ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2011, p. 5676–5684 Vol. 55, No. 12
0066-4804/11/$12.00 doi:10.1128/AAC.00101-11

Role of the MexEF-OprN Efflux System in Low-Level Resistance of *Pseudomonas aeruginosa* to Ciprofloxacin▽

Catherine Llanes,[1] Thilo Köhler,[2] Isabelle Patry,[1] Barbara Dehecq,[1] Christian van Delden,[2] and Patrick Plésiat[1]*

Centre National de Référence de la Résistance aux Antibiotiques, Laboratoire associé P. aeruginosa, Hôpital Jean Minjoz, Besançon, France,[1] and Service of Infectious Diseases, University Hospital Geneva, 1211 Geneva, Switzerland[2]

Received 24 January 2011/Returned for modification 18 March 2011/Accepted 3 July 2011

In this study, we investigated the resistance mechanisms to fluoroquinolones of 85 non-cystic fibrosis strains of *Pseudomonas aeruginosa* exhibiting a reduced susceptibility to ciprofloxacin (MICs from 0.25 to 2 μg/ml). In addition to MexAB-OprM (31 of 85 isolates) and MexXY/OprM (39 of 85), the MexEF-OprN efflux pump (10 of 85) was found to be commonly upregulated in this population that is considered susceptible or of intermediate susceptibility to ciprofloxacin, according to current breakpoints. Analysis of the 10 MexEF-OprN overproducers (*nfxC* mutants) revealed the presence of various mutations in the *mexT* (2 isolates), *mexS* (5 isolates), and/or *mvaT* (2 isolates) genes, the inactivation of which is known to increase the expression of the *mexEF-oprN* operon in reference strain PAO1-UW. However, these genes were intact in 3 of 10 of the clinical strains. Interestingly, ciprofloxacin at 2 μg/ml or 4 μg/ml preferentially selected *nfxC* mutants from wild-type clinical strains ($n = 10$ isolates) and from first-step mutants ($n = 10$) overexpressing Mex pumps, thus indicating that MexEF-OprN represents a major mechanism by which *P. aeruginosa* may acquire higher resistance levels to fluoroquinolones. These data support the notion that the *nfxC* mutants may be more prevalent in the clinical setting than anticipated and strongly suggest the involvement of still unknown genes in the regulation of this efflux system.

Fluoroquinolones such as ciprofloxacin and levofloxacin are widely used in the treatment of chronic and acute infections caused by *Pseudomonas aeruginosa* (9). However, the efficacy of these antibiotics may be compromised by the emergence of resistant mutants exhibiting drug target alterations (GyrA, ParC, GyrB, ParE) (1, 44, 49) or overproducing drug efflux mechanisms (47). Four active efflux pumps belonging to the resistance nodulation cell division (RND) family of transporters may be responsible for an increased (2- to 16-fold) resistance to these agents when overexpressed as a result of mutations, namely, MexAB-OprM, MexXY/OprM, MexCD-OprJ, and MexEF-OprN (32, 42, 64). Other efflux systems of the RND (MexHI-OpmD, MexPQ-OpmE) (43, 51), the MATE (multidrug and toxic compound extrusion) (PmpM) (20), or the ABC (Orf12) (56) family of transporters have also been reported to export fluoroquinolones in laboratory mutants of *P. aeruginosa*, but their relevance in the clinical setting remains unknown.

The MexEF-OprN pump is expressed at very low levels in wild-type bacteria and does not significantly contribute to the natural resistance of this species to antibiotics (32). Its stable upregulation (*nfxC*-type mutants) is typically associated with a higher resistance to fluoroquinolones, chloramphenicol, trimethoprim, and carbapenems. In addition, the resultant phenotype shows hypersusceptibility to various β-lactams (ticarcillin, cefepime, ceftazidime, aztreonam) and aminoglycosides (32, 40). To date, only a few clinical strains have been identified as *nfxC* mutants (45, 48). Data from animal models of infection seem to indicate that such mutants are less efficient at providing fluoroquinolone resistance *in vivo* than *in vitro* (17) and that they have an impaired virulence (28, 31, 34).

Expression of the MexEF-OprN-encoding operon is positively regulated by a LysR-type transcriptional activator (MexT) encoded by a gene (*mexT*) adjacent to the *mexEF-oprN* operon (32). The protein MexT has additional regulatory functions as it downregulates expression of *oprD*, the gene for the carbapenem-selective porin OprD, and the *mexAB-oprM* operon that determines the efflux system MexAB-OprM (40). In several PAO1 laboratory subclones, overexpression of the *mexEF-oprN* operon has been found to result from mutations in the *mexT* gene, that surprisingly revert or suppress mutations inactivating this gene (39). However, this is not the case in other susceptible reference strains, such as PA14 (http://www.pseudomonas.com), which contain the "active" form of MexT. Some *nfxC* mutants exhibit alterations in the *mexS* flanking gene (21, 53), which codes for a putative oxidoreductase positively regulated by MexT (31). Whether the inactivation of MexS eventually leads to accumulation of secondary metabolites which may serve as effector molecules for MexT is still hypothetical (31, 36). Downstream of the *mexT* gene, the *mexT-mexE* intergenic DNA contains two *nod* boxes (31), which were recently identified as a MexT-binding site (*nod* box 1) and as part of the *mexEF-oprN* promoter (*nod* box 2) (41). Adding to this complex regulation of MexEF-OprN, it was finally reported that mutations in the *mvaT* gene enhanced *mexEF-oprN* expression (61). MvaT belongs to the histone-like nucleoid structuring protein (H-NS) family and is a global

* Corresponding author. Mailing address: Laboratoire de Bactériologie, Hôpital Jean Minjoz, 25030 Besançon Cedex, France. Phone: (33) 3 81 66 82 86. Fax: (33) 3 81 66 89 14. E-mail: patrick.plesiat@univ-fcomte.fr.

▽ Published ahead of print on 12 September 2011.

regulator involved in quorum-sensing-dependent responses and biofilm formation (8).

The goal of the present work was to gain information about the mechanisms involved in low-level resistance to fluoroquinolones in *P. aeruginosa* with emphasis on the characteristics of *nfxC* mutants found in the clinical setting.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Eighty-five nonredundant clinical strains of *P. aeruginosa* collected between 2004 and 2007, and exhibiting a low-level resistance to ciprofloxacin (MIC from 0.25 to 2 μg/ml) were selected from our laboratory collection. According to the Clinical and Laboratory Standards Institute (CLSI) breakpoints (7), the strains are considered susceptible (MIC, ≤1 μg/ml) or of intermediate susceptibility (MIC, 2 μg/ml) to ciprofloxacin. These bacteria were isolated from blood ($n = 25$), sputum ($n = 23$), superficial body sites ($n = 18$), urine ($n = 13$), bronchoalveolar lavage fluid ($n = 2$), and other sources ($n = 4$). The well-characterized mutants PT629 (10), MutGR1 (60), EryR (18), and PAO7H (32) that all derive from wild-type reference strain PAO1-UW (PAO1 University of Washington) (55) were used as positive controls in reverse transcription real-time quantitative PCR (RT-qPCR) experiments for identification of gain-of-efflux mutants overexpressing MexAB-OprM, MexXY/OprM, MexCD-OprJ, and MexEF-OprN pumps, respectively. Plasmid pMEQR1 (this study) which carries the wild-type *mexS* gene from PAO1-UW, cloned in the broad-host range vector pME6001 (4), was employed to complement clinical *nfxC* strains. Three wild-type susceptible reference strains (PAO1-UW, PA14, and ATCC 27853) as well as 7 wild-type susceptible clinical strains collected in our hospital (MAR-1, BOU-1, SCL-1, SCH-1, VIT-1, LAU-1, and PUB-1), and 10 efflux overproducers selected from the present collection (32.1, 37.1, 40.1, 43.1, 3994, 4070, 4016, 4173, 4175, and 4177) were used for selection of ciprofloxacin-resistant mutants *in vitro*. Unless otherwise specified, all of the bacterial strains were cultivated on Mueller-Hinton agar (MHA) or in Mueller-Hinton broth (MHB) (Bio-Rad, Marne-la-Vallée, France) at 37°C.

Drug susceptibility testing. The MICs of selected antibiotics were determined by the conventional macrodilution technique (6) in Mueller-Hinton agar containing adjusted concentrations of divalent cations (BBL, Cockeysville, MD). Since variations in MIC due to efflux pumps can be within a 2-fold range, MIC values were determined at least twice, in two independent experiments. The categorization of strains in S, I, and R was performed according to CLSI breakpoints (7).

Characterization of gain-of-efflux mutants. Constitutive upregulation of efflux pumps known to extrude fluoroquinolones (MexAB-OprM, MexXY/OprM, MexCD-OprJ, MexEF-OprN, MexGHI-OpmD, MexPQ-OpmE, MexVW/OprM, and PmpM) was assessed for the 85 clinical strains by RT-qPCR. The transcript levels of *mexB*, *mexY*, *mexC*, *mexE*, *mexG* and *mexV* genes were determined as reported previously (10, 23, 27, 35). Primers were designed by Primer3 software v.0.4.0 (http://frodo.wi.mit.edu/primer3) to quantify the mRNAs of the *mexQ* and *pmpM* genes (Table 1). The mRNA levels were normalized in each strain to that of the *rpsL* housekeeping gene (46) and expressed as ratios to the values of strain PAO1 (by definition, set at 1). The strains were considered overproducing mutants when their transcript levels of the *mexB*, *mexY*, *mexC*, or *mexE* gene were higher than the respective thresholds defined by Hocquet et al. (24). In the absence of positive controls or defined thresholds available for the *mexG*, *mexQ*, and *pmpM* genes, we arbitrarily considered these strongly repressed genes (in PAO1) (63) as overexpressed when their respective mRNA levels were at least 5-fold more than in PAO1. The concordance between the RT-qPCR results and the drug resistance phenotype was considered for each strain. In a previous study, MexAB-OprM overproduction (*mexB* gene transcripts ≥2-fold that of PAO1) was found to be correlated with a ≥4-fold increase in resistance to ticarcillin (MIC, ≥64 μg/ml) and aztreonam (MIC, ≥16 μg/ml) (37). Similarly, MexXY/OprM overproduction (*mexY* gene levels ≥5-fold that of PAO1) correlated either with a ≥2-fold increase in aminoglycoside resistance or with a cefepime/ceftazidime MIC ratio of ≥4 (23). MexEF-OprN and MexCD-OprJ overproducers exhibited *mexE* and *mexC* gene transcript levels ≥100-fold more than in PAO1, respectively (24). Compared to wild-type bacteria, both types of mutants showed a >2-fold hypersusceptibility to ticarcillin, aztreonam, and aminoglycosides (26, 32). Finally, overproduction of MexEF-OprN was specifically associated with a ≥4-fold increase in imipenem resistance, while upregulation of MexCD-OprJ resulted in a 2- to 4-fold-higher resistance to cefepime. The mRNA amounts of the *mexT* activator gene were assessed with *nfxC* mutants as previously described (61).

TABLE 1. Primers used for DNA sequencing, gene cloning, and RT-qPCR

Function and oligonucleotide name	Primer sequence (5′ to 3′)	Size (bp)	Reference
Sequencing			
nfxB gene (564 bp)			
nfxB-1	ACGCGAGGCCAGTTTTCT	731	26
nfxB-2	ACTGATCTTCCCGAGTGTCG		26
inter-nfxB1	GTCGCAGGCCTGGATGAT	502[a]	This study
inter-nfxB2	CTGCACCGTCAGGACCTC		This study
mexS (1,020 bp)			
PA2491-F	CGGATACAGTCACAACCCATGAG	1,157	53
PA2491-R	CGGTCAACGATCTGTGGATCTG		53
seqmexS-1	CAAGGGCGTCAATGTCATC		This study
seqmexS-2	GATGACATTGACGCCCTTG		This study
seqmexS-3	GGAGGCCAGCGTCTACTA		This study
seqmexS-4	GACACCGACGAGACCTTCAT		This study
mexT gene (915 bp)			
MexT-1	GTAGTAGACGCTGGCCTCCAC	1,921[b]	57
MexT-2	GTGAATTCGTCCCACTCGTTC		57
seqmexT-1	CTATTGATGCCGAACCTGCT		This study
seqmexT-2	AATAGTCGTCGAGGGTCAGC		This study
seqmexT-3	TGATGAAAACGGATCACTCG		This study
seqmexT-4	GGGAACTAATCGAACGAC		This study
mvaT gene (374 bp)			
MvaT-1	CGCGGTTTACTTACAGTTTCG	347	This study
MvaT-2	AACGCTATTCGCTGGAGACT		This study
Gene cloning			
mexS gene			
qrh-PB	GGCATAGGATCCCTGACAGGCA TAGC		This study
qrh-MH	GACCGGTATAAGCTTCGCGCA ATGG		This study
RT-qPCR			
mexQ gene			
mexQ-1	GCAGGTGACTACCGCCTATC	104	This study
mexQ-2	ATGTACAGCATCCCCTCGAC		This study
pmpM gene			
pmpM-3	AACATCCCGATCAACTACGC	131	This study
pmpM-4	CTTGTTCACCCAGAACAGCA		This study

[a] Intergenic region between the *nfxB* and *mexC* genes.
[b] Contains the *mexS-mexT* and *mexT-mexE* intergenic regions.

PCR conditions and DNA sequencing. A search for mutations in the quinolone resistance-determining regions (QRDRs) of GyrA, GyrB, ParC, and ParE was performed for the 85 clinical strains and for the ciprofloxacin-resistant mutants selected *in vitro*, according to the method described by Hocquet et al. (22). The *mexXY* operon was sequenced (58) for five strains exhibiting wild-type or increased susceptibility levels to aminoglycosides while overexpressing the *mexY* gene significantly. The *nfxB*, *mexT*, *mexS*, and *mvaT* genes and the *nfxB-mexC* and *mexT-mexE* intergenic regions were sequenced on both strands in strains with *mexCD-oprJ* or *mexEF-oprN* overexpression, respectively (Table 1).

Construction of pMEQR1 and complementation experiments. To construct plasmid pMEQR1, a 1.4 kb DNA fragment carrying the *mexS* gene from PAO1-UW was amplified with primers qrh-PB and qrh-MH (Table 1). After restriction with BamHI and SphI, the resulting fragment was inserted into BamHI-SphI-cleaved pUC119 (59) to yield plasmid pQRH1. A 1.4-kb KpnI-HindIII fragment from the latter construct was then subcloned into the broad-host range expression vector pME6001, yielding pMEQR1. In gene complementation experiments, pMEQR1 and pME6001 (Gm^r) were transferred by triparental mating into *nfxC* strains 40.1, 93.1, 3936, 3974, 4099, and 4198 by using *Escherichia coli* HB101(pRK2013) (Km^r) as a helper strain (30). Transconjugants were selected on MHA supplemented with gentamicin (30 μg/ml) and ampicillin (40 μg/ml) to counterselect *E. coli* donor strains.

Genotype analysis. To determine whether the 10 MexEF-OprN overproducers were clonally related, we analyzed their single-nucleotide polymorphism (SNP) types by using Clondiag chips (Alere, Sèvres, France), as previously described (62).

Serotype determination. Production of band B LPS, carrying the O antigen of *P. aeruginosa*, was assessed by slide agglutination of fresh colonies with specific antisera (Bio-Rad, Marne-la-Vallée, France). The O serotype was restored in nonagglutinable isolate 66.1 by culturing bacteria in MHB supplemented with amikacin at a 1:6 MIC (54).

TABLE 2. Susceptibility rates of the 85 clinical isolates of *P. aeruginosa*

Antibiotic	MIC for clinical isolates					MICs of PAO1-UW (μg/ml)
	MIC range (μg/ml)	Categorization (%)[a]			MIC_{50}/MIC_{90} (μg/ml)	
		S	I	R		
Ciprofloxacin	0.25–2	85.9	14.1	0	0.5/2	0.12
Levofloxacin	0.5–8	80	16.5	3.5	2/4	0.5
Ticarcillin	1–1,024	81.2	—	18.8	32/128	16
Ceftazidime	0.5–64	90.6	5.9	3.5	4/8	2
Cefepime	0.5–16	89.4	10.6	0	4/16	2
Aztreonam	1–64	54.1	30.6	15.3	8/32	4
Imipenem	0.5–16	85.9	7.0	7.1	2/8	1
Meropenem	0.03–16	96.5	2.3	1.2	1/2	0.5
Gentamicin	≤0.125–1,024	41.2	37.6	21.2	8/32	4
Tobramycin	0.125–256	90.6	3.5	5.9	2/4	1
Amikacin	0.5–64	95.3	2.3	2.4	8/16	8
Apramycin	1–256	—	—	—	32/64	16

[a] As defined by the CLSI breakpoints. S, susceptible; I, intermediate; R, resistant; —, the CLSI has not defined this category for this antibiotic.

***In vitro* selection of ciprofloxacin-resistant mutants.** Ten wild-type strains (MIC of ciprofloxacin, 0.12 μg/ml) and 10 characterized gain-of-efflux mutants (MIC, 0.5 μg/ml) were compared for their ability to become resistant to higher concentrations of ciprofloxacin. Approximately 5×10^9 bacteria collected at the logarithmic phase (optical density at 600 nm [OD_{600}], ~1) and at the stationary phase (OD_{600}, >3) of growth in MHB were plated on MHA plates containing 2 μg/ml or 4 μg/ml ciprofloxacin. In parallel, the initial inoculum was quantified by spreading appropriate dilutions on drug-free MHA and subsequent colony counting. After 36 h of incubation at 37°C, the resistant clones were counted, replicated on drug-free MHA, and finally streaked on ciprofloxacin (2 μg/ml or 4 μg/ml). Only those colonies developing on drug-containing plates after the second transfer were scored as resistant mutants. Their numbers were compared to those of the initial inoculum to calculate mutant frequencies. In each experiment, the resistance profiles of 10 randomly chosen colonies were established by the diffusion method in MHA with 16 antibiotic disks (Bio-Rad). Those exhibiting different resistance profiles were subsequently frozen at −70°C for further analysis (RT-qPCR determination of the transcript levels of *mexB*, *mexC*, *mexE* and *mexY* genes and analysis of the QRDR sequences). All of the experiments were carried out in triplicate from independent batches of culture.

RESULTS

***P. aeruginosa* strains with decreased susceptibility to ciprofloxacin.** In order to characterize the resistance mechanisms responsible for low-level fluoroquinolone resistance in *P. aeruginosa*, 85 nonrepetitive, non-cystic fibrosis (non-CF) clinical strains with ciprofloxacin MICs ranging from 0.25 to 2 μg/ml were selected from our laboratory collection. According to the CLSI breakpoints, 73 of 85 were "susceptible" (MIC, ≤1 μg/ml) and 12 of 85 were of "intermediate susceptibility" (MIC, 2 μg/ml) to this antibiotic. As reported previously (19), levofloxacin was 2- to 4-fold less effective than ciprofloxacin (Table 2). Ceftazidime, meropenem, tobramycin, and amikacin were the most active antibiotics tested (≥90% susceptible strains). Of note, only 19.8% of the isolates were susceptible to all the products tested except ciprofloxacin. Seventy of the 85 isolates displayed a significant increase in resistance (≥4-fold the MIC for reference strain PAO1-UW) to one or several aminoglycosides (n = 21 isolates), β-lactams (n = 52), and/or carbapenems (n = 32).

Mechanisms responsible for low resistance to ciprofloxacin. RT-qPCR experiments revealed a high prevalence (86%) of mutants overexpressing one (n = 64) or two (n = 9) efflux systems (Table 3). MexXY/OprM (n = 39) and MexAB-OprM

TABLE 3. Mechanisms involved in low-level ciprofloxacin resistance in the 85 clinical isolates

Resistance mechanisms[a]							No. of strains (n = 85)	Ciprofloxacin MIC range (μg/ml)
Efflux systems overproduced				Mutations in QRDRs		Unknown mechanism		
MexAB-OprM	MexXY	MexCD-OprJ	MexEF-OprN	GyrA	GyrB			
+	−	−	−	−	−	−	21	0.25–2
+	−	−	−	−	S466F	−	1	2
+	+	−	−	−	−	−	7	0.5–1
+	−	−	+	−	−	−	1	1
+	−	+*	−	−	−	−	1	0.5
−	+	−	−	−	−	−	32	0.25–2
−	−	+**	−	−	−	−	1	2
−	−	−	+	−	−	−	9	0.5–2
−	−	−	−	D87N	−	−	1	1
−	−	−	−	T83I	−	−	3	0.5–2
−	−	−	−	−	S466Y	−	1	1
−	−	−	−	−	S466F	−	1	2
−	−	−	−	−	−	+	6	0.25–2

[a] Sequencing of the *nfxB* gene, the repressor gene of MexCD-OprJ, revealed a A24E substitution in strain 4070 (indicated with an asterisk) located in the DNA binding domain of the NfxB protein, as well as a G180S change in strain 4044 (indicated with a double asterisk).

($n = 31$) were by far the most prevalent pumps derepressed in this collection. Confirming previous data on CF (25) and non-CF (26) strains, MexCD-OprJ overproducers were quite rare ($n = 2$). One of the two *nfxB* mutants (strain 4070) exhibited a low-level resistance to ticarcillin (MIC, 64 μg/ml) because of concomitant overproduction of MexAB-OprM (i.e., ticarcillin is a good substrate for the latter system). Expression of the *mexG*, *mexQ*, and *pmpM* genes were from 0.1 to 4.5-fold that of reference strain PAO1-UW, strongly suggesting that MexGHI-OpmD, MexPQ-OpmE, and PmpM, respectively were not activated in the selected strains.

Sequencing of the QRDRs revealed single mutations in the *gyrA* or *gyrB* gene in seven strains, with ciprofloxacin MICs ranging from 0.5 to 2 μg/ml (Table 3), but no mutations in the *parC* and *parE* genes. In strain 4065 (ciprofloxacin MIC, 2 μg/ml), the S466F alteration of subunit GyrB was concurrent with MexAB-OprM upregulation. In six strains (MICs ranging from 0.25 to 2 μg/ml), no fluoroquinolone resistance determinants could be identified despite complete sequencing of the *gyrA* gene (14) and a PCR search for *qnr* genes (5) (data not shown). Four of these strains exhibited a wild-type resistance phenotype, except with a lower susceptibility (2- to 4-fold) to fluoroquinolones compared with PAO1-UW. The other two strains (3990 and 4068) were nontypeable, suggesting LPS alterations (38).

Resistance profiles of the *nfxC* mutants. Interestingly, we found a relatively high number ($n = 10$ isolates) of MexEF-OprN overproducers in the collection, coming from superficial body sites (3963, 3974, 4099, 4177, 4198), blood cultures (40.1, 93.1, 4088), or respiratory samples (3936, 4076). Genotyping of these strains using the Clondiag chips showed that none of them shared the same SNP profile (Table 4). Similar to typical *in vitro*-selected *nfxC* mutants, the 10 isolates exhibited a 4- to 16-fold-higher resistance to ciprofloxacin and imipenem than PAO1-UW. Furthermore, 6/10 strains were hypersusceptible to the other β-lactams tested owing to impaired *mexAB-oprM* expression (from 2- to 100-fold less than in PAO1) (Table 4). In the four remaining bacteria, the hypersusceptibility to β-lactams was masked by MexAB-OprM being overproduced (Table 4, strain 3974) or by derepression of intrinsic cephalosporinase AmpC (strains 93.1, 4076, and 4177). This was demonstrated by the complete reversion of β-lactam resistance by growth in the presence of AmpC inhibitor cloxacillin at 1,000 μg/ml (data not shown). As already noted for *in vitro* selected mutants, 7 of 10 strains were hypersusceptible to aminoglycosides and exhibited decreased expression of the *mexXY* operon (*mexY* gene levels ≤2-fold that of PAO1-UW) (16, 53). The 22-fold-higher expression of the *mexY* gene in one strain (3974) was not associated with a higher resistance to aminoglycosides possibly because of multiple mutations affecting protein MexY (Y181D, Q282R, Q866H, S874P) in this isolate.

Role of MexT in MexEF-OprN upregulation. Nucleotide sequencing of the *mexT* gene revealed that all of the 10 *nfxC* mutants had the 8-bp (119- to 126-bp) deletion known to result in reversion of the nonsense *mexT* gene (in PAO1-UW) to the sense open reading frame (like in PAO7H and PA14). In two strains (4088 and 4177), the MexT amino acid sequence was found to exhibit mutations (G257S and R166H compared with PAO7H, respectively), which were not predicted by the SIFT

TABLE 4. Characterization of the MexEF-OprN overproducers[a]

Strain	MIC (μg/ml)											Genotype (SNP)	Alteration(s) in regulators of MexEF-OprN[b]			mRNA levels for indicated gene			
	CIP	LVX	GEN	TOB	AMK	APR	FEP	TIC	CAZ	ATM	IMP	MEM		MexS	MexT	MvaT	*mexE*	*mexT*	*mexB*	*mexY*
Reference strains																				
PAO1-UW	0.12	0.5	4	1	8	16	2	16	2	4	1	0.5	ND	‡[d]		‡	1	1	1	1
PAO7H	2	8	4	0.5	4	16	2	8	2	1	4	2	0002	‡	‡[e]	‡	1,075	1	0.5	2.1
Clinical strains																				
40.1	0.5	2	4	1	4	16	1	8	1	2	4	1	239A		*	‡	446	0.4	0.01	1
93.1	1	4	2	0.5	4	8	8	64	32	16	4	2	2812	E54G, G78S, †[c]	*	‡	1,081	0.4	0.5	0.1
3936	2	4	2	0.5	2	8	0.5	4	0.5	≤2	4	0.5	859A	A75V, †	*	‡	1,760	0.3	0.4	0.5
3963	1	2	4	1	2	16	1	8	1	1	4	0.5	E882	†	*	‡	324	0.2	0.4	0.6
3974	1	4	4	1	8	16	8	64	4	16	4	4	6992	A75V, †	*	‡	128	0.3	2.3	22
4076	2	4	0.5	0.12	1	8	4	32	16	16	8	2	882A		*	‡	2,281	0.3	0.6	0.2
4088	2	4	2	0.5	2	8	1	8	1	1	8	0.25	B421	†	G257S,*	‡	678	0.3	0.6	0.6
4099	0.5	1	2	0.25	1	4	1	8	0.5	2	4	0.5	A671	T152A, †	*	Δ1bp (230)	1,035	0.2	0.5	0.5
4177	0.5	1	8	0.5	2	8	4	64	16	32	16	2	802A	†	R166H,*	‡	510	0.2	0.4	0.4
4198	2	4	1	0.25	1	8	1	8	1	1	4	1	F42A	K17T, A175V, E181D, †, S289T, V308I, V318I	*	A115T	1,013	0.5	0.4	0.6

[a] CIP, ciprofloxacin; LVX, levofloxacin; GEN, gentamicin; TOB, tobramycin; AMK, amikacin; APR, apramycin; FEP, cefepime; TIC, ticarcillin; CAZ, ceftazidime; ATM, aztreonam; IMP, imipenem; MEM, meropenem. ND, not determined.

[b] Amino acid positions refer to strain PAO1-UW (http://www.pseudomonas.com).

[c] †, the D249N substitution was considered nonsignificant because it was found also in PA14 and in seven environmental susceptible strains (data not shown).

[d] ‡, identical to the PAO1-UW sequence.

[e] *, active *mexT* gene missing the 8-bp insert (34) found in PAO1-UW.

TABLE 5. Complementation of the MexEF-OprN overproducers with the *mexS* gene[a]

Strain	MIC (μg/ml)											*mexE* gene expresssion level
	CIP	LVX	TOB	AMK	APR	FEP	TIC	CAZ	ATM	IMP	MEM	
40.1 (pME6001)	0.5	2	1	4	8	1	8	1	2	4	1	2,115
40.1 (pMEQR1)	**0.25**	**1**	2	8	16	1	8	1	2	4	1	420
93.1 (pME6001)	1	4	1	4	8	16	64	32	16	4	2	1,912
93.1 (pMEQR1)	**0.25**	**1**	2	8	16	32	128	64	32	4	2	290
3936 (pME6001)	2	4	2	2	8	0.5	4	1	1	4	0.5	2,301
3936 (pMEQR1)	**0.5**	**1**	2	4	16	1	8	1	2	2	0.25	469
3974 (pME6001)	1	4	2	8	16	8	64	4	16	4	4	94
3974 (pMEQR1)	1	4	2	8	16	8	64	4	16	4	4	81
4099 (pME6001)	0.5	**1**	0.5	1	4	1	8	0.5	2	4	0.5	1,653
4099 (pMEQR1)	**0.25**	**0.5**	1	2	4	1	8	0.5	2	4	0.5	240
4198 (pME6001)	2	4	1	1	4	1	8	1	2	4	1	2,084
4198 (pMEQR1)	2	4	1	1	4	1	8	1	2	4	1	2,192

[a] Values in boldface indicate that complementation with the *mexS* gene succeeded in restoring the susceptibility to fluoroquinolones. CIP, ciprofloxacin; LVX, levofloxacin; TOB, tobramycin; AMK, amikacin; APR, apramycin; FEP, cefepime; TIC, ticarcillin; CAZ, ceftazidime; ATM, aztreonam; IMP, imipenem; MEM, meropenem.

algorithm (http://sift.jcvi.org/www/SIFT_BLink_submit.html) to affect the protein's function.

All the 10 *nfxC* mutants appeared to express the *mexT* gene 2- to 5-fold less than PAO1-UW or PAO7H (Table 4), which cannot account for *mexEF-oprN* upregulation, since the protein MexT basically acts as an activator (31). More interestingly, two strains (4088 and 4099) exhibited a *mexT*-proximal G156A mutation in *nod* box 2. This mutation is located in the conserved DNA motif ATCA(N5)GTCGTA(N4)ACYAT predicted *in silico* to be a regulatory region of *P. aeruginosa* genes whose expression is induced by MexT (57).

Role of MexS in MexEF-OprN upregulation. Sequence analysis of the *mexS* gene predicted a D249N substitution in protein MexS in all of our *nfxC* mutants compared with the PAO1-UW sequence (Table 4). However, this amino acid variation, which also occurs in wild-type strain PA14, was found in seven drug-susceptible environmental strains of *P. aeruginosa* from our laboratory collection (data not shown). More interestingly, 5 of 10 *nfxC* mutants exhibited additional amino acid changes in MexS (Table 4). The A175V, E181D, and V308I substitutions in strain 4198 were also identified in the multidrug resistant isolate PA7 (http://www.pseudomonas.com). To evaluate the impact of these different sequence variations on MexEF-OprN expression, we tried to complement the mutants with a plasmid-borne *mexS* gene from strain PAO1-UW (Table 5). Plasmid pMEQR1 was successfully transferred into strains 40.1, 93.1, 3936, 3974, 4099, and 4198. In 40.1, 93.1, 3936 (which harbors a wild-type *mexS* gene), and 4099, the wild-type susceptibility to fluoroquinolones was partially restored and the *mexE* mRNA levels decreased about 6-fold upon complementation (Table 5). Futhermore, 93.1 and 3936 displayed a 2-fold increase in resistance to specific substrates of the MexXY/OprM (tobramycin, amikacin, apramycin) and MexAB-OprM pumps (ticarcillin, aztreonam). In 40.1 and 4099, the gain in resistance was limited to aminoglycosides. Contrasting with these results, transfer of plasmid pMEQR1 had virtually no effect on the resistance profiles and *mexE* levels of isolates 3974 and 4198. Note that for unclear reasons, pMEQR1 was unable to influence the bacterial resistance to carbapenems except in strain 3936.

Mutations in MvaT. One *nfxC* mutant (4099) appeared to harbor an inactive *mvaT* gene because of a frameshifting deletion while another mutant (4198) contained a A115T substitution in the MvaT protein, which was predicted to be tolerated by the SIFT algorithm (Table 4). As indicated in Table 5, overexpression of the wild-type *mexS* gene from pMEQR1 allowed the partial recovery of wild-type susceptibility in 4099, thus overriding the possible effect of the inactivated *mvaT* gene on *mexEF-oprN* expression.

Selection of MexEF-OprN mutants by ciprofloxacin. As shown above, activation of drug extrusion mechanisms constitutes a very common way of allowing *P. aeruginosa* to achieve low-level resistance to fluoroquinolones. An important clinical issue is to know whether these first-step mutants are more prone to generate highly resistant bacteria than wild-type strains when exposed to these agents *in vivo* (3). To test this hypothesis, we first compared the ability of 10 wild-type strains (MIC of ciprofloxacin, 0.12 μg/ml) and 10 efflux pump overproducers (MIC, 0.5 μg/ml) to give rise to mutants able to grow on ciprofloxacin 2 μg/ml. Mutation frequencies ranging from 10^{-10} to 10^{-8} were observed in both cases, suggesting a weak impact of preexisting efflux mechanisms on the emergence of more highly resistant *P. aeruginosa* (mean mutation frequencies of 3.1×10^{-9} for wild-type strains versus 4.4×10^{-9} for efflux overproducers) (Table 6). Of note, two strains (3994 and 4070) exhibiting relatively high mutation frequencies were isolated from chronic infections, as noted previously (21). Growth on ciprofloxacin (2 μg/ml) selected MexEF-OprN gain-of-efflux mutants in 8 of 10 wild-type strains, regardless of the growth conditions (exponential or stationary phase). One strain (SCH1) exhibited a substitution in GyrA in addition to MexEF-OprN-mediated efflux. Starting with efflux pump-overproducing mutants, growth on 2 μg/ml ciprofloxacin led mainly to the recovery of mutants coexpressing simultaneously two or three Mex systems (Table 6). Again, most of these secondary mutants (6 of 10) were of the *nfxC* type, but the emergence of alterations in QRDRs was higher than in the wild-type population (8 mutants versus 2), especially when the mutants were selected from stationary-phase bacteria (6 of 10 strains). Emergence of both drug target alterations and additional drug efflux

TABLE 6. Analysis of mutants selected on ciprofloxacin, 2 μg/ml[a]

Strain	Background	Exponentially growing cells			Stationary-phase cells		
		Mutation frequencies[b]	Additional resistance mechanisms	CIP MIC (μg/ml)	Mutation frequencies[b]	Additional resistance mechanisms	CIP MIC (μg/ml)
Wild-type strains (CIP MICs, 0.12 μg/ml)							
PAO1		$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
PA14		1×2.10^{-8}	EFN	4	3.5×10^{-8}	EFN	4
CIP76110		6.6×10^{-10}	EFN	4	$<2 \times 10^{-10}$		
BOU1		1.0×10^{-9}	EFN	4	4.6×10^{-9}	EFN	4
LAU1		9.3×10^{-9}	EFN	4	4.0×10^{-10}	EFN	8
MAR1		1.3×10^{-10}	CDJ	4	$<2 \times 10^{-10}$		
PUB1		1.2×10^{-9}	Unknown	4	4.2×10^{-8}	Unknown	8
SCL1		4.2×10^{-9}	EFN	4	2.7×10^{-8}	EFN	4
SCH1		1.6×10^{-9}	EFN	4	4.2×10^{-9}	EFN + T83I (GyrA)	16
			EFN + D87N (GyrA)	8			
VIT1		1.6×10^{-9}	EFN	4	8.0×10^{-10}	EFN	4
Efflux overproducers (CIP MICs, 0.5 μg/ml)							
32.1	ABM	3.3×10^{-10}	EFN	4	4.0×10^{-10}	EFN + D87Y (GyrA)	16
37.1	ABM + XY	2.6×10^{-9}	EFN	4	4.0×10^{-10}	EFN	4
40.1	EFN	1.3×10^{-10}	T83I (GyrA)	8	6.0×10^{-10}	S466Y (GyrB)	6
43.1	XY	1.2×10^{-9}	EFN	4	4.8×10^{-9}	T83I (GyrA)	8
3994	ABM + XY	1.7×10^{-8}	CDJ[c]	4	1.4×10^{-8}	EFN[c]	4
			EFN[c]	4			
4016	XY	3.4×10^{-9}	CDJ	4	3.4×10^{-9}	T83I (GyrA)	8
			ABM + D87Y (GyrA)	8			
4070	CDJ	7.2×10^{-9}	EFN	4	2.0×10^{-10}	EFN	4
4173	XY	6.6×10^{-10}	EFN	4	8.0×10^{-10}	T83I (GyrA)	16
4175	ABM	$<2 \times 10^{-10}$			6.0×10^{-10}	T83I (GyrA)	8
4177	EFN	1.1×10^{-8}	No[d]	4	3.9×10^{-9}	No[d]	4

[a] CIP, ciprofloxacin; ABM, MexAB-OprM; CDJ, MexCD-OprJ; EFN, MexEF-OprN; XY, MexXY(OprM). Mutations in the QRDR regions are underlined.
[b] Calculated from an initial inoculum of 5×10^9 bacteria (experiments performed in triplicate). The value $<2 \times 10^{-10}$ indicates that no colonies were obtained on selective plates.
[c] In these mutants, the *mexXY* operon was no longer overexpressed.
[d] No additional mechanism.

mechanisms was observed for strains 32.1 and 4016, indicating an accumulation of independent mutational events during the selection. The MexEF-OprN-overproducing strain 4177 was the only one able to increase its resistance to ciprofloxacin without the cooperation of other known mechanisms (Table 6). In the 4177-derived mutants (MIC of ciprofloxacin, >2 μg/ml), the transcript levels of the *mexE* gene were ca. 5-fold higher than in the parental strain 4177 (MIC, 0.5 μg/ml). This further increase in *mexEF-oprN* expression was associated with a nucleotide change in the *mexS* gene leading to a C269Y substitution.

With 4 μg/ml ciprofloxacin (Table 7), only 3 of 10 wild-type strains and 6 of 10 efflux overproducers gave rise to more resistant mutants (mean mutation frequency of 8.4×10^{-10}). While efflux mechanisms were systematically selected from exponentially growing cells, thus increasing the MICs of ciprofloxacin to 8 μg/ml, only QRDR mutants arose from stationary-phase cells, resulting in MICs of up to 32 μg/ml. Interestingly, in both situations, two mechanisms were needed to grow on such a high concentration of ciprofloxacin.

DISCUSSION

This work shows that a number of strains considered susceptible to ciprofloxacin with respect to the CLSI breakpoints (≤1 μg/ml) actually express one or several low-level resistance mechanisms to fluoroquinolones. According to some pharmacokinetic/pharmacodynamic (PK/PD) studies, MICs of 0.25 and 0.5 μg/ml would be sufficient to impair the therapeutic efficacy of ciprofloxacin (11). For the first time, we demonstrate that in addition to MexAB-OprM and MexXY/OprM, the MexEF-OprN efflux pump contributes significantly to this low-level resistance in non-CF clinical strains of *P. aeruginosa*. Previous studies of *nfxC* mutants selected *in vitro* from various PAO1 sublines have shown that alterations in the *mexT*, *mexS*, or *mvaT* gene may result in MexEF-OprN overproduction (34, 53, 61). All of the clinical *nfxC* mutants reported here turned out to harbor a *mexT* gene lacking the frameshifting 8-bp insert found in PAO1-UW and a synonymous D249N change in the putative oxidoreductase MexS. To which extent the other amino acid variations found in MexT (strains 4088 and 4177), in MexS (strains 40.1, 93.1, 3974, 4099, and 4198), and/or in MvaT (strain 4198) may account for the *nfxC* resistance profile is still unclear. Indeed, overexpression of the *mexS* gene from plasmid pMEQR1 was sufficient by itself to partially restore a wild-type susceptibility to fluoroquinolones, β-lactams, and aminoglycosides in isolate 3936 that harbors wild-type *mexT*, *mexS*, and *mvaT* genes. Of note, none of these genes (except the *mvaT* gene in isolate 4099) appeared to be disrupted by frameshift deletions or insertions in the *nfxC*

TABLE 7. Analysis of mutants selected on ciprofloxacin, 4 μg/ml[a]

Strain	Background	Exponentially growing cells			Stationary-phase cells		
		Mutation frequencies[b]	Additional resistance mechanisms	CIP MIC (μg/ml)	Mutation frequencies[b]	Additional resistance mechanisms	CIP MIC (μg/ml)
Wild-type strains (CIP MICs, 0.12 μg/ml)							
PAO1		$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
PA14		3.5×10^{-10}	EFN + XY	8	$<2 \times 10^{-10}$		
CIP76110		$<2 \times 10^{-10}$			2×10^{-10}	EFN + T83I (GyrA)	8
BOU1		$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
LAU1		$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
MAR1		$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
PUB1		$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
SCL1		$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
SCH1		$<2 \times 10^{-10}$			4×10^{-10}	EFN + T83I (GyrA)	16
VIT1		$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
Efflux overproducers (CIP MICs, 0.5 μg/ml)							
32.1	ABM	$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
37.1	ABM + XY	$<2 \times 10^{-10}$			2×10^{-10}	T83I (GyrA)	16
40.1	EFN	$<2 \times 10^{-10}$			2×10^{-10}	T83I (GyrA)	32
43.1	XY	1.8×10^{-9}	EFN	8	2×10^{-10}	T83I (GyrA)	8
3994	ABM + XY	2.3×10^{-9}	EFN	8	$<2 \times 10^{-10}$		
4016	XY	$<2 \times 10^{-10}$			1.8×10^{-9}	T83I (GyrA)	16
4070	CDJ	$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
4173	XY	$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
4175	ABM	$<2 \times 10^{-10}$			$<2 \times 10^{-10}$		
4177	EFN	9.3×10^{-10}	No[c]	8	$<2 \times 10^{-10}$		

[a] CIP, ciprofloxacin; ABM, MexAB-OprM; CDJ, MexCD-OprJ; EFN, MexEF-OprN; XY, MexXY(OprM). Mutations in the QRDR regions are underlined.
[b] Calculated from an initial inoculum of 5×10^9 bacteria (experiments performed in triplicate). The value $<2 \times 10^{-10}$ indicates that no colonies were obtained on selective plates.
[c] No additional mechanism.

strains, as it is often the case for regulatory loci in mutants overproducing MexAB-OprM, MexXY/OprM, or MexCD-OprJ (36). Furthermore, according to previous data (61), disruption of the *mvaT* gene should result in supersusceptibility to carbapenems instead of resistance, as in 4099 (Table 4). Recently, sequencing analysis of *in vitro*-selected *nfxC* mutants from PAO1-UW allowed us to identify of a P19S change in regulator MexT as a cause of *mexEF-oprN* overexpression and multidrug resistance (D. Fournier, unpublished data). The mutated position is located near the helix-turn-helix (HTH) motif (positions 26 to 38) of the DNA binding domain in MexT. In contrast, the G257S and R166H substitutions found in strains 4088 and 4177, respectively, map outside this HTH motif as well as outside the putative LysR substrate-binding domain of MexT (from amino acid 48 to 113). The regulation of MexEF-OprN expression thus appears much more complex than anticipated and requires further investigation.

nfxC mutants have rarely been reported in the clinical setting (45, 48), perhaps because of their impaired virulence/cytotoxicity or because they were underrecognized. Indeed, as noted here, the typical *nfxC* phenotype may be masked in some strains by additional resistance mechanisms involving efflux systems, β-lactamase production, and/or drug target alterations. The negative impact of *nfxC* mutations on bacterial virulence is complex, as it involves MexT-dependent and MexT-independent pathways (57). Our observation that three of our *nfxC* isolates were involved in bacteremic episodes is not sufficient by itself to rule out these findings, because susceptibility to pseudomonal infections depends primarily on the patient's condition (e.g., low leukocyte counts) (2). We are currently analyzing the virulence phenotype of the mutants identified in this study.

Our *in vitro* selection experiments demonstrate that when overproduced, MexEF-OprN allows *P. aeruginosa* to develop higher resistance levels to fluoroquinolones (MICs of ciprofloxacin, up to 8 μg/ml), either from wild-type bacteria or from first-step resistant mutants. Why *nfxC* mutants are selected at higher rates than *nfxB* mutants on ciprofloxacin is still unclear, as MexCD-OprJ tends to provide *P. aeruginosa* with higher resistance levels to this agent than MexEF-OprN (33). A trivial explanation could be that in addition to its role in drug efflux, MexEF-OprN may alleviate the oxidative stress induced by ciprofloxacin (12). In support of this hypothesis, MexEF-OprN expression is activated by the nitrosative (13) or oxidative stress (15, 29). In stationary-phase cells, the SOS response triggered by fluoroquinolones may incidentally increase the mutation rates in QRDRs and thus antibiotic resistance levels (50).

In conclusion, the prevalence of *nfxC* mutants has probably been underestimated in non-cystic fibrosis patients. As indicated in Table 3, the clinical *nfxC* mutants were found to exhibit a resistance range to ciprofloxacin similar to that of the MexAB-OprM or MexXY/OprM surexpressors. According to this, such *nfxC* mutants are not expected to have a selective advantage over the other Mex mutants in patients under treatment. On the other hand, our *in vitro*-selected *nfxC* mutants appeared to be much more resistant than the *nfxC* clinical

strains (MIC of 4 to 8 μg/ml versus 0.5 to 2 μg/ml) (Tables 6 and 7). A plausible explanation could be that different *nfxC* mutations may result in different resistance levels to ciprofloxacin. Whether these different hypothetical mutations may have the same impact on the virulence of *P. aeruginosa* warrants further studies, since it is possible that some mutants are not clinically relevant.

REFERENCES

1. **Akasaka, T., M. Tanaka, A. Yamaguchi, and K. Sato.** 2001. Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. Antimicrob. Agents Chemother. **45:**2263–2268.
2. **Baltch, A. L.** 1994. *Pseudomonas aeruginosa* bacteremia, p. 73–128. *In* A. L. Baltch and R. P. Smith (ed.), *Pseudomonas aeruginosa* infections and treatment. Marcel Dekker, New York, NY.
3. **Baquero, F.** 2001. Low-level antibacterial resistance: a gateway to clinical resistance. Drug Resist Updat. **4:**93–105.
4. **Blumer, C., S. Heeb, G. Pessi, and D. Haas.** 1999. Global GacA-steered control of cyanide and exoprotease production in *Pseudomonas fluorescens* involves specific ribosome binding sites. Proc. Natl. Acad. Sci. U. S. A. **96:**14073–14078.
5. **Cattoir, V., L. Poirel, V. Rotimi, C. J. Soussy, and P. Nordmann.** 2007. Multiplex PCR for detection of plasmid-mediated quinolone resistance *qnr* genes in ESBL-producing enterobacterial isolates. J. Antimicrob. Chemother. **60:**394–397.
6. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 7th ed., vol. 26. M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
7. **Clinical and Laboratory Standards Institute.** 2008. Performance standards for antimicrobial susceptibility testing, 18th informational supplement, vol. 27. M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
8. **Diggle, S. P., K. Winzer, A. Lazdunski, P. Williams, and M. Camara.** 2002. Advancing the quorum in *Pseudomonas aeruginosa*: MvaT and the regulation of *N*-acylhomoserine lactone production and virulence gene expression. J. Bacteriol. **184:**2576–2586.
9. **Drlica, K., M. Malik, R. J. Kerns, and X. Zhao.** 2008. Quinolone-mediated bacterial death. Antimicrob. Agents Chemother. **52:**385–392.
10. **Dumas, J. L., C. van Delden, K. Perron, and T. Kohler.** 2006. Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. FEMS Microbiol. Lett. **254:**217–225.
11. **Dupont, P., D. Hocquet, K. Jeannot, P. Chavanet, and P. Plésiat.** 2005. Bacteriostatic and bactericidal activities of eight fluoroquinolones against MexAB-OprM-overproducing clinical strains of *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **55:**518–522.
12. **Dwyer, D. J., M. A. Kohanski, B. Hayete, and J. J. Collins.** 2007. Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. Mol. Syst. Biol. **3:**91.
13. **Fetar, H., et al.** 2011. *mexEF-oprN* multidrug efflux operon of *Pseudomonas aeruginosa*: regulation by the MexT activator in response to nitrosative stress and chloramphenicol. Antimicrob. Agents Chemother. **55:**508–514.
14. **Friedman, S. M., T. Lu, and K. Drlica.** 2001. Mutation in the DNA gyrase A gene of *Escherichia coli* that expands the quinolone resistance-determining region. Antimicrob. Agents Chemother. **45:**2378–2380.
15. **Frisk, A., et al.** 2004. Transcriptome analysis of *Pseudomonas aeruginosa* after interaction with human airway epithelial cells. Infect. Immun. **72:**5433–5438.
16. **Fukuda, H., M. Hosaka, K. Hirai, and S. Iyobe.** 1990. New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **34:**1757–1761.
17. **Griffith, D. C., et al.** 2006. Pharmacodynamics of levofloxacin against *Pseudomonas aeruginosa* with reduced susceptibility due to different efflux pumps: do elevated MICs always predict reduced in vivo efficacy? Antimicrob. Agents Chemother. **50:**1628–1632.
18. **Hamzehpour, M. M., J. C. Pechère, P. Plésiat, and T. Kohler.** 1995. OprK and OprM define two genetically distinct multidrug efflux systems in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**2392–2396.
19. **Hansen, G. T., X. Zhao, K. Drlica, and J. M. Blondeau.** 2006. Mutant prevention concentration for ciprofloxacin and levofloxacin with *Pseudomonas aeruginosa*. Int. J. Antimicrob. Agents **27:**120–124.
20. **He, G. X., et al.** 2004. An H^+-coupled multidrug efflux pump, PmpM, a member of the MATE family of transporters, from *Pseudomonas aeruginosa*. J. Bacteriol. **186:**262–265.
21. **Henrichfreise, B., I. Wiegand, W. Pfister, and B. Wiedemann.** 2007. Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. Antimicrob. Agents Chemother. **51:**4062–4070.
22. **Hocquet, D., X. Bertrand, T. Kohler, D. Talon, and P. Plésiat.** 2003. Genetic and phenotypic variations of a resistant *Pseudomonas aeruginosa* epidemic clone. Antimicrob. Agents Chemother. **47:**1887–1894.
23. **Hocquet, D., P. Nordmann, F. El Garch, L. Cabanne, and P. Plésiat.** 2006. Involvement of the MexXY-OprM efflux system in emergence of cefepime resistance in clinical strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **50:**1347–1351.
24. **Hocquet, D., M. Roussel-Delvallez, J. D. Cavallo, and P. Plésiat.** 2007. MexAB-OprM- and MexXY-overproducing mutants are very prevalent among clinical strains of *Pseudomonas aeruginosa* with reduced susceptibility to ticarcillin. Antimicrob. Agents Chemother. **51:**1582–1583.
25. **Jalal, S., O. Ciofu, N. Hoiby, N. Gotoh, and B. Wretlind.** 2000. Molecular mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrob. Agents Chemother. **44:**710–712.
26. **Jeannot, K., et al.** 2008. Resistance and virulence of *Pseudomonas aeruginosa* clinical strains overproducing the MexCD-OprJ efflux pump. Antimicrob. Agents Chemother. **52:**2455–2462.
27. **Jeannot, K., M. L. Sobel, F. El Garch, K. Poole, and P. Plésiat.** 2005. Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribosome interaction. J. Bacteriol. **187:**5341–5346.
28. **Jin, Y., H. Yang, M. Qiao, and S. Jin.** 2011. MexT regulates the type III secretion system through MexS and PtrC in *Pseudomonas aeruginosa*. J. Bacteriol. **193:**399–410.
29. **Juhas, M., et al.** 2004. Global regulation of quorum sensing and virulence by VqsR in *Pseudomonas aeruginosa*. Microbiology **150:**831–841.
30. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70:**191–197.
31. **Kohler, T., S. F. Epp, L. K. Curty, and J. C. Pechère.** 1999. Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. J. Bacteriol. **181:**6300–6305.
32. **Kohler, T., et al.** 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. **23:**345–354.
33. **Kohler, T., M. Michea-Hamzehpour, P. Plésiat, A. L. Kahr, and J. C. Pechère.** 1997. Differential selection of multidrug efflux systems by quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **41:**2540–2543.
34. **Kohler, T., C. van Delden, L. K. Curty, M. M. Hamzehpour, and J. C. Pechère.** 2001. Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. J. Bacteriol. **183:**5213–5222.
35. **Li, Y., et al.** 2003. A new member of the tripartite multidrug efflux pumps, MexVW-OprM, in *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **52:**572–575.
36. **Lister, P. D., D. J. Wolter, and N. D. Hanson.** 2009. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin. Microbiol. Rev. **22:**582–610.
37. **Llanes, C., et al.** 2004. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. Antimicrob. Agents Chemother. **48:**1797–1802.
38. **Masecar, B. L., R. A. Celesk, and N. J. Robillard.** 1990. Analysis of acquired ciprofloxacin resistance in a clinical strain of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **34:**281–286.
39. **Maseda, H., K. Saito, A. Nakajima, and T. Nakae.** 2000. Variation of the *mexT* gene, a regulator of the MexEF-OprN efflux pump expression in wild-type strains of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **192:**107–112.
40. **Maseda, H., et al.** 2004. Enhancement of the *mexAB-oprM* efflux pump expression by a quorum-sensing autoinducer and its cancellation by a regulator, MexT, of the *mexEF-oprN* efflux pump operon in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **48:**1320–1328.
41. **Maseda, H., M. Uwate, and T. Nakae.** 2010. Transcriptional regulation of the *mexEF-oprN* multidrug efflux pump operon by MexT and an unidentified repressor in *nfxC*-type mutant of *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **311:**36–43.
42. **Masuda, N., et al.** 2000. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:**3322–3327.
43. **Mima, T., H. Sekiya, T. Mizushima, T. Kuroda, and T. Tsuchiya.** 2005. Gene cloning and properties of the RND-type multidrug efflux pumps MexPQ-OpmE and MexMN-OprM from *Pseudomonas aeruginosa*. Microbiol. Immunol. **49:**999–1002.
44. **Mouneimne, H., J. Robert, V. Jarlier, and E. Cambau.** 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**62–66.
45. **Oh, H., J. Stenhoff, S. Jalal, and B. Wretlind.** 2003. Role of efflux pumps and mutations in genes for topoisomerases II and IV in fluoroquinolone-resistant *Pseudomonas aeruginosa* strains. Microb. Drug Resist. **9:**323–328.
46. **Perron, K., et al.** 2004. CzcR-CzcS, a two-component system involved in

heavy metal and carbapenem resistance in *Pseudomonas aeruginosa*. J. Biol. Chem. **279:**8761–8768.
47. **Poole, K.** 2000. Efflux-mediated resistance to fluoroquinolones in gram-negative bacteria. Antimicrob. Agents Chemother. **44:**2233–2241.
48. **Pumbwe, L., and L. J. Piddock.** 2000. Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **44:**2861–2864.
49. **Rejiba, S., A. Aubry, S. Petitfrere, V. Jarlier, and E. Cambau.** 2008. Contribution of ParE mutation and efflux to ciprofloxacin resistance in *Pseudomonas aeruginosa* clinical isolates. J. Chemother. **20:**749–752.
50. **Riesenfeld, C., M. Everett, L. J. Piddock, and B. G. Hall.** 1997. Adaptive mutations produce resistance to ciprofloxacin. Antimicrob. Agents Chemother. **41:**2059–2060.
51. **Sekiya, H., et al.** 2003. Functional cloning and characterization of a multidrug efflux pump, MexHI-OpmD, from a *Pseudomonas aeruginosa* mutant. Antimicrob. Agents Chemother. **47:**2990–2992.
52. Reference deleted.
53. **Sobel, M. L., S. Neshat, and K. Poole.** 2005. Mutations in PA2491 (*mexS*) promote MexT-dependent *mexEF-oprN* expression and multidrug resistance in a clinical strain of *Pseudomonas aeruginosa*. J. Bacteriol. **187:**1246–1253.
54. **Soler, C. P., S. Gidenne, P. Saint-Blancard, A. Kerleguer, and P. Gerome.** 2004. Recovery method of serotypable character in non serotypable *Pseudomonas aeruginosa* strains. Pathol. Biol. (Paris) **52:**33–38. (In French.)
55. **Stover, C. K., et al.** 2000. Complete genome sequence of *Pseudomonas aeruginosa* PA01, an opportunistic pathogen. Nature **406:**959–964.
56. **Szczepanowski, R., et al.** 2004. Antibiotic multiresistance plasmid pRSB101 isolated from a wastewater treatment plant is related to plasmids residing in phytopathogenic bacteria and carries eight different resistance determinants including a multidrug transport system. Microbiology **150:**3613–3630.
57. **Tian, Z. X., et al.** 2009. MexT modulates virulence determinants in *Pseudomonas aeruginosa* independent of the MexEF-OprN efflux pump. Microb. Pathog. **47:**237–241.
58. **Vettoretti, L., et al.** 2009. Efflux unbalance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrob. Agents Chemother. **53:**1987–1997.
59. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.
60. **Vogne, C., J. R. Aires, C. Bailly, D. Hocquet, and P. Plésiat.** 2004. Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Antimicrob. Agents Chemother. **48:**1676–1680.
61. **Westfall, L. W., et al.** 2006. *mvaT* mutation modifies the expression of the *Pseudomonas aeruginosa* multidrug efflux operon *mexEF-oprN*. FEMS Microbiol. Lett. **255:**247–254.
62. **Wiehlmann, L., et al.** 2007. Population structure of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U. S. A. **104:**8101–8106.
63. **Yang, L., L. Chen, L. Shen, M. Surette, and K. Duan.** 2011. Inactivation of MuxABC-OpmB transporter system in *Pseudomonas aeruginosa* leads to increased ampicillin and carbenicillin resistance and decreased virulence. J. Microbiol. **49:**107–114.
64. **Zhang, L., X. Z. Li, and K. Poole.** 2001. Fluoroquinolone susceptibilities of efflux-mediated multidrug-resistant *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia cepacia*. J. Antimicrob. Chemother. **48:**549–552.