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Susceptibility of *Xanthomonas maltophilia* to Six Quinolones and Study of Outer Membrane Proteins in Resistant Mutants Selected In Vitro

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The in vitro susceptibilities of 75 clinical isolates of *Xanthomonas maltophilia* to nalidixic acid, five fluoroquinolones, latamoxef, and doxycycline were determined. Spontaneous mutants were selected, at a frequency of about 10^{-5} to 10^{-7} from four strains by culturing the strains in the presence of each quinolone. Selection in the presence of nalidixic acid provided mutants that were either resistant only to that compound or that exhibited cross-resistance to all the fluoroquinolones tested. Cross-resistance was always observed for mutants selected on any of the five fluoroquinolones. It was always associated with chloramphenicol resistance and, frequently, with doxycycline resistance. The electrophoretic alterations of the outer membrane proteins of the mutants suggest that different mechanisms may be involved in quinolone resistance in *X. maltophilia*.

Xanthomonas maltophilia has recently been isolated with increasing frequency and is mainly responsible for nosocomial infections in compromised hosts (17). Its natural resistance restricts the choice of antibiotics for use in the treatment of infected patients (1, 2, 11, 16), and quinolones have been considered as a possible option for therapy (17). The agar diffusion method has revealed a particular phenotype of quinolone susceptibility for this species (12). In the current study, we determined the MICs of nalidixic acid, pefloxacin, ciprofloxacin, ofloxacin, temafloxacin, sparfloxacin, latamoxef, and doxycycline for 75 clinical isolates of *X. maltophilia*. Since the emergence of mutants resistant to quinolones and unrelated drugs has been described in several species of gram-negative bacilli (5–7, 14, 18, 20), especially *Pseudomonas aeruginosa* (8, 15), from 4 of the 75 strains of *X. maltophilia*, we selected mutants that were resistant to six quinolones. The resistance patterns of the mutants were studied, and the electrophoretic profiles of the outer membrane proteins (OMPs) were analyzed for each type of mutant.

MICs were determined by a twofold serial agar dilution method on Mueller-Hinton agar by using a replicating spot device, with an inoculum of about 10^4 CFU per spot. Spontaneous mutants were selected from four susceptible strains by spreading approximately 2×10^8 CFU onto plates of Mueller-Hinton agar containing each of the six quinolones at concentrations of 4, 8, and 16 times the respective MICs for the strains. After 48 h at 37°C, the frequencies of mutation were evaluated and the MICs of the six quinolones, doxycycline, latamoxef, and chloramphenicol were determined. The mutants were classified according to their patterns of resistance to these nine antibiotics. From each type of mutant, OMP fractions were prepared as described previously (6) and were submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation (13.5% acrylamide, 0.36% bisacrylamide).

Results of susceptibility testing for the clinical isolates are presented in Table 1. Sparfloxacin was the most active compound, with an MIC for 90% of the strains tested of 2 μg/ml. Ofloxacin, temafloxacin, and ciprofloxacin had equal activities and were consistently more active than pefloxacin. Taking a breakpoint of 2 μg/ml for pefloxacin, none of the strains could be considered susceptible.

Five types of mutants were selected (Table 2). Mutants N and Nm had increased levels of resistance to nalidixic acid, and there was an increased susceptibility to latamoxef in Nm mutants. NP mutants were resistant to nalidixic acid and pefloxacin. QD and QC mutants exhibited cross-resistance to all the quinolones tested; this was associated with resistance either to doxycycline and chloramphenicol (QD) or to chloramphenicol alone (QC).

The different quinolones selected mutants at similar frequencies (8.9×10^{-6} to 6.3×10^{-8}) for the four strains (Table 3). However, nalidixic acid selected a larger variety of mutants than did the fluoroquinolones. Among the five types of mutants which were defined, three types (N, Nm, and NP) were selected only by nalidixic acid, while the two

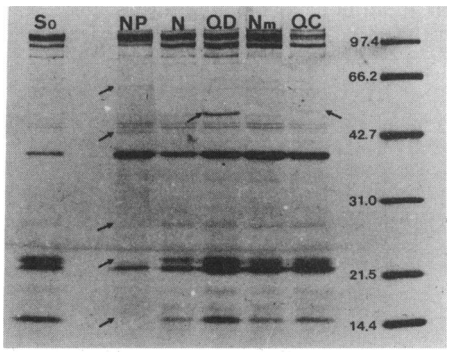

FIG. 1. OMP profiles of the parental strain of *X. maltophilia* (S_0) and its derivatives (N, Nm, NP, QD, and QC mutants). Arrows indicate modifications in OMPs compared with the OMPs of the parental strain. Molecular weights are given in thousands. Outer membrane extract from 75 μg of whole membrane proteins was applied to each well.

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TABLE 1. In vitro susceptibilities of 75 clinical isolates of *X. maltophilia*

Antimicrobial agent	MIC (μg/ml)[a]		
	Range	50%	90%
Nalidixic acid	8–128	8	16
Pefloxacin	2–32	4	8
Ciprofloxacin	0.5–16	2	4
Ofloxacin	0.5–16	1	4
Temafloxacin	0.25–32	1	8
Sparfloxacin	0.06–16	0.25	2
Doxycycline	1–8	4	4
Latamoxef	1–64	4	32

[a] 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

other types (QD and QC) were selected by all the compounds tested, including nalidixic acid (Table 2). N, Nm, and NP mutants were isolated at lower frequencies (fewer than 10% of the clones selected by nalidixic acid), and for these mutants there was a lesser increase in the MICs than there was for the other mutants; NP mutants, which were selected with nalidixic acid, despite their cross-resistance to pefloxacin, were not selected by pefloxacin. In contrast to what was found for the N, Nm, and NP mutant types selected by nalidixic acid, all the mutants selected in the presence of a fluoroquinolone were resistant to all the quinolones tested. More than 50% of the selected mutants were of the QD type (Table 2). None of the mutants showed cross-resistance to latamoxef; moreover, Nm clones appeared to be hypersusceptible to this antibiotic (Table 2). Resistance in all of the mutants was stable after five passages on drug-free medium.

Electrophoresis of OMP fractions (Fig. 1) revealed no change for the mutants that were resistant only to nalidixic acid (N and Nm mutants). In contrast, NP clones demonstrated many differences when they were compared with the parental strain. Two major bands (15 and 23 kDa) and a minor band (27.5 kDa) disappeared, while two faint bands (42.5 and 59.5 kDa) appeared. For the QD mutant, a significant enhancement of the 48-kDa protein was observed. The 48-kDa band was not seen in OMPs of QC clones, but in this case, a 50.5-kDa protein appeared.

In this study, the mutation frequency in *X. maltophilia* was slightly higher than that observed by Felmingham et al. (4) for *P. aeruginosa*. Acquired resistance to fluoroquinolones has been well documented in members of the family *Enterobacteriaceae* and *P. aeruginosa*. Several mutations have been identified; these mutations affect the A or B subunit of the gyrase or the permeability for quinolones (9).

TABLE 2. Resistance phenotypes of the mutant clones selected from one strain of *X. maltophilia*

Mutant types	Fold change in MIC of the following antimicrobial agents[a]:								
	NA (8)	P (2)	C (1)	O (1)	T (1)	S (0.25)	D (2)	L (16)	C (8)
N	+2	–	–	–	–	–	–	–	–
Nm	+2	–	–	–	–	–	–	−2	–
NP	+3	+2	–	–	–	–	–	–	–
QD	+3	+4	+4	+4	+4	+4	+2	–	+2
QC	+4	+4	+4	+4	+4	+4	–	–	+2

[a] Values in parentheses are MICs (in micrograms per milliliter) for the parental strain. (–), no change; NA, nalidixic acid; P, pefloxacin, C, ciprofloxacin; O, ofloxacin; T, temafloxacin; S, sparfloxacin; D, doxycycline; L latamoxef; C, chloramphenicol.

TABLE 3. Mutation rates for four strains of *X. maltophilia* and types of mutants selected by six quinolones at concentrations of four times the MIC

Selective agent	Mutation rate (10^{-6})	Type of mutants[a]
Nalidixic acid	0.33–8.9	N, Nm, NP, QD, QC
Pefloxacin	0.06–2.6	QD, QC
Ciprofloxacin	0.78–4.6	QD, QC
Ofloxacin	0.12–1.8	QD, QC
Temafloxacin	0.14–1.5	QD, QC
Sparfloxacin	0.15–0.6	QD, QC

[a] Defined by their phenotypes given in Table 2.

N clones, which did not present any cross-resistance to nonquinolone antibiotics and exhibited OMP profiles identical to those of the parent, may be phenotypically similar to the *gyrA* type mutant.

As for the NP clones, resistance to nalidixic acid and pefloxacin might be due to a pleotropic mutation rather than to a specific mechanism, as has been described in *Salmonella* species (5) and *Escherichia coli* (19).

Cross-resistance to unrelated antibiotics was found to be linked to reduced permeability by the loss of or a decrease in the OMP(s) of approximately 30 to 40 kDa in *P. aeruginosa* (3), *E. coli* (10), *Klebsiella* species (6, 18), *Salmonella* species (5), and *Enterobacter* and *Serratia* species (6). In contrast, Hirai et al. (8) and Legakis et al. (13) have observed the appearance of an additional OMP of 54 kDa in *P. aeruginosa* for mutants which exhibited cross-resistance to quinolones, tetracycline, and chloramphenicol. It was suggested that the new protein would act as a permeability barrier (8). The same role could be expected for the proteins of 48 and 50.5 kDa observed in QD and QC mutants of *X. maltophilia*. However, further studies will be necessary to elucidate the roles of these proteins.

In conclusion, quinolone-resistant mutants of *X. maltophilia* can be isolated in vitro at a high frequency. Resistance appears to be supported by various mechanisms. The most frequent types of mutants exhibited cross-resistance to all quinolones and to nonrelated antibiotics associated with quantitative or qualitative changes in at least one OMP.

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