Outer Membrane Alterations in Multiresistant Mutants of *Pseudomonas aeruginosa* Selected by Ciprofloxacin

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Spontaneous mutants of *Pseudomonas aeruginosa* selected by ciprofloxacin were studied for outer membrane alterations. Acquisition of ciprofloxacin resistance was at least partially related to defects in lipopolysaccharide synthesis. When ciprofloxacin resistance was combined with resistance to β-lactams and aminoglycosides, several alterations in outer membrane proteins were noted.

Recent findings with *Pseudomonas aeruginosa* mutants selected with norfloxacin (14) or ciprofloxacin (8, 24) implicate an altered DNA gyrase A, an impaired cellular permeability, or both as the mechanism of resistance. In the present report, we describe outer membrane (OM) alterations induced upon acquisition of antibiotic resistance in spontaneous mutants of *P. aeruginosa* selected by ciprofloxacin.

Three clinical isolates of *P. aeruginosa* and a number of mutants were studied (Table 1). The bacteriophage-propagating strains and the respective phages were a generous gift from T. L. Pitt (Central Public Health Laboratory, London, United Kingdom). Mutants resistant to ciprofloxacin were selected by plating bacterial suspensions on Mueller-Hinton agar containing ciproflocaxin at 4, 8, and 16 times the MIC (25). The mutational frequency was calculated after incubation for 48 h at 37°C.

Antibiotic susceptibility testing was done by an agar dilution method with Mueller-Hinton agar. A single disk diffusion method was also used for a series of antibiotics. OM proteins were prepared from mid-log-phase cultures in nutrient broth by mild ultrasonic treatment and differential solubilization of the cytoplasmic membrane with sarcosyl. They were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6). Lipopolysaccharide (LPS) profiles were taken by electrophoresis of crude whole-cell extracts on urea gels (4 M urea, 15% acrylamide) (15).

The uptake of ciprofloxacin by the cells was measured by a bioassay with *Escherichia coli* K-12, as previously described for norfloxacin (13).

Serum susceptibility was assayed by using active normal human serum (28). O serotyping was performed as described previously (19). Detection of β-lactamase activity was done as described previously (18). Aminoglycoside-modifying enzymes were examined by determining the relative susceptibility to a series of aminoglycosides (27). Phage susceptibility testing (2) was done by using a phage concentration 10 times the routine dilution test concentration (26).

Ciprofloxacin-resistant mutants occurred at a very low frequency (10^{-8} to 10^{-10}). Higher frequencies were observed with lower drug concentrations. In agreement with previously reported results (25, 29), an 8- to 16-fold increase in resistance to ciprofloxacin compared with that of wild-type (WT) strains was observed in over 60% of the mutants. Table 1 shows representative mutants of each WT strain.

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The ciprofloxacin-selected mutants were resistant to other quinolones. Although studies related to the affinity of DNA gyrase have not been done, the fact that the increments in resistance of the mutants relative to their parent strains were similar for ciprofloxacin, norfloxacin, and nalidixic acid might be taken as indicating one-step mutations resulting in altered DNA gyrase A (14, 24). The cross-resistance between quinolones, aminoglycosides, and β-lactams that was observed with LK-5 and LK-7 mutants of *P. aeruginosa* has not been previously reported (7, 25). Moreover, norfloxacin-resistant mutants exhibiting hypersusceptibility to aminoglycosides and β-lactams have been described (14). Cross-resistance between fluoroquinolones-imipenem (21) and fluoroquinolones-moxalactam (8) was observed but it was not expanded to aminoglycosides and other β-lactams. Ciprofloxacin-resistant mutants have also been reported to be resistant to carbenicillin, chloramphenicol, and novobiocin (24).

The multiply resistant mutants were found to be resistant to LPS-specific phages. They were also rendered nontypable

TABLE 1. Antibiotic susceptibility patterns of *P. aeruginosa* mutants selected with ciprofloxacin

Strain	MIC (μg/ml)[a]					Additional antibiotics with diminished activity[b]
	CIP	NOR	NAL	CB	GM	
P703 (WT)	0.5	1.0	1,024	25	2	None
LK-3	4	8	2,048	25	2	None
P706 (WT)	0.5	2.0	1,024	100	2	None
LK-5	8	16	8,192	800	16	AMK, NET, TOB, MOX
LK-6	4	8	4,096	50	2	None
LK-7	16	16	8,192	1,600	32	AMK, NET, TOB, MOX
P850 (WT)	4	8	2,048	>1,600	>128	AMK, NET, TOB, MOX
LK-9	8	16	8,192	>1,600	>128	AMK, NET, TOB, MOX
LK-12	16	16	8,192	>1,600	>128	AMK, NET, TOB, MOX

[a] CIP, Ciprofloxacin; NOR, norfloxacin; NAL, nalidixic acid; CB, carbenicillin; GM, gentamicin.
[b] From results of disk diffusion tests with amikacin (AMK), netilmicin (NET), tobramycin (TOB), and moxalactam (MOX). Drugs were considered to have altered activity if the zone of inhibition differed in the mutants by at least 5 mm.
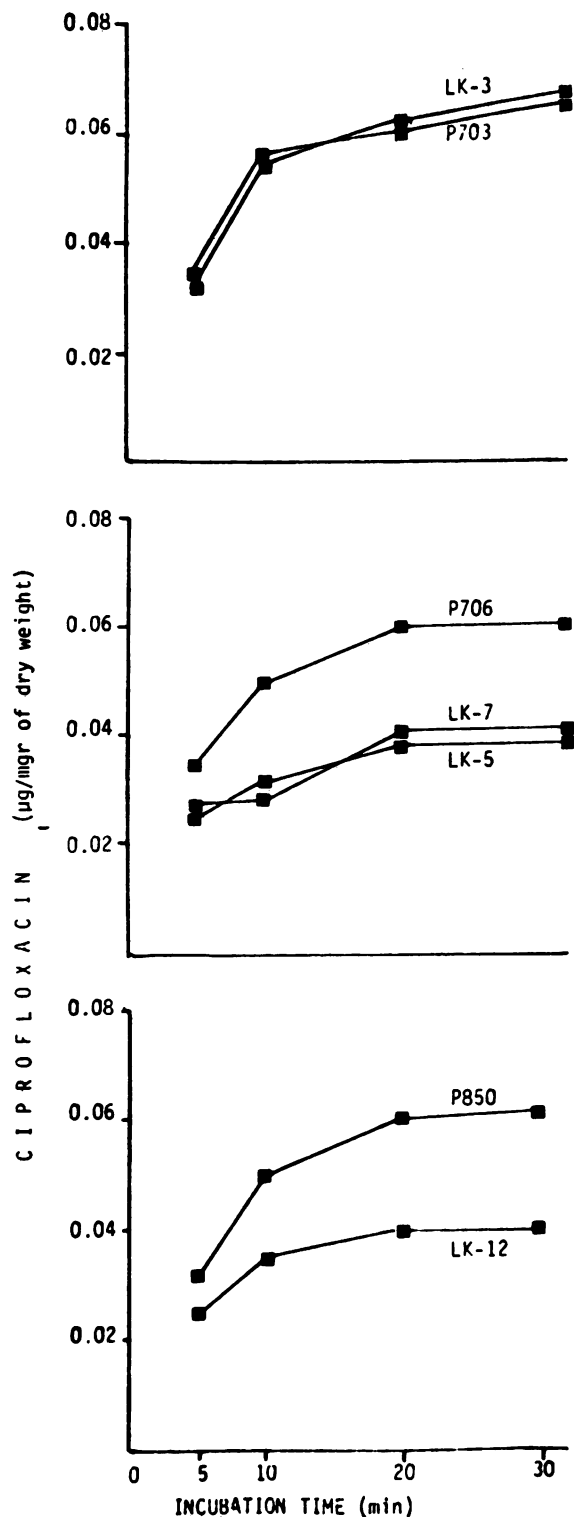
FIG. 1. Uptake of ciprofloxacin by mutants of *P. aeruginosa*. Points represent the mean values of three different experiments differing less than 10%. The mutants of P706 and the LK-12 mutant of P850 displayed 30% lower ciprofloxacin uptake than did the WT strains.
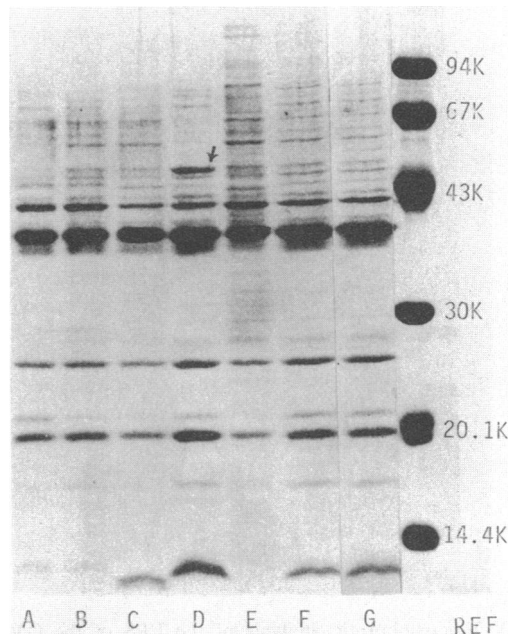

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins of ciprofloxacin-resistant mutants of *P. aeruginosa*. Lanes: A, P703 (WT); B, LK-3 (mutant of P703); C, P706 (WT); D, LK-5 (mutant of P706); E, LK-7 (mutant of P706); F, P850 (WT); G, LK-12 (mutant of P850). Ref, Reference proteins. Molecular sizes (in kilodaltons) are indicated. Approximately 50 μg of protein from each sample was loaded onto the gels. The arrow designates the 54-kilodalton protein in the LK-5 mutant.

by O antisera as compared with their parent strains belonging to the O3 (strain P706) or O12 (strain P850) serogroup. The WT strain P850 and its mutant LK-12 possessed β-lactamase and 2'-O"-nucleotidyltransferase, whereas neither β-lactamases nor aminoglycoside-modifying enzymes were detected in any other WT or mutant strain studied. The observations described above, taken together with the multiple nature of antibiotic resistance, suggest the involvement of an impaired cellular permeability (12, 19, 23, 24). This has been investigated by measuring the uptake of ciprofloxacin (Fig. 1). Mutants LK-5, LK-7, and LK-12 showed about a 30% reduction of ciprofloxacin uptake compared with that of the WT strains P706 and P850, whereas LK-3 displayed a mode of drug uptake similar to that of its parent strain.

Impaired permeability has been reported (3, 16) to broaden the spectrum and convey a high level of resistance. In that respect it might be reasonable to suggest that a defective outer membrane permeability operates synergistically with probably an altered DNA gyrase, affording a high level of ciprofloxacin resistance in LK-5, LK-7, and LK-12 mutants.

It has been suggested that impaired permeability might reflect differences in the detailed organization of the OM (4, 16, 22).

Previous reports have shown OM protein alterations to be associated with quinolone and β-lactam resistance in *Klebsiella*, *Enterobacter*, and *Serratia* species (12, 25). Our results (Fig. 2) demonstrate some changes in OM proteins, including the increased production of a 54-kilodalton protein by the LK-5 mutant. However, since this mutant exhibited resistance to multiple antibiotics, it was not possible to assign a specific role of the 54-kilodalton OM protein for the impaired cellular permeability to ciprofloxacin as has been
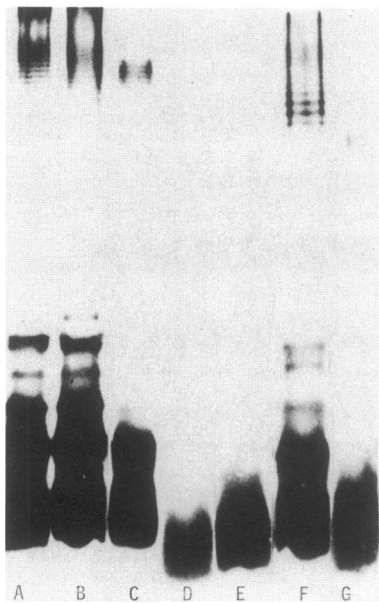
FIG. 3. Electrophoretic analysis of the LPS of the OM of *P. aeruginosa* mutants. Lanes are as described in the legend to Fig. 2. Discrepancies between the WT strains P706 and P850 and their respective mutants were revealed in the O antigen and the core region.

shown for *E. coli* norfloxacin-resistant mutants (1, 13, 16). Interestingly, this protein was not overexpressed in the other mutants. It appears that quinolone resistance involving OM permeability in *P. aeruginosa* may differ from the one mentioned above for *E. coli* (14).

In LK-7 additional high-molecular-weight bands appeared, whereas a remarkable decrease in a 12-kilodalton protein was noted. None of the mutants examined presented substantial changes in porin F (37 kilodaltons), although a relative reduction of this protein might be considered in the LK-7 mutant. When resistance was restricted to quinolones, no OM protein changes were observed. However, a diminution or loss of a 31- to 32-kilodalton OM protein has been correlated with quinolone resistance in *P. aeruginosa* (8).

LPS is known to contribute to resistance to β-lactams (10, 11) and aminoglycosides (4, 5, 9) and also to act as a receptor to phages (17, 20). Therefore, LPSs were examined by polyacrylamide gel electrophoresis (Fig. 3). The LPS of LK-3 resembled that of the WT P703, whereas LK-5 and LK-7 LPSs differed markedly from that of the parent strain, P706; the ladder pattern was absent, with the defect being most enhanced in the LK-5 strain. Also, LK-12 was characterized by a severe truncation of LPS compared to the WT P850 LPS. This finding suggests that the involvement of LPS in the expression of ciprofloxacin resistance is likely.

All of the characteristics of the strains were stable. Although the hypersusceptibility of multiply resistant mutants (LK-5, LK-7) to human serum suggests that emergence of such mutants is unlikely to cause systematic infections, mutants like LK-3 that retain parental OM characteristics might require some consideration.

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