ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1990, p. 281–286
0066-4804/90/020281-06$02.00/0

Vol. 34, No. 2

# Analysis of Acquired Ciprofloxacin Resistance in a Clinical Strain of *Pseudomonas aeruginosa*

BARBARA L. MASECAR, ROGER A. CELESK, AND NORMAN J. ROBILLARD*

*Pharmaceutical Division, Miles Inc., West Haven, Connecticut 06516*

Received 31 July 1989/Accepted 14 November 1989

**Decreasing susceptibility to ciprofloxacin was investigated in sequential clinical isolates of *Pseudomonas aeruginosa* from a patient on ciprofloxacin therapy. All isolates were verified as the same strain by DNA probe. MICs of all quinolones tested were 16- to 32-fold higher for the posttherapy isolates; nonquinolone MICs were unchanged. The isolates were compared by analyses of outer membrane proteins and lipopolysaccharide composition, antimicrobial susceptibilities, measurement of accumulation of ciprofloxacin, and inhibition of DNA gyrase activity by ciprofloxacin and nalidixic acid. No significant changes in outer membrane proteins or ciprofloxacin accumulation were observed; however, both posttherapy isolates lost the long chain O-polysaccharide component of lipopolysaccharide. Preparations of DNA gyrase from the quinolone-resistant posttherapy isolates were 16- to 32-fold less sensitive to inhibition of supercoiling by ciprofloxacin and nalidixic acid than was gyrase from the pretherapy isolate. Inhibition studies on combinations of heterologous gyrase subunits showed that decreased inhibition was conferred by the resistant gyrase A subunits. Thus, acquired resistance to ciprofloxacin in this strain involved an alteration in the A subunit of DNA gyrase and was associated with changes in lipopolysaccharide.**

Ciprofloxacin, one of the most active new quinolone antimicrobial agents, is bactericidal against a broad spectrum of gram-positive and gram-negative microorganisms, including *Pseudomonas aeruginosa*. Although *P. aeruginosa* is intrinsically less susceptible to most antibiotics than other clinically significant gram-negative organisms, 90% of *P. aeruginosa* strains are inhibited by ciprofloxacin at 0.5 μg/ml (7).

There have been reports of decreased susceptibility to ciprofloxacin during therapy in clinical studies of *P. aeruginosa* infections (9, 10, 27, 35). These reports did not specify whether the decrease in susceptibility was due to the selection of naturally occurring resistant strains or the mutation of the original strain to a resistant variant.

Kaatz and Seo (24), however, have described a *P. aeruginosa* strain whose identity was based on serotype, pyocin type, and plasmid profile. The MIC of ciprofloxacin for this strain increased from 0.57 to 5.42 μg/ml during parenteral therapy, with no significant change in susceptibility to other antibiotics. Resistance involved a decrease in the sensitivity of DNA synthesis to inhibition by ciprofloxacin, but the basis of this decreased sensitivity was not investigated further. Ogle et al. (31) analyzed 25 pairs of pre- and posttherapy isolates of *P. aeruginosa* from patients treated with imipenem, norfloxacin, or ciprofloxacin. Southern hybridization showed clonal identity between 23 of the 25 paired isolates, confirming the development of quinolone resistance rather than superinfection in these strains. The mechanism of this acquired resistance was not studied.

Quinolones interfere with the activity of DNA gyrase (12, 39), an essential bacterial topoisomerase that converts relaxed DNA to the supercoiled form. DNA gyrase has been isolated from a variety of bacteria (3, 11, 26, 40) including *P. aeruginosa* (28). In vitro studies with *P. aeruginosa* PAO have shown that alterations in DNA gyrase (*gyrA cfxA nalA* mutations) or permeability (*cfxB nfxB*) can result in decreased susceptibility to quinolones, including ciprofloxacin (32), norfloxacin (20), and nalidixic acid (22).

To further understand the factors which mediate quinolone susceptibility in *P. aeruginosa*, we studied three clinical isolates of *P. aeruginosa* from a patient with a complicated infection. The MIC of ciprofloxacin increased from 0.5 μg/ml at the initiation of parenteral ciprofloxacin therapy to 16 μg/ml during therapy. All isolates were verified as the same strain by Southern hybridization with a strain-specific *P. aeruginosa* DNA probe (John Ogle, University of Colorado School of Medicine, Denver). To characterize the mechanisms involved in the acquisition of resistance to ciprofloxacin in vivo, we examined the outer membrane proteins (OMPs), lipopolysaccharide (LPS) content, antimicrobial susceptibilities, accumulation of ciprofloxacin, and DNA gyrase of these isolates. We provide evidence that the acquired resistance to quinolones in this clinical strain is primarily due to an alteration in the DNA gyrase subunit A and is associated with a change in the LPS content of the cell envelope. This is the first report of the isolation and characterization of quinolone-resistant DNA gyrase from clinical isolates of *P. aeruginosa*.

(This paper was presented in part at the 89th Annual Meeting of the American Society for Microbiology [B. L. Masecar, R. A. Celesk, and N. J. Robillard, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, A-80, p. 14].)

## MATERIALS AND METHODS

**Bacterial isolates.** *P. aeruginosa* MP-001 was isolated in 1986 prior to ciprofloxacin therapy from a patient with mediastinitis and empyema. *P. aeruginosa* MP-002 and MP-003 were isolated from chest tube drainage and sputum, respectively, approximately 3 weeks after initiation of parenteral ciprofloxacin therapy. All were submitted to Miles Inc., Pharmaceutical Div., West Haven, Conn., by Steven Seidenfeld (R. H. Dedman Medical Center, Dallas, Tex.).

The isolates were verified as one strain by John Ogle (University of Colorado School of Medicine, Denver), using

* Corresponding author.

Southern hybridization with an epidemiological DNA probe (31).

**Antimicrobial susceptibilities.** Cation-supplemented Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) was used to determine MICs by standard methods (30).

**Antibiotics.** Antibiotics were obtained as follows: ciprofloxacin (Miles Inc., Pharmaceutical Div.); tetracycline and novobiocin (Sigma Chemical Co., St. Louis, Mo.); carbenicillin (Beecham Laboratories, Bristol, Tenn.); norfloxacin (Merck Sharp & Dohme, Rahway, N.J.); ofloxacin (Ortho Diagnostics, Inc., Raritan, N.J.); enoxacin (Warner-Lambert Co., Ann Arbor, Mich.); and tobramycin (Eli Lilly & Co., Indianapolis, Ind.).

**Isolation of DNA gyrase.** Cells were grown, harvested, and frozen as described previously (32). Approximately 10 g of cells was lysed for each gyrase isolation. Cell lysis and streptomycin sulfate precipitation were performed as described previously (36). Solid ammonium sulfate was added to the lysate to a final concentration of 0.4 g/ml; after being stirred on ice for 30 min, the lysate was centrifuged at 10,000 × *g* for 20 min at 4°C. The pellets were suspended in approximately 20 ml of buffer B (0.05 M KCl–25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–1 mM EDTA–10% [wt/vol] glycerol–1 mM dithiothreitol) (38) and dialyzed overnight against two changes of the same buffer. Gyrase subunits A and B were purified from the dialysate by the novobiocin-Sepharose procedure of Stadenbauer and Orr (38) with the following modifications. After baseline was achieved with buffer B as determined by an ISCO UA-5 Absorbance/Fluorescence Detector (ISCO Inc., Lincoln, Nebr.), gyrase A was eluted from the novobiocin-Sepharose column with a 0.05 to 1.0 M KCl gradient in buffer B. This was followed by an extensive wash with 3 M KCl in buffer B. Buffer B was then passed over the column to regain baseline. Gyrase B was eluted with 5 M urea in buffer B. Fractions containing the A and B subunits were dialyzed and concentrated against buffer B in a Micro-ProDiCon negative pressure microprotein dialysis concentrator (Bio-Molecular Dynamics, Beaverton, Oreg.).

**DNA supercoiling assay and $IC_{50}$ determinations.** Relaxed plasmid pBR322 substrate DNA was prepared by treatment with calf thymus topoisomerase I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) according to the recommended protocol of the manufacturer. The DNA supercoiling assay was performed as previously described (29). One unit of gyrase is defined as that amount of enzyme which catalyzes the conversion of one-half of the relaxed closed circular DNA to the supercoiled form in 30 min at 37°C in the standard gyrase reaction containing 0.4 μg of DNA. Approximately 6 U each of gyrase A and B per reaction was used.

The $IC_{50}$ is defined as the concentration of antibiotic that inhibits 50% of the supercoiling activity of gyrase in a standard gyrase reaction. The $IC_{50}$s of ciprofloxacin and nalidixic acid were determined for each of the gyrase preparations. Antibiotic was added to a series of gyrase reactions before the addition of enzyme to achieve a final concentration range of either 0.25 to 16.0 μg of ciprofloxacin per ml or 25 to 1,600 μg of nalidixic acid per ml. A control reaction without drug was included. The enzyme was removed by extraction with chloroform-isoamyl alcohol (24:1), and the substrate DNA was electrophoresed through a 1% agarose gel in TPE buffer (0.08 M Tris phosphate–0.002 M EDTA, pH 8) overnight at 30 V. After the gel was stained in 0.5 μg of ethidium bromide per ml, the $IC_{50}$s were determined by visual comparison with the control reaction lacking antibiotic.

**Accumulation of ciprofloxacin.** Cell cultures grown overnight in basal salts medium (BSM) (33) were diluted 1:20 in fresh BSM and incubated for 4 to 5 h with aeration at 35°C. The cells were harvested by centrifugation and suspended to a turbidity of 260 Klett units in cold BSM. Cell volumes of 5 ml were added to 50-ml flasks and temperature equilibrated at 37°C for 5 min in a shaking water bath. When used, 50 μl of 25 mM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added just before the temperature equilibration period. Fifty microliters of a 100× ciprofloxacin stock solution was added and incubated for 5 min. One-milliliter samples were then centrifuged through 0.5 ml of silicon oil at 15,000 × *g* for 3 min to remove extracellular ciprofloxacin as described previously (5, 23) and frozen at −70°C. The bottom of each microcentrifuge tube was cut off below the water-oil interface with a heated scalpel. Tube bottoms were inverted over absorbent paper to remove residual oil. Cell pellets were suspended in 0.2 ml of phosphate-buffered saline (pH 7.2), transferred to new 1.5-ml microcentrifuge tubes, and boiled for 7 min to extract cell-associated drug (19). Cell debris was removed by centrifugation at 15,000 × *g* for 5 min. The concentrations of ciprofloxacin in the supernatants were determined by an agar well diffusion bioassay (16), using *Klebsiella pneumoniae* ATCC 10031 as the assay organism.

**Membrane protein isolation.** Late-log-phase cells grown with aeration at 35°C in BSM were diluted 1:50 into 2-liter flasks containing 400 ml of fresh BSM. After overnight incubation with aeration at 35°C, the cells were harvested by centrifugation. Cell lysates containing OMPs were produced, using a modification of the method of Ames (1). Cells were suspended in cold 0.0625 M Tris hydrochloride (pH 6.8), maintained on ice, and disrupted by sonic oscillation, using the maximum output of a VirTis Virsonic 300 microprobe (The VirTis Co., Inc., Gardiner, N.Y.). The cells were subjected to 10 bursts of 30 s each with a 30-s pause between bursts. Debris from the lysate was removed by centrifugation at 6,000 × *g* for 20 min in a Sorvall SS34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.). Resultant supernatants were centrifuged in a Beckman Ti70 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 111,000 × *g* for 35 min at 10°C. OMPs contained in the pellets were purified, using the method of Sawai et al. (34), incorporating an overnight incubation of OMPs in 2% lauroyl sarcosine–sodium salt. The purified OMPs were suspended in 0.01 M phosphate buffer (pH 7.0). Protein content was estimated, using the method of Smith et al. (37).

**LPS preparation.** LPS were extracted from whole cell lysates, using the method of Hitchcock and Brown (21). After growth in BSM with aeration at 35°C, cells were harvested in late log phase, washed in Dulbecco phosphate-buffered saline (pH 7.2), and suspended to a density of 300 Klett units. For each isolate, a 1.5-ml sample was centrifuged in a microcentrifuge tube and the supernatant was discarded. Cell pellets were suspended in 50 μl of a lysing buffer containing 2% sodium lauryl sulfate, 4% 2-mercaptoethanol, 10% glycerol, 0.1 mg of bromphenol blue per ml, and 1 M Tris hydrochloride (pH 6.8). The lysate was boiled for 10 min. Contaminating protein in samples of LPS was digested with proteinase K at 60°C for 1 h.

## RESULTS

**Susceptibility to quinolones and other antibiotics.** Posttherapy isolates MP-002 and MP-003 exhibited 16- and 32-fold decreases in susceptibility to ciprofloxacin, respectively,

TABLE 1. Susceptibilities of pre- and posttherapy isolates of a *P. aeruginosa* clinical strain to quinolone antibiotics

| Isolate no. | MIC (μg/ml) | | | | |
|---|---|---|---|---|---|
| | Ciprofloxacin | Norfloxacin | Ofloxacin | Enoxacin | Nalidixic acid |
| MP-001 | 0.5 | 2 | 4 | 2 | 64 |
| MP-002 | 8 | 32 | 32 | 32 | >256 |
| MP-003 | 16 | 32 | 32 | 32 | >256 |

compared with the pretherapy isolate MP-001 (Table 1). Complete cross-resistance to other quinolones tested was also observed for MP-002 and MP-003. Susceptibilities to several nonquinolone antibiotics were within one dilution of the values obtained for MP-001 (data not shown). The MIC of novobiocin, whose target is the B subunit of DNA gyrase (13), remained unchanged at 200 μg/ml.

**OMP composition.** Comparison of the composition of OMPs from the pre- and posttherapy isolates (Fig. 1A) showed some variation in the density of the Coomassie-stained proteins which migrated similarly to the D1 and H1 OMPs described by Hancock and Carey (17). Variation was also observed among the isolates in several lightly staining bands that migrated between the 45,000- and 66,000-dalton markers. These differences were not seen consistently and could not be correlated with quinolone resistance.

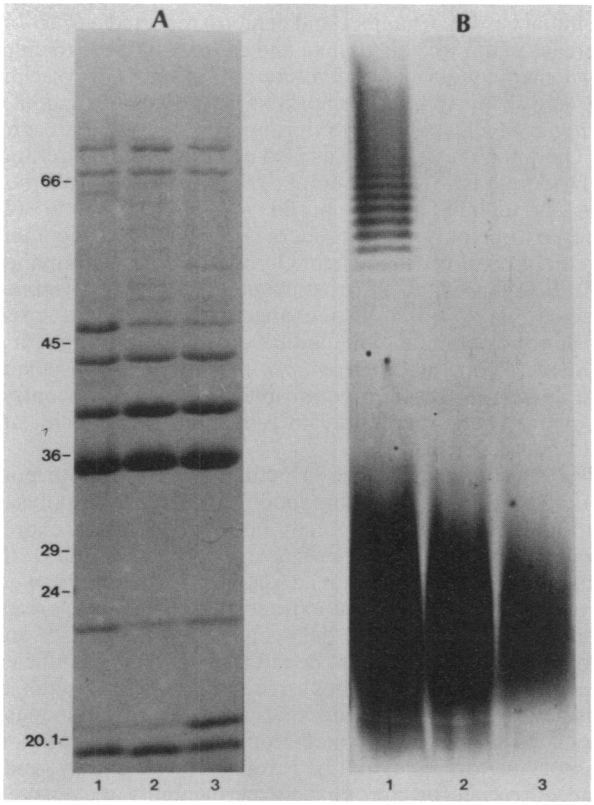


FIG. 1. Analyses of outer membranes of pre- and posttherapy isolates of a *P. aeruginosa* clinical strain. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OMPs. Lanes: 1, isolate MP-001; 2, isolate MP-003; 3, isolate MP-002. Molecular masses (in kilodaltons) are indicated to the left. (B) Sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis of extracted LPS, visualized by silver stain. Lanes: 1, isolate MP-001; 2, isolate MP-003; 3, isolate MP-002.

TABLE 2. Accumulation of ciprofloxacin by pre- and posttherapy isolates of a *P. aeruginosa* clinical strain exposed to 40 μg of drug per ml

| Isolate no. | Ciprofloxacin (ng/mg) accumulated (mean[a] ± SE) | |
|---|---|---|
| | Without CCCP | With CCCP[b] |
| MP-001 | 337 ± 96 | 2,054 ± 321 |
| MP-002 | 382 ± 104 | 1,970 ± 200 |
| MP-003 | 186 ± 47 | 1,759 ± 140 |

[a] Each value is the mean of four separate determinations.
[b] CCCP final concentration, $2.5 \times 10^{-4}$ M.

**LPS analysis.** Polyacrylamide gel analysis of LPS showed that both posttherapy isolates had lost the long chain O-polysaccharide core complexes (Fig. 1B) present in MP-001 and characteristic of *P. aeruginosa*.

**Accumulation of ciprofloxacin.** MP-003 accumulated less ciprofloxacin than either MP-001 or MP-002 (Table 2). All three isolates responded similarly to the presence of the metabolic inhibitor CCCP, with proportional increases in ciprofloxacin accumulation.

**Analysis of DNA gyrase.** To determine whether resistance in the posttherapy isolates was due to an altered DNA gyrase, each enzyme preparation was assayed for inhibition of supercoiling ($IC_{50}$) by ciprofloxacin and nalidixic acid (Table 3). DNA gyrase preparations from the posttherapy isolates were 16- to 32-fold more resistant to inhibition of supercoiling by ciprofloxacin. The $IC_{50}$s of ciprofloxacin for gyrase from MP-002 and MP-003 were 8 and 16 μg/ml, respectively, while the $IC_{50}$ of nalidixic acid was 400 μg/ml for both isolates.

$IC_{50}$ assays were repeated with combinations of heterologous DNA gyrase subunit preparations derived from MP-001 combined with either MP-002 or MP-003. When the A subunit of MP-001 was replaced by the A subunit from either MP-002 or MP-003, there was a significant increase in resistance to inhibition of supercoiling by both antibiotics (Table 4). This is illustrated in Fig. 2, where the A subunit of MP-001 (lanes A through H) was replaced with the A subunit from MP-003 (lanes I through P). Conversely, substitution of the A subunit of either posttherapy gyrase enzyme with the A subunit from MP-001 resulted in decreased $IC_{50}$s comparable to those of the MP-001 holoenzyme.

## DISCUSSION

In vitro resistance to ciprofloxacin in *P. aeruginosa* PAO can occur by a mutation in the gene for DNA gyrase subunit A (*nalA gyrA cfxA*) resulting in a quinolone-resistant enzyme (20, 22, 32). *cfxA* mutants of *P. aeruginosa* PAO exhibit 8- to 16-fold increases in MICs of ciprofloxacin and cross-resistance to other quinolones, while nonquinolone antibiotic susceptibilities remain unchanged. *cfxB* mutants of *P. aerug-*

TABLE 3. Concentrations of ciprofloxacin and nalidixic acid which inhibit *P. aeruginosa* DNA gyrase supercoiling activity

| Source of DNA gyrase | $IC_{50}$ (μg/ml) | |
|---|---|---|
| | Ciprofloxacin | Nalidixic acid |
| MP-001 | 0.5 | 25 |
| MP-002 | 8 | 400 |
| MP-003 | 16 | 400 |

TABLE 4. Concentrations of ciprofloxacin and nalidixic acid which inhibit supercoiling activity of *P. aeruginosa* reconstituted DNA gyrases

| Source of reconstituted DNA gyrase[a] | $IC_{50}$ (μg/ml) | |
|---|---|---|
| | Ciprofloxacin | Nalidixic acid |
| As + Br1 | 0.5 | 25 |
| Ar1 + Bs | 8.0 | 400 |
| As + Br2 | 1.0 | 50 |
| Ar2 + Bs | 8.0 | 400 |

[a] Abbreviations: As and Bs, gyrase A and B subunits from isolate MP-001; Ar1 and Br1, gyrase A and B subunits from isolate MP-002; Ar2 and Br2, gyrase A and B subunits from isolate MP-003.

*inosa* PAO are resistant to ciprofloxacin and cross-resistant to novobiocin, tetracycline, chloramphenicol, and carbenicillin (32). *cfxB* mutants are associated with the appearance of an additional OMP (5, 32) and appear altered in permeability. Outer membrane alterations also occur in quinolone-resistant *nfxB* mutants of PAO, which exhibit decreased norfloxacin accumulation and hypersusceptibility to β-lactam and aminoglycoside antibiotics (20).

In this report, we have described the mechanism of resistance to ciprofloxacin which occurred within a single clinical strain of *P. aeruginosa*. The quinolone-specific decrease in susceptibility observed in the posttherapy isolates is characteristic of *cfxA* mutants of *P. aeruginosa* PAO2 isolated in vitro (32).

As with *cfxA* mutants of PAO2, DNA gyrase from the posttherapy isolates was significantly more resistant to inhibition of supercoiling by ciprofloxacin and nalidixic acid. The gyrase A subunits from either of the resistant isolates conferred resistance to both antibiotics in heterologous gyrase subunit assays. Therefore, alterations in the A subunit of DNA gyrase significantly contributed to quinolone resistance in this strain.

To determine whether other factors contribute to resistance, we analyzed the accumulation of ciprofloxacin by these organisms and the composition of their outer membranes. Although MP-003 appeared to accumulate less ciprofloxacin than either MP-001 or MP-002, this decrease was not readily apparent in the presence of CCCP. In a recent report, we demonstrated that *P. aeruginosa* PAO2 accumulates up to sevenfold more ciprofloxacin when deenergized by the presence of CCCP, indicating the possible presence of an active efflux system similar to that proposed for *Escherichia coli* (8); however, *cfxB* mutants of PAO2 accumulate up to fourfold less ciprofloxacin than PAO2 and its *cfxA* mutants under these same conditions (5).

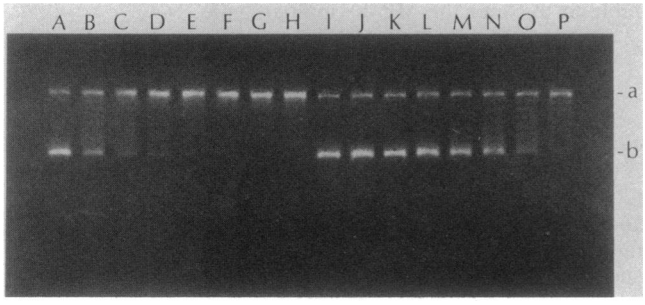


FIG. 2. Inhibition of supercoiling activity of reconstituted gyrase by ciprofloxacin. A reaction mixture of the standard supercoiling assay contained 6 U each of DNA gyrase A and B subunits from MP-001 (pretherapy) (lanes A through H) or 6 U of DNA gyrase subunit A from MP-003 (posttherapy) with 6 U of gyrase subunit B from MP-001 (lanes I through P). The upper band (a) represents relaxed pBR322 DNA. The lower band (b) represents supercoiled pBR322 DNA. Ciprofloxacin concentrations (μg/ml): lanes A and I, 0 (controls); lanes B and J, 0.25; lanes C and K, 0.50; lanes D and L, 1.0; lanes E and M, 2.0; lanes F and N, 4.0; lanes G and O, 8.0; and lanes H and P, 16.0.

The OMP compositions of the pre- and posttherapy isolates were similar but not identical. Both posttherapy isolates showed variable expression of the D1 and H1 OMPs which have been implicated in permeability-associated antibiotic resistance (4, 18). These OMP variations were associated with growth phase and medium composition (gels not shown) and did not appear to be involved in resistance in this strain. Together with the lack of any decrease in CCCP-induced ciprofloxacin accumulation compared with MP-001, these observations suggest that neither a *cfxB* nor an *nfxB* class mutation is involved in the decreased susceptibility of MP-002 and MP-003.

The long chain O-polysaccharide component of LPS, which was not observed in the posttherapy isolates, was easily visualized in LPS preparations of the pretherapy isolate. Unlike similar LPS alterations in other quinolone-resistant isolates of *P. aeruginosa* (6, 25), LPS alterations could not be correlated with decreased accumulation of ciprofloxacin. While both posttherapy isolates lost the O-polysaccharide side chains, only MP-003 exhibited an apparent decrease in accumulated drug. MP-002 accumulated amounts of drug similar to those accumulated by MP-001, the initial isolate. Chamberland et al. (6) reported a 67 to 74% decrease in norfloxacin uptake and cross-resistance to chloramphenicol associated with alterations in the O-polysaccharide side chains of LPS in a norfloxacin-resistant isolate of *P. aeruginosa* selected in vivo during experimental endocarditis. Quinolone resistance was also attributed to an alteration in DNA gyrase because total DNA synthesis was more resistant to inhibition by norfloxacin. Legakis et al. (25) observed not only slightly decreased quinolone accumulation and loss of the long chain O-polysaccharide component of LPS in ciprofloxacin-resistant mutants of *P. aeruginosa* selected in vitro but also changes in OMPs and cross-resistance to nonquinolone antibiotics. Decreased length of LPS side chains in *P. aeruginosa* has also been associated with decreased β-lactam permeability (14, 15) and, contrarily, with hypersusceptibility to β-lactams, gentamicin, and hydrophobic agents (2).

The major change in the structure of LPS in both posttherapy isolates suggests that modification of the O-polysaccharide component of LPS may be involved in the expression of resistance to quinolones; however, because the accumulation assay does not adequately distinguish between intracellular and adherent extracellular drug, the precise nature of the relationship between alterations of LPS and quinolone resistance cannot be inferred. There is insufficient evidence that saline washes or centrifugation through oil satisfactorily removes drug that may be instantaneously associated with the outer membrane. Also, small variations in associated drug may not be resolvable by bioassay, and a small difference in ciprofloxacin permeability may have a large impact on cell viability.

The increase in resistance of DNA gyrase-mediated supercoiling to ciprofloxacin within this strain, conferred by the gyrase A subunits from the resistant posttherapy isolates, correlates very well with the decrease in ciprofloxacin susceptibility in these isolates. Therefore, we conclude that an alteration in the gyrase A subunit is the major contributor

to quinolone resistance in this clinical strain of *P. aeruginosa*. Loss of the O-polysaccharide side chains of LPS may play a minor role as well.


## ACKNOWLEDGMENTS

We thank Holly Godin, Janet Herrington, Denise Rasimas, and Barbara Thurberg for determining the MICs presented in this paper. We also thank Steven Seidenfeld for providing the clinical isolates and John Ogle for the epidemiological typing of these isolates.


## LITERATURE CITED


1. **Ames, G. F.-L.** 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. **248:** 634–644.
2. **Angus, B. L., J. A. M. Fyfe, and R. E. W. Hancock.** 1987. Mapping and characterization of two mutations to antibiotic supersusceptibility in *Pseudomonas aeruginosa*. J. Gen. Microbiol. **133:**2905–2914.
3. **Aoyama, H., K. Sato, T. Fujii, K. Fujimaki, M. Inoue, and S. Mitsuhashi.** 1988. Purification of *Citrobacter freundii* DNA gyrase and inhibition by quinolones. Antimicrob. Agents Chemother. **32:**104–109.
4. **Büscher, K.-H., W. Cullmann, W. Dick, and W. Opferkuch.** 1987. Imipenem resistance in *Pseudomonas aeruginosa* resulting from diminished expression of an outer membrane protein. Antimicrob. Agents Chemother. **31:**703–708.
5. **Celesk, R. A., and N. J. Robillard.** 1989. Factors influencing the accumulation of ciprofloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **33:**1921–1926.
6. **Chamberland, S., A. S. Bayer, T. Schollaardt, S. A. Wong, and L. E. Bryan.** 1989. Characterization of mechanisms of quinolone resistance in *Pseudomonas aeruginosa* strains isolated in vitro and in vivo during experimental endocarditis. Antimicrob. Agents Chemother. **33:**624–634.
7. **Chin, N.-X., and H. C. Neu.** 1984. Ciprofloxacin, a quinolone carboxylic acid compound active against aerobic and anaerobic bacteria. Antimicrob. Agents Chemother. **25:**319–326.
8. **Cohen, S. P., D. C. Cooper, J. S. Wolfson, K. S. Souza, L. M. McMurry, and S. B. Levy.** 1988. Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. Antimicrob. Agents Chemother. **32:**1187–1191.
9. **Eron, L. J., L. Harvey, D. L. Hixon, and D. M. Poretz.** 1985. Ciprofloxacin therapy of infections caused by *Pseudomonas aeruginosa* and other resistant bacteria. Antimicrob. Agents Chemother. **28:**308–310.
10. **Follath, F., M. Bindschedler, M. Wenk, R. Frei, H. Stadler, and H. Reber.** 1986. Use of ciprofloxacin in the treatment of *Pseudomonas aeruginosa* infections. Eur. J. Clin. Microbiol. **5:** 236–240.
11. **Gellert, M., K. Mizuuchi, M. O'Dea, and H. Nash.** 1976. DNA gyrase: an enzyme that introduces superhelical turns into DNA. Proc. Natl. Acad. Sci. USA **73:**3872–3876.
12. **Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. Tomizawa.** 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. USA **74:**4772–4776.
13. **Gellert, M., M. H. O'Dea, T. Itoh, and J. Tomizawa.** 1976. Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. USA **73:**4474–4478.
14. **Godfrey, A. J., and L. E. Bryan.** 1984. Resistance of *Pseudomonas aeruginosa* to new β-lactamase-resistant β-lactams. Antimicrob. Agents Chemother. **26:**485–488.
15. **Godfrey, A. J., L. Hatleid, and L. E. Bryan.** 1984. Correlation between lipopolysaccharide structure and permeability resistance in β-lactam-resistant *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **26:**181–186.
16. **Gonzalez, M. A., A. H. Moranchel, S. Duran, A. Pichardo, J. L. Magana, B. Painter, A. Forrest, and F. L. Drusano.** 1985. Multiple-dose pharmacokinetics of ciprofloxacin administered intravenously to normal volunteers. Antimicrob. Agents Chemother. **28:**235–239.
17. **Hancock, R. E. W., and A. M. Carey.** 1979. Outer membrane of *Pseudomonas aeruginosa*: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. **140:**902–910.
18. **Hancock, R. E. W., V. J. Raffle, and T. I. Nicas.** 1981. Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **19:**777–785.
19. **Hirai, K., H. Aoyama, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1986. Differences in susceptibility to quinolones of outer membrane mutants of *Salmonella typhimurium* and *Escherichia coli*. Antimicrob. Agents Chemother. **29:**535–538.
20. **Hirai, K., S. Suzue, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1987. Mutations producing resistance to norfloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **31:**582–586.
21. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
22. **Inoue, Y., K. Sato, T. Fujii, K. Hirai, M. Inoue, S. Iyobe, and S. Mitsuhashi.** 1987. Some properties of subunits of DNA gyrase from *Pseudomonas aeruginosa* PAO1 and its nalidixic acid-resistant mutant. J. Bacteriol. **169:**2322–2325.
23. **Johnson, J. D., W. L. Hand, J. B. Francis, N. King-Thompson, and R. W. Corwin.** 1980. Antibiotic uptake by alveolar macrophages. J. Lab. Clin. Med. **95:**429–439.
24. **Kaatz, G. W., and S. M. Seo.** 1988. Mechanism of ciprofloxacin resistance in *Pseudomonas aeruginosa*. J. Infect. Dis. **158:** 537–541.
25. **Legakis, N. L., L. S. Tzouvelekis, A. Makris, and H. Kotsifaki.** 1989. Outer membrane alterations in multiresistant mutants of *Pseudomonas aeruginosa* selected by ciprofloxacin. Antimicrob. Agents Chemother. **33:**124–127.
26. **Liu, L. F., and J. C. Wang.** 1978. *Micrococcus luteus* DNA gyrase. Active components and a model for its supercoiling of DNA. Proc. Natl. Acad. Sci. USA **5:**2098–2102.
27. **Mehtar, S., Y. Drabu, and P. Blakemore.** 1986. Ciprofloxacin in the treatment of infections caused by gentamicin resistant gram-negative bacteria. Eur. J. Clin. Microbiol. **5:**248–251.
28. **Miller, R. V., and T. R. Scurlock.** 1985. DNA gyrase (Topoisomerase II) from *Pseudomonas aeruginosa*. Biochem. Biophys. Res. Commun. **110:**694–700.
29. **Mizuuchi, K., M. Mizuuchi, M. H. O'Dea, and M. Gellert.** 1984. Cloning and simplified purification of *Escherichia coli* DNA gyrase A and B proteins. J. Biol. Chem. **259:**9199–9201.
30. **National Committee for Clinical Laboratory Standards.** 1985. Approved standard M7-A. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. National Committee for Clinical Laboratory Standards, Villanova, Pa.
31. **Ogle, J. W., L. B. Reller, and M. L. Vasil.** 1988. Development of resistance in *Pseudomonas aeruginosa* to imipenem, norfloxacin, and ciprofloxacin during therapy proved by typing with a DNA probe. J. Infect. Dis. **158:**743–748.
32. **Robillard, N. J., and A. L. Scarpa.** 1988. Genetic and physiological characterization of ciprofloxacin resistance in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **32:** 535–539.
33. **Roehl, R. A., and P. V. Phibbs, Jr.** 1982. Characterization and genetic mapping of fructose phosphotransferase mutations in *Pseudomonas aeruginosa*. J. Bacteriol. **149:**897–905.
34. **Sawai, T., R. Hiruma, N. Kawana, M. Kaneko, F. Taniyasu, and A. Inami.** 1982. Outer membrane permeation of β-lactam antibiotics in *Escherichia coli*, *Proteus mirabilis*, and *Enterobacter cloacae*. Antimicrob. Agents Chemother. **22:**585–592.
35. **Scully, B. F., H. C. Neu, M. F. Parry, and W. Mandell.** 1986. Oral ciprofloxacin therapy of infections due to *Pseudomonas aeruginosa*. Lancet **i:**819–822.
36. **Scurlock, T. R., and R. V. Miller.** 1979. PaeExo IX: a unique deoxyribonuclease from *Pseudomonas aeruginosa* active in the presence of EDTA. Nucleic Acids Res. **7:**167–177.
37. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using

bicinchoninic acid. Anal. Biochem. **150**:76–85.
38. **Stadenbauer, W. L., and E. Orr.** 1981. DNA gyrase: affinity chromatography on novobiocin-Sepharose and catalytic properties. Nucleic Acids Res. **9**:3589–3603.
39. **Sugino, A., C. L. Peebles, K. N. Kreuzer, and N. R. Cozzarelli.** 1977. Mechanism of action of nalidixic acid: purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. USA **74**:4767–4771.
40. **Takahata, M., and T. Nishino.** 1988. DNA gyrase of *Staphylococcus aureus* and inhibitory effect of quinolones on its activity. Antimicrob. Agents Chemother. **32**:1192–1195.