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NOTES

Differential Selection of Multidrug Efflux Systems by Quinolones in *Pseudomonas aeruginosa*

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Resistance mechanisms selected after in vitro exposure to 12 quinolones were analyzed for *Pseudomonas aeruginosa*. Efflux-type mutants were predominant. Quinolones differed in their ability to select a particular efflux system. While the newer fluoroquinolones favored the MexCD-OprJ system, the older quinolones selected exclusively the MexEF-OprN or MexAB-OprM systems. A protonable C-7 substituent in combination with a C-6 fluorine atom is a structural determinant of quinolones involved in efflux pump substrate specificity.

Pseudomonas aeruginosa is known for its intrinsic resistance to a variety of antimicrobial agents and its ability to develop multidrug resistance (MDR). Several reports have documented that quinolones were especially prone to select MDR phenotypes in *P. aeruginosa* in vitro as well as in vivo (2, 4, 9, 14, 20). Referring to the selective agent, the mutations responsible for these phenotypes were designated *nalB*, *nfxB*, and *nfxC*. The finding that these mutations resulted in the overexpression of multidrug efflux systems, however, became obvious only recently, following the identification of three distinct multidrug efflux operons.

The *nalB* mutants have been shown to overexpress the MexAB-OprM efflux system (19), which confers resistance to quinolones, β-lactams, tetracycline, chloramphenicol, and trimethoprim (7, 10–12). Two further multidrug efflux systems which share a similar genetic organization as well as sequence similarity with the *mexAB-oprM* operon have been described recently. The *mexCD-oprJ* operon, which is overexpressed in *nfxB*-type strains (18), confers resistance to quinolones (4), erythromycin (16), zwitterionic cephems (13), and trimethoprim (7). The *mexEF-oprN* efflux operon (8) endows resistance to quinolones, chloramphenicol (2, 3), and trimethoprim (8) and is overexpressed in *nfxC*-type mutants. Here we show that quinolones select preferentially and differentially active efflux systems, and we identify structural determinants which are involved in substrate specificity.

Quinolone-resistant mutants were selected by plating of approximately 10^9 to 10^{10} CFU of Luria-Bertani (LB) medium-grown overnight culture of strain PAO1 (laboratory collection) on LB plates containing a single quinolone at concentrations of 1 to 8 times the MIC. Spontaneously resistant colonies appeared after incubation at 37°C for 24 to 48 h. Unless otherwise stated, eight colonies were chosen randomly from each plate and inoculated in LB medium without antibiotic. Overnight cultures were used to determine susceptibilities to antimicrobial agents (obtained from their respective manufacturers or Sigma) in Mueller-Hinton broth by the microdilution method (6). The MICs of the quinolones for PAO1 used in the selection were as follows: nalidixic acid, 80 mg/liter; norfloxacin, 1.0 mg/liter; ciprofloxacin, 0.125 mg/liter; ofloxacin, 1.5 mg/liter; pefloxacin, 2.0 mg/liter; trovafloxacin, 1.0 mg/liter; CP-415,145, 2.5 mg/liter; sparfloxacin, 0.5 mg/liter; enoxacin, 0.8 mg/liter; pipemidic acid, 10 mg/liter; flumequine, 10 mg/liter; and oxolinic acid, 10 mg/liter. Where indicated, MICs were also determined with LB medium-antibiotic gradient plates (1), which allowed a more subtle comparison between the individual mutants. The MICs were defined by the boundary between confluent and nonconfluent growth.

For a given antibiotic concentration, the frequencies at which resistant colonies appeared were comparable for most of the quinolones tested. At 2× the MIC, frequencies varied from 1.2×10^{-6} to 1.3×10^{-7}, while at 4× the MIC, the frequencies were between 1.1×10^{-7} and 1.1×10^{-8}. With trovafloxacin, the frequencies were between 5×10^{-8} (2× the MIC) and 4×10^{-10} (4× the MIC). Among the 321 colonies analyzed, 7 that were isolated on nalidixic acid (4× and 6× the MIC) and 11 that were isolated on CP-415,145 (Pfizer AG, Zurich, Switzerland) presented MIC changes only for quinolones and were

TABLE 1. MIC profiles of the PAO1 wild-type strain and MDR derivatives selected by quinolones

Antimicrobial agent	MIC (μg/ml) for:			
	PAO1 wild type	MDR derivative		
		MexAB-OprM	MexCD-OprJ	MexEF-OprN
Erythromycin	256	128–256	512–2,048	128–256
Cefpirome	1	4–8	8–16	1–2
Chloramphenicol	64	256–512	128–256	≥1,024
Carbenicillin	32	128–256	8–32	32–64
Ciprofloxacin	0.125	0.25–0.5	0.5–1	0.5–1
Trimethoprim	128	1,024–2,048	256–1,024	512–1,024
Ceftazidime	2	4–8	1–2	0.5–1

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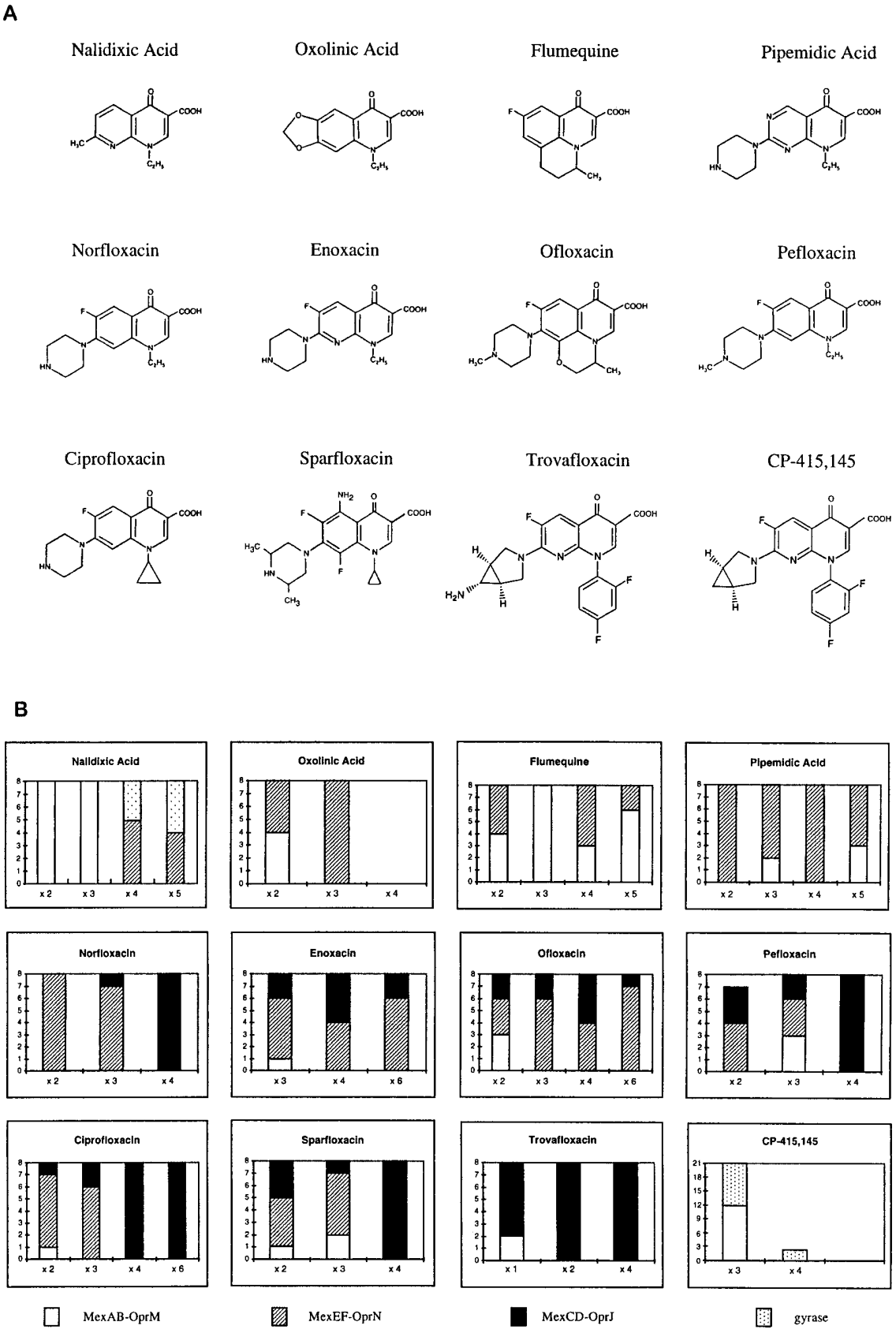

FIG. 1. (A) Chemical structures of quinolones used for the selection of resistant mutants of *P. aeruginosa* PAO1. (B) Selection of efflux-type systems in *P. aeruginosa* in relation to structurally different quinolones and their various concentrations. The ordinate indicates the number of colonies overexpressing one of the three efflux systems or showing a gyrase phenotype. The abscissa shows the quinolone concentrations (fold MIC) used for the selection of resistant colonies. Bars indicate mutant phenotypes.

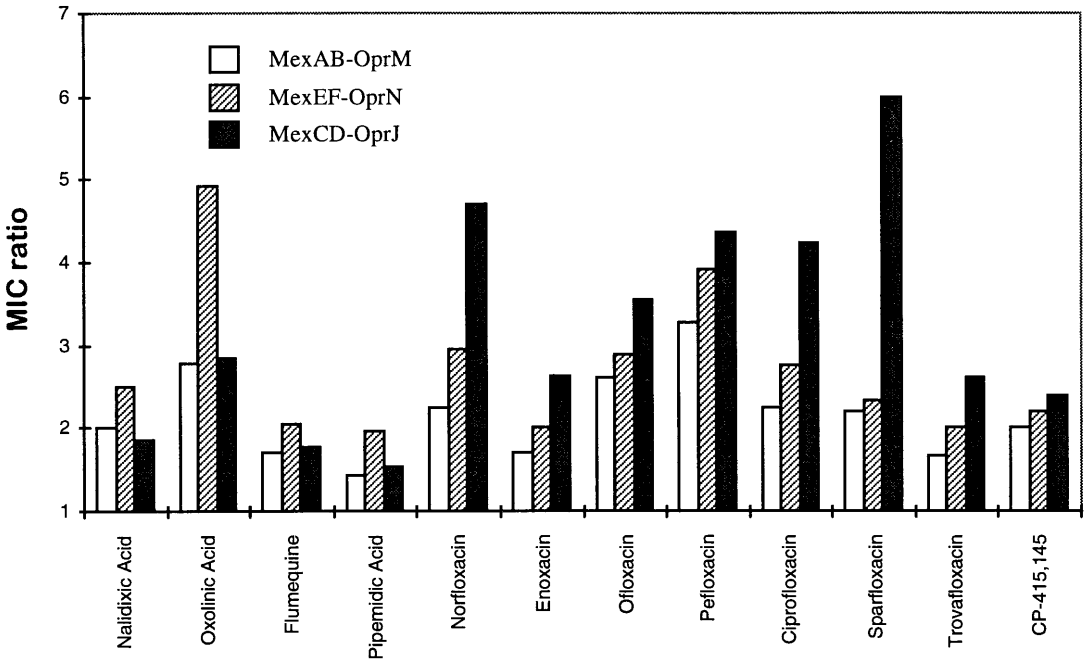

FIG. 2. MIC ratios of quinolones for MDR mutants as determined with antibiotic gradient plates. Ratios were obtained by dividing the MICs for representative MDR mutants selected on sparfloxacin (2× MIC) by those for the wild-type strain, PAO1. Bars indicate the overexpressed efflux system.

considered to be gyrase mutants. The remaining 303 colonies exhibited antibiotic profiles (Table 1) corresponding to those of *P. aeruginosa* strains overexpressing one of the three efflux systems. To confirm the efflux-related nature of the mutants, one set of eight colonies selected on sparfloxacin (2× the MIC), containing representatives of all three efflux phenotypes, were further examined by complementation with plasmids carrying the corresponding regulator gene *nfxB* (18) or *mexR* (19) and by analysis of outer membrane protein profiles (data not shown). In all cases, the results confirmed the MIC-based classification and allowed identification of the MDR strains as either *nalB*, *nfxB*, or *nfxC* mutants.

A first important conclusion is that in vitro, the preferred resistance mechanism selected by *P. aeruginosa* in response to a single exposure to quinolones, at concentrations close to the MIC, is antibiotic efflux and not alteration of the gyrase gene. Although gyrase mutations confer a higher level of resistance, they seem to occur less frequently than the efflux-type mutations, even in clinical isolates of *P. aeruginosa* (5). Activation of efflux systems either by mutation or by a transient increase in their expression could prevent significant cytosolic accumulation of the quinolones and therefore increase the life expectancy of the bacterial cell. In case of reexposure to the drug, subsequent mutations, as for example in the gyrase gene, would then allow a further increase in the resistance level. Such efflux-gyrase double mutants have been isolated from patients (21) and, after quinolone treatment, from mice infected with *P. aeruginosa* (15).

Classification of MDR mutants revealed that quinolones (Fig. 1A) had different abilities in terms of selecting efflux systems. Previous observations showed that *nalB* mutants were obtained after exposure to nalidixic acid (20), and *nfxB* or *nfxC* mutants were selected by norfloxacin (2, 4). Our results extend this view, since we found that all of the older quinolones (nalidixic, oxolinic, and pipemidic acids and flumequine) selected exclusively MexAB-OprM and MexEF-OprN overproducers, while the newer fluoroquinolones selected strains overproducing any of the three efflux systems (Fig. 1B). The occurrence of the three efflux mutants obtained on sparfloxacin and pefloxacin at 2× to 3× the MIC indicates that *nalB*-, *nfxB*-, and *nfxC*-type mutants might have been present at comparable frequencies in the initial cell population of the wild-type strain. We therefore hypothesized that any bias of this distribution would indicate a preference of a given quinolone for a particular efflux system and hence be an indication of the efficiency of the selected efflux system in extruding this compound.

To determine whether the three efflux systems indeed conferred different levels of resistance, the MICs for the eight MDR mutants described above were analyzed with quinolone-containing gradient plates. For all of the older quinolones, the MexEF-OprN efflux system yielded the highest MIC ratios (Fig. 2). In contrast, for all of the newer quinolones tested, the MexCD-OprJ system provided the largest increase in MIC ratios, sparfloxacin and norfloxacin being the most affected substrates. Essentially the same results were obtained with eight MDR mutants selected on pefloxacin (3× the MIC). With a few exceptions, the results from the MIC ratios might explain why at the highest antibiotic concentrations used, the older quinolones generally selected the MexEF-OprN system, while among the newer fluoroquinolones, the MexCD-OprJ efflux system was preferentially selected. Apparently at low antibiotic concentrations (1× to 2× the MIC), any of the three systems provides ample efflux activity; however, at higher antibiotic concentrations, only the most efficient efflux system for a given quinolone will be selected.

It was recently suggested that the MexCD-OprJ efflux system expels amphiphilic substrates that insert themselves into the lipid bilayer and carry a positive charge, such as for example, erythromycin (17) and the zwitterionic cephems (13). Indeed, our results show that nalidixic acid, oxolinic acid, and flumequine, which all lack a positive charge (Fig. 1A), never selected the *nfxB*-type mutants. More strikingly, with CP-415,145, a trovafloxacin derivative lacking the amino group at the C-7 substituent, no *nfxB*-type mutants could be selected, al-

though these were predominant with trovafloxacin. On the other hand, pipemidic acid, which does carry a positive charge on the piperazine ring, did not select *nfxB*-type mutants either, suggesting that in the case of quinolones, a positive charge is necessary but not sufficient as a determinant of substrate specificity. However, once a fluorine atom is present at the C-6 position in addition to the piperazine ring, the selection switches from *nalB*- and *nfxC*- to *nfxB*-type mutants. This suggests that in addition to the positive charge, the electronegative fluorine atom at the C-6 position is a structural determinant for the substrate specificity of the MexCD-OprJ efflux system.

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