of operon *mexXY* (18, 27, 42, 43). Since upregulation of this three-component pump only produces a modest twofold enhancement of aminoglycosides MICs in non-CF *P. aeruginosa* strains, additional mechanisms have been proposed to contribute to the very low susceptibility of some CF isolates to these polycationic agents (e.g., a tobramycin MIC of ≥8 μg/ml) (37, 42). For instance, production of a defective lipopolysaccharide (LPS) may impair the uptake of aminoglycosides across the outer membrane (5, 10, 22). Similarly, complex quantitative or qualitative alterations in the electron transport chain may perturb the active uptake process of these drugs across the cytoplasmic membrane and limit their intracellular accumulation to levels below that required for ribosome inhibition (41). However, the relevance of all of these mechanisms to the CF context remains to be established.

In an attempt to better understand the stepwise evolution of *P. aeruginosa* isolates toward high aminoglycoside resistance in CF, we examined the interplays and cumulative effects of different nonenzymatic mechanisms (i.e., not involving the production of aminoglycoside-modifying enzymes) engineered in reference strain PAO1. Our data show that the accumulation of mechanisms in mutants leads to a gradual increase in the resistance to aminoglycosides, as seen in CF patients.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in the present study are listed in Table 1. Strain PAO1, the genome of which has been entirely sequenced, was used throughout the study as the reference *P. aeruginosa* (40). *Escherichia coli* DH5α was the bacterial host in all cloning experiments. Bacterial cultures were performed in Mueller-Hinton broth with adjusted concentrations of Ca^{2+} and Mg^{2+} (MHB; BBL, Cockeysville, MD) or on Mueller-Hinton agar plates (MHA; Bio-Rad, Marnes-La-Coquette, France). In mating experiments with *E. coli* SM10, S17.1, or S17.1 λpir, *P. aeruginosa* transconjugants were selected on M9 minimal agar medium (M9MAM) (3) supplemented with 0.2% (wt/vol) sodium citrate as the sole carbon source or on MHA containing 200 μg of cetrimide/ml. The media were rendered selective by the addition of antimicrobials at the following concentrations: 100 μg of ampicillin/ml (AMP 100) or 10 μg of gentamicin/ml (GEN 10) for *E. coli* and 250 μg of ticarcillin/ml (TIC 250), 200 μg of gentamicin/ml (GEN 200), or 12 μg of mercuric chloride ($HgCl_2$)/ml for *P. aeruginosa*. Anaerobic growth of *P. aeruginosa* was assessed on MHA plates containing 0.3% (wt/vol) KNO_3 as a terminal electron acceptor in an atmosphere composed of (ratio) N_2, H_2, and CO_2 (85:10:5). All of the cultures were incubated at 37°C. LPS O-serotyping was performed by slide agglutination with overnight colonies and specific antisera provided by Bio-Rad.

Antibiotic susceptibility tests. The MICs of antimicrobial agents were determined by the conventional serial twofold macrodilution method in MHB with inocula of approximately 10^4 bacteria per spot, as recommended (21).

Transposon insertion mutagenesis. A library of 12,000 insertion mutants was constructed in strain PAO1 with mini-transposon Tn*5*-Hg as described previously (11). Suicide plasmid pUTHg (ampicillin resistant [Amp^r], $HgCl_2^r$) that carries Tn*5*-Hg was mobilized from *E. coli* donor strain S17.1 λpir. The insertional mutants were selected on MHA supplemented with $HgCl_2$ (marker of Tn*5*-Hg) and cetrimide and then replicated on MHA containing 4 μg of gentamicin/ml to screen for clones resistant to aminoglycosides. To localize the insertion sites of Tn*5*-Hg in these resistant bacteria, a SalI genomic library from each mutant was constructed in *E. coli* strain DH5α with the versatile plasmid vector pUC18 (Amp^r) (44) and screened by colony blotting with a *merA* biotinylated probe prepared by PCR amplification with the PCR DIG labeling mix kit from Roche Diagnostics (primers mer1 and mer2 in Table 2). The DNA regions flanking Tn*5*-Hg in each positive recombinant plasmid (pUCNuoG, pUCRplY, and

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TABLE 1. Bacterial strains

Strain	Description[a]	Source or reference
P. aeruginosa		
PAO1	Wild type	B. W. Holloway
FE10	PAO1 *nuoG*::Tn*5*-Hg	This study
FE49	PAO1 *galU*::Tn*5*-Hg	This study
FE53	PAO1 *mexZ*::Tn*5*-Hg	This study
FE57	PAO1 *rplY*::Tn*5*-Hg	This study
FE10U	FE10 *galU*::*FRT*	This study
FE53U	FE53 *galU*::*FRT*	This study
FE57U	FE57 *galU*::*FRT*	This study
FE53G	FE53 *nuoG*::*FRT*	This study
FE57G	FE57 *nuoG*::*FRT*	This study
FE57Z	FE57 *mexZ*::*FRT*	This study
FE53GU	FE53G *galU*::*FRT*	This study
FE57GU	FE57G *galU*::*FRT*	This study
FE57UZ	FE57U *mexZ*::*FRT*	This study
FE57GZ	FE57G *mexZ*::*FRT*	This study
FE57GUZ	FE57GU spontaneous mutant with an Ala_{20}-to-Val change in MexZ	This study
KJ7106	PAO1 Δ*PA5471*	This study
FE60	PAO1 Δ*mexXY*	This study
FE63	FE57 Δ*PA5471*	This study
FE64	FE57 Δ*mexXY*	This study
E. coli		
DH5α	*supE44 endA1 hsdR17*(r_K^- m_K^+) *thi-1 recA1* Δ(*argF-lacZYA*)*U169* ϕ 80d*lacZ*ΔM15 *phoA gyrA96 relA1 deoR* λ^-	Gibco-BRL
SM10	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Kan^r)	8
S17.1	*recA thi pro hsdR*$^-$*M*$^+$ RP4-2-Tc::Mu Km::Tn*7* (Tmp^r Str^r)	38
S17.1λpir	*recA thi pro hsdR*$^-$*M*$^+$ RP4-2-Tc::Mu Km::Tn*7* λpir (Tmp^r Str^r)	29

[a] Abbreviations: Kan^r, resistance to kanamycin; Str^r, resistance to streptomycin; Tmp^r, resistance to trimethoprim.

pUCGalU) were sequenced with the Sanger's dideoxynucleotide method by using M13F and M13R universal primers (44) and compared to the *Pseudomonas* genome database (http://v2.pseudomonas.com/) for identification.

DNA techniques. Restriction enzymes, T4 DNA ligase, and alkaline phosphatase were obtained from Roche Diagnostics (Meylan, France) and used as recommended by the supplier. Blunt-ending of restricted DNA fragments was accomplished by the addition of 1 U of Klenow enzyme (Roche) and 40 μM deoxynucleoside triphosphates (3) or 1 U mung bean nuclease (Promega, Charbonnières-Les-Bains, France) to the reaction mixture at the end of enzymatic digestion. Chromosomal DNA suitable for PCR amplification was extracted and purified with the Wizard genomic DNA purification kit (Promega). Plasmid DNA was prepared by the standard alkaline lysis method (3) or by using the Plasmid Midi Preps kit from QIAGEN (Courtaboeuf, France), as recommended by the manufacturer. Selected restriction fragments were purified from agarose electrophoretic gels with the QIAquick gel extraction kit (QIAGEN). Transformation of strains of *E. coli* or *P. aeruginosa* with plasmid DNA has been described previously (3, 39). PCR amplifications were performed according to the manufacturer's protocol for RedTaq DNA polymerase (Sigma-Aldrich, Saint Quentin Fallavier, France), with 5% dimethyl sulfoxide in the reaction mixture when *P. aeruginosa* chromosomal DNA was used as the template. Sequences of the primers used in the present study are listed in Table 2. Nucleotide sequences of the PCR products were determined on both strands by the Sanger method with an Applied Biosystems 3130 automatic sequencer (Applied Biosystems, Courtaboeuf, France) and edited with the BLASTN algorithm available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

To complement mutant FE57, gene *rplY* was amplified on a 1-kb XbaI PCR fragment and cloned in proper orientation downstream of the arabinose-inducible promoter of XbaI-linearized vector pARA6 (ticarcillin resistant; T. Köhler, unpublished data). Expression of *rplY* from the resultant plasmid pARAL25 was induced in FE57 by culture in the presence of 0.2% (wt/vol) arabinose.

Quantitative real-time-PCR. Specific gene expressions were assessed by quantitative real-time PCR after retrotranscription (RT-PCR) as described previously (9, 14). Briefly, 2 μg of total RNA was reverse transcribed with ImpromII reverse transcriptase as specified by the supplier (Promega). The amounts of specific cDNA were evaluated in a Rotor Gene RG3000 RealTime PCR machine (Corbett Research, Sydney, Australia) by using the SybrGreen Quantitect kit (QIAGEN), primers designed from the sequence of the *Pseudomonas* database (Table 2), and *uvrD* transcripts as an internal control (19). The mRNA levels of a given target gene in a given strain were normalized with those of *uvrD* and expressed as ratios (fold change) to that of wild-type strain PAO1 used as a reference.

Gene inactivation. Gene inactivation was accomplished with the *sacB*-based strategy developed by Hoang et al. (13). Inactivation of genes *nuoG*, *mexZ*, and *galU* was carried out as follows: a 1-kb BamHI-HindIII or XbaI-EcoRI PCR fragment carrying the gene of interest was cloned into BamHI-HindIII- or XbaI-EcoRI-restricted vector pEX100Tlink (Amp^r) (33), yielding recombinant plasmids pEXNG, pEXZ, and pEXGU, respectively. Each construct was cleaved in the middle of the insert (endonucleases PstI, XhoI, and EcoRV, respectively) to produce a single fragment subsequently blunt ended with Klenow enzyme or mung bean nuclease. The 1.7-kb SmaI fragment which contains the FRT cassette [the gentamicin resistance gene *aac(3)-I*, the *gfp* gene and the *FRT* sequences] from plasmid pPS858 (13) was then ligated to each of the DNA fragments, yielding new constructs named pEXNGR, pEXZR, and pEXGUR, respectively. These plasmids were individually transferred by overnight conjugation from *E. coli* strain S17.1 to *P. aeruginosa* as described previously (32). The recombinant clones were selected on M9MAM plates supplemented with GEN 200, and merodiploids from these clones were resolved by subculture on MHA GEN 200 medium containing 5% (wt/vol) sucrose. Flippase-promoted excision of the chromosomally integrated FRT cassette (gentamicin-resistant [Gen^r]-green fluorescent protein markers) was achieved by conjugational transfer of plasmid pFLP2 (Amp^r) (13) from donor *E. coli* SM10 and selection of the *P. aeruginosa* transconjugants on M9MAM TIC 250. Plasmid pFLP2 was subsequently cured by streaking selected recipient clones on MHA medium supplemented with 5% (wt/vol) sucrose. Sequencing analysis showed that *nuoG*, *mexZ*, and *galU* open reading frames were interrupted in the resultant mutants by DNA fragments of about 145-bp carrying a single *FRT* sequence. Despite repeated attempts, we were unable to inactivate gene *rplY* with this method.

Construction of *mexXY* deletion mutants (Δ*mexXY*) was yielded by allelic exchange with plasmid pEXΔXYR. To construct this vector, a 1.6-kb HindIII-BamHI fragment carrying *mexZ* and the 5′ part of *mexX* and a 1.4-kb BamHI-EcoRI fragment carrying the 3′-part of *mexY* were amplified by PCR from strain

TABLE 2. Primers used in this study

Function and primer	Nucleotide sequence (5′–3′) (endonuclease)[a]	Source or reference
Gene expression		
uvrD1	CAC GCC TCG CCC TAC AGC A	20
uvrD2	GGA TCT GGA AGT TCT GCT CAG C	20
mexB1	ATC CGC CAG ACC ATC GCC A	15
mexB2	CAT CAC CAG GAA CAC GAG GAG G	15
mexY1a	TTA CCT CCT CCA GCG GC	19
mexY1b	GTG AGG CGG GCG TTG TG	19
PA5471-A	AGC TAC AGC AGG TCG AGA CG	This study
PA5471-B	TTG ATG TCG AGC AGT TCC AG	This study
nuoH1	GGT CGG CTC GTT CAA CA	This study
nuoH2	TGG CGG CGA AGT AGA AG	This study
pth-1	CGT GGC TGA CCG CAA GTA	This study
pth-2	GGG CAT GTC GAG TTC GTC	This study
Cloning		
L25-1	CTC TCT AGA CGA AAA CGC CCC GCT CC (XbaI)	This study
L25-2	CTC TCT AGA TTG CGG TCA GCC ACG AG (XbaI)	This study
Gene inactivation		
galUrecA	CTC GAA TTC CTT CGT CCA TGT CGA AGG AT (EcoRI)	This study
galUrecB	CTC TCT AGA CGG GCA ACT ATG TGG AGT TC (XbaI)	This study
nuoGrecA	CTC GGA TCC ACC CAC CAG AAC CAG GAA CT (BamHI)	This study
nuoGrecB	CTC AAG CTT GAG TTG CCG GAC ACT ACC AG (HindII)	This study
mexZrecA	CTC GGA TCC GGC GCG ACA GTA GCA TAT AA (BamHI)	This study
mexZrecB	CTC AAG CTT CGT GAA GCT ACC GTG ACA GA (HindIII)	This study
mexZb-1	AAG CTT AAG CTT GCG TTC GCA CTT GAG GTA GAG (HindIII)	6
mexGb-1	ACG CGG ATC CGT TCT CGA CGA TCA CCC ACTC (BamHI)	6
mexHb1	ACC GGA ATT CCA CCA GGA AGA ACA GCG GTAC (EcoRI)	6
mexHf1	ACG CGG ATC CCT GGA TGC TGG TCT ACA CCCT (BamHI)	6
In5471A3	CTC GAG CTC GAG CTG GAT TTC TTC CTC GAC (SacI)	This study
In5471A4	CTC GGA TCC TCC TCG ATT ACC TCG ACC TC (BamHI)	This study
Sequencing		
M13F	GTA AAA CGA CGG CCA GT	44
M13R	CAG GAA ACA GCT ATG AC	44
merA1	CCA CGG GAC ACG GTG AA	This study
merA2	CGG CAT CGC GGT TAG AT	This study
SeqmexZ1	GCA GCC CAG CAG GAA TAG	24
SeqmexZ2	GCC TGT CGG TGC TCT ACA TC	24

[a] Restriction sites were introduced into primers; these are underlined in the sequences, with the corresponding endonucleases indicated in parentheses). All amplification reactions were conducted at 61°C except in sequencing experiments (50°C).

PAO1 and cloned together into HindIII-EcoRI-restricted gene replacement vector pEX18Ap (Amp^r) (13), yielding construct PEXΔXY. Then, a 1.7-kb BamHI fragment from pPS858 containing the FRT cassette was inserted into the BamHI site linking the two cloned PCR fragments, yielding construct pEXΔXYR (Amp^r, Gen^r). To construct PA5471 deletion strains, a 1.9-kb SacI-BamHI PCR fragment carrying gene PA5471 was cloned into SacI-BamHI-restricted vector pEX100Tlink (Amp^r), yielding pEX5471. The SmaI FRT cassette from pPS858 was next cloned into the insert of pEX5471 previously linearized with BstEII and treated with Klenow enzyme to produce pEXΔ5471 (Amp^r, Gen^r). Both pEXΔXYR and pEXΔ5471 were transferred to *P. aeruginosa* and selected as described above, resulting in mutants lacking 1.7 kb from *mexXY* and 0.7 kb from PA5471, respectively.

Vectors pEXNGR, pEXGUR, and pEXZR allowed the construction of six double mutants, four triple mutants, and one quadruple mutant deficient in the genes *galU*, *nuoG*, and *mexZ* in PAO1 or FE57 (*rplY*::Tn*5*-Hg). The FRT cassette (Gen^r) was not excised by the flippase enzyme in the triple and quadruple mutants to avoid the loss of large genomic regions as reported by Hoang et al. (13). The quadruple mutant FE57GUZ (overexpressing *mexXY*) was obtained from the triple-mutant FE57GU by selection on MHA plates supplemented with ciprofloxacin (1 μg/ml) and cefepime (16 μg/ml), which are good MexXY-OprM substrates (15, 26). DNA sequencing revealed a C-to-T change at position 59 in gene *mexZ* of FE57GUZ corresponding to an Ala-to-Val change in the MexZ repressor protein.

Immunodetection of MexY. Bacterial membranes were isolated and analyzed by Western blotting with a MexY specific polyclonal antiserum, as described previously (16).

RESULTS AND DISCUSSION

Isolation of insertion mutants of PAO1. In an attempt to identify new resistance mechanisms to aminoglycosides in *P. aeruginosa*, we generated a set of 12,000 Tn*5*-Hg insertional mutants in strain PAO1, the genome of which has been sequenced (40) and annotated (http://v2.pseudomonas.com/). Ten mutants were able to grow on 4 μg of gentamicin/ml (fourfold the MIC for PAO1) after replica plating. Comparison of the DNA regions flanking Tn*5*-Hg in each of these clones to the whole genomic sequence of PAO1 identified four disrupted genes, namely, *nuoG* (PA2642; mutant FE10), *galU* (PA2023; mutant FE49), *mexZ* (PA2020; mutant FE53), and *rplY* (PA4671; mutant FE57) (Table 1). Compared to wild-type parent PAO1, all of these mutants exhibited a general twofold

TABLE 3. Drug susceptibilities of the various mutants constructed in strain PAO1

Bacterial strain	Inactivated gene(s)	MIC (μg/ml)[a]						
		GEN	AMK	TOB	NET	KAN	CIP	FEP
PAO1	Wild type	1	4	0.5	4	64	0.25	4
FE10	*nuoG*	2	8	1	8	256	0.25	4
FE49	*galU*	2	8	1	8	128	0.25	4
FE53	*mexZ*	4	8	1	8	128	1	16
FE57	*rplY*	2	8–16	1	8	256	0.5	4
FE57	*rplY* complemented with pARAL25[b]	1	4	0.25	ND	ND	0.25	ND
FE10U	*nuoG*, *galU*	**4**	8	**2**	8	256	0.25	4
FE53U	*mexZ*, *galU*	**8**	**16**	**2**	**16**	**256**	0.5–1	16
FE57U	*rplY*, *galU*	**8**	16	**2**	**16**	256	0.5	4–8
FE53G	*mexZ*, *nuoG*	**8**	**16**	**2**	**16**	256	1	16
FE57G	*rplY*, *nuoG*	**8**	**32**	**2**	**16**	**512**	0.5	4
FE57Z	*rplY*, *mexZ*	**8**	16	**2**	**16**	256	1	16
FE53GU	*mexZ*, *nuoG*, *galU*	ND	**32**	**4**	**64**	**512**	1	16
FE57GU	*rplY*, *nuoG*, *galU*	ND	32	**4**	**64**	512	0.5	8
FE57UZ	*rplY*, *galU*, *mexZ*	ND	**32**	**4**	**64**	**512**	1	16
FE57GZ	*rplY*, *nuoG*, *mexZ*	ND	32	**4**	**128**	**1,024**	1	16
FE57GUZ	*rplY*, *nuoG*, *galU*, *mexZ*	ND	**64**	**8**	**256**	1,024	1	16
KJ7106	*PA5471*	0.125	1	0.25	ND	ND	0.25	4
FE60	*mexXY*	0.125	1	0.25	ND	ND	0.25	4
FE63	*rplY*, *PA5471*	0.25	2	0.5	ND	ND	0.25	2
FE64	*rplY*, *mexXY*	0.25	2	0.5	ND	ND	0.25	2

[a] Results of two independent determinations are given. The MICs in boldface correspond to additive (2-fold increase) or multiplicative (>2-fold increase) effects of combined mutations on resistance. GEN, gentamicin; AMK, amikacin; TOB, tobramycin; NET, netilmicin; KAN, kanamycin; CIP, ciprofloxacin; FEP, cefepime. ND, not determined.

[b] Gene *rplY* cloned on arabinose-inducible plasmid pARA6. Transformants were grown in the presence of 150 μg per ml ticarcillin for plasmid maintenance and 0.2% arabinose. Control strain FE57(pARA6) cultivated under the same conditions exhibited resistance levels similar to those of FE57 in MHA.

increase in resistance to aminoglycosides, including gentamicin, amikacin, tobramycin, netilmicin, and kanamycin (Table 3). The susceptibilities to antibiotics of other classes were unchanged in these bacteria except for FE53 (ciprofloxacin and cefepime) and FE57 (ciprofloxacin).

Mutants FE10, FE49, and FE53. Expression of gene *nuoG* downstream of *nuoG* was found to be abolished in mutant FE10 confirming that Tn*5*-Hg promoted the disruption of the *nuoABDEFGHIJKLMN* operon which codes for proton-translocating type I NADH oxidoreductase in this strain. Supporting the notion that inactivation of NADH dehydrogenase, an enzymatic complex that significantly contributes to the proton electrochemical gradient, impairs membrane energetics and thereby the uptake of aminoglycosides (41), mutant FE10 was unable to grow anaerobically with KNO_3 as the terminal electron acceptor. Of note, gene *mexY* expression in FE10 was comparable to that of parental strain PAO1, ruling out a direct role for MexXY-OprM in the increased resistance of this mutant (not shown).

The *galU* gene product (UDP-glucose pyrophosphorylase) catalyzes the conversion of glucose-1-phosphate to UDP-glucose, which is essential for the synthesis of a complete LPS outer core. Consistent with earlier results showing that *galU* knockout results in the production of truncated (rough) LPS molecules lacking both A- and B-band polysaccharides in *P. aeruginosa* (7), mutant FE49 was autoagglutinable and was found to lack the O:5 antigen of parent PAO1 when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with a specific monoclonal antibody (data not shown). Accounting for the resistance phenotype of FE49, loss of the A- and B-band LPS was reported to impair the antibacterial activity of aminoglycosides by compromising their binding to the cell surface (22).

In agreement with other results (42, 43), inactivation of repressor gene *mexZ* in FE53 resulted in increased expression of gene *mexY* (40-fold greater than in PAO1) and constitutive overproduction of multidrug transporter MexY (data not shown). The resistance of FE53 to the structurally unrelated antibiotics cefepime and ciprofloxacin in addition to aminoglycosides provided additional evidence for the system MexXY-OprM being stably upregulated in this mutant (Table 3) (26).

Mutant FE57. Mini-transposon Tn*5*-Hg was found to be integrated in the fifth gene (PA4671) of an operon predicted to contain at least six coding sequences (from PA4667 to PA4672) by the *Pseudomonas* Genome Project (http://v2.pseudomonas.com/). The disrupted gene named *rplY* encodes a putative product of 204 amino acids (21,962 Da) identified as L25, a ribosome-associated protein widely distributed in bacteria and which binds to the 5S rRNA loop E via a highly conserved N-terminal domain (Protein Families Database of Alignments, accession number Pfam01386). This domain shares significant sequence homologies with the N-terminal part of the general stress protein Ctc from *Bacillus subtilis*, a factor presumed to ensure accurate ribosomal translation under stress conditions (e.g., exposure to high salt concentrations) (36). Whether L25-like proteins are genuine ribosomal proteins or shock factors remains to be elucidated (1). However, consistent with a dispensable role for L25 in protein synthesis, inactivation of the *rplY* gene in mutant FE57 impacted only moderately the growth kinetics of *P. aeruginosa* (generation time of 44.9 min versus 35.8 min for PAO1).

Forty nucleotides downstream of *rplY* in the PAO1 genome

is a gene designated *pth* (PA4672) expected to code for a product of 194 amino acids (20,803 Da) homologous to peptidyl-tRNA hydrolase from *E. coli* (58% identities, 72% similarities, pfam01195). This enzyme (Pth) is known to catalyze the hydrolysis of peptidyl-tRNA species that dissociate from the ribosome when premature termination occurs during protein synthesis (2). Therefore, Pth plays a crucial role in the recycling of essential tRNA molecules such as $tRNA^{Lys}$ sequestered as peptidyl-tRNAs (12), especially when the translation process aborts, for instance, because of the action of antibiotics targeting the ribosome (28). RT-PCR experiments showed that the transcript levels of *pth* were not abolished in mutant FE57 but just slightly reduced compared to that of PAO1 (ratio of 0.34 as a mean value of two distinct experiments), probably because of a polar effect of Tn*5*-associated promoters (4). To determine whether the low *pth* activity would be responsible for the resistance phenotype of FE57, we complemented this mutant with a plasmid-borne copy of *rplY* expressed from tightly controlled arabinose promoter *pBAD* (plasmid pARAL25). As indicated in Table 3, arabinose-induced FE57 cells harboring pARAL25 exhibited wild-type drug susceptibilities despite reduced *pth* expression (i.e., similar to that of FE57 transformed with vector pARA6), thereby indicating that *rplY* knockout was the major cause of drug resistance in FE57.

***PA5471*-dependent overexpression of MexXY in mutant FE57.** It has recently been reported that expression of operon *mexXY* is induced as a result of protein synthesis inhibition by antibiotics targeting the ribosome, whether these inhibitors are substrates (tetracycline, macrolides, and aminoglycosides) or not (chloramphenicol) of the MexXY-OprM pump (19, 26). The antibiotic-induced expression of *mexXY* was subsequently shown to be dependent on the transcription levels of a housekeeping gene named *PA5471* coding for a product of unknown function and cotranscribed with another gene (*PA5470*), itself predicted to encode a factor carrying signature sequences of peptidyl-tRNA hydrolases (30). Evidence was provided that the product of PA5471 impacts *mexXY* expression directly or indirectly via modulation of repressor MexZ activity (30). Since FE57 displayed a reduced expression of another presumed peptidyl-tRNA hydrolase gene (*PA4672*) and an enhanced resistance to ciprofloxacin (Table 3), a substrate (26, 34) but not an inducer of the MexXY-OprM pump (19), we examined the role of *mexXY* in the resistance of this mutant. The genes *mexY* and *PA5471* were found to be upregulated 9.4- and 3-fold, respectively, in FE57 compared to PAO1. On the other hand, *mexY* activity appeared to be 4.4 lower in FE57 than in *mexZ*-null mutant FE53 despite comparable resistance levels to aminoglycosides (Table 3). Assessment of protein MexY production by Western blotting confirmed these RT-PCR results (data not shown). To evaluate more accurately the relevance of MexXY upregulation in FE57, we inactivated *PA5471* and the operon *mexXY* in PAO1 (mutants KJ7106 and FE60, respectively) and in FE57 (mutants FE63 and FE64, respectively). As indicated in Table 3 and in agreement with other data (30), elimination of *PA5471* or *mexXY* dramatically increased the susceptibility of parental strain PAO1 to aminoglycosides. Interestingly, suppression of these genes left a higher baseline resistance to aminoglycosides in the *rplY*-defective background (compare mutants FE63 and KJ7106 and mutants FE64 and FE60). These findings clearly indicated that the primary mechanism of aminoglycoside resistance in FE57 was the alteration of ribosomal protein L25, the modest resistance to ciprofloxacin likely resulting from the slight upregulation of MexXY-OprM system.

The fact that FE63 and FE64 were more susceptible to cefepime (as well as carbenicillin, meropenem, and aztreonam [data not shown]) than their PAO1 counterparts (KJ7106 and FE60, respectively) strongly suggests that the lack of L25 negatively impacts the resistance to β-lactams in *P. aeruginosa*. Supporting this notion, cefepime (in contrast to ciprofloxacin) exhibited the same activity on FE57 as on PAO1, despite MexXY being moderately upregulated in FE57 (Table 3). RT-PCR experiments showed that FE57 hypersusceptibility to β-lactams such as carbenicillin was not associated with reduced expression of MexAB-OprM, the pump which strongly contributes to the natural resistance of *P. aeruginosa* to this antibiotic (23) (data not presented).

Double, triple, and quadruple mutants. To get an insight into the mechanisms able to potentiate the efflux-based resistance to aminoglycosides in clinical strains of *P. aeruginosa*, we generated a set of double, triple, and quadruple mutants of PAO1 exhibiting poor outer membrane permeability (Δ*galU*), reduced active drug transport (Δ*nuoG*), increased active drug efflux (Δ*mexZ*), and/or drug target alteration (Δ*rplY*). Sequential accumulation of these mechanisms whatever their nature produced cumulative effects on the resistance to aminoglycosides with each mechanism addition, leading to a general twofold (sometimes fourfold) increase in the MIC of these antibiotics (Table 3). This was also applicable to kanamycin, the resistance levels of which are determined by chromosomally encoded APH[3′] enzyme in wild-type strains of *P. aeruginosa* (31).

Ultimately, inactivation of the four genes, *nuoG*, *galU*, *mexZ*, and *rplY*, as in mutant FE57GUZ, resulted in an elevated resistance to widely prescribed products such as amikacin, tobramycin, and netilmicin, thereby illustrating how clinical strains of *P. aeruginosa* may step by step become very recalcitrant to clinically important drugs without the need for extrinsic enzymes. Whether *nuoG*-, *galU*-, or *rplY*-null mutants occur among CF strains of *P. aeruginosa* warrants further investigation.

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