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Clinical Isolate of *Citrobacter freundii* Highly Resistant to New Quinolones

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About 30% of 150 recent clinical isolates of *Citrobacter freundii* were resistant to ≥3.13 μg of norfloxacin and ofloxacin per ml. Study of one quinolone-resistant strain for which the norfloxacin MIC was 100 μg/ml suggested that resistance was associated with both an altered A subunit of DNA gyrase and reduction in drug uptake accompanied by a decrease in an outer membrane protein.

There has been interest recently in the development of new quinolones, some of which have much greater in vitro activity and, hence, broader spectra than nalidixic acid (23). The development of resistance can significantly shorten the useful lifetime of antimicrobial agents (17, 18). Resistance to nalidixic acid and new quinolones in bacteria is the result of chromosomal mutations, some of which have been identified and mapped in *Escherichia coli* K-12 (7, 9–12).

Citrobacter freundii resistant to quinolones has previously been isolated from clinical material (14, 20). Also, *C. freundii* can rapidly acquire resistance to nalidixic acid; the MIC rose to 1,600 μg/ml after five transfers (8). We reported earlier that one of the resistance mechanisms of the spontaneous nalidixic acid-resistant mutant M2-5 was an altered DNA gyrase A subunit (1).

In this study, we surveyed the in vitro susceptibilities of clinical isolates of *C. freundii* and investigated the resistance mechanisms of one strain which was highly resistant, focusing both on DNA gyrase and drug uptake.

A total of 150 strains of *C. freundii* were tested for quinolone susceptibility. They were collected from several hospitals throughout Japan between 1984 and 1986. The majority of the strains were isolated from upper urinary tract infections, and some of the patients involved had been treated with new quinolones (including some administered during clinical trials). Clinical isolate *C. freundii* GN16598 was selected for further study. *C. freundii* IID976 (wild type), M2-5 (Nal^r) (1), and C-2 (Chl^r) (this study) were also used. Previous reports have already correlated decreased susceptibility to chloramphenicol with reduction in amounts of either the OmpF porin protein of *E. coli* (4, 5, 13, 16) or the 37-, 39-, and 40-kilodalton outer membrane proteins of *Enterobacter cloacae* (21). We applied this method to *C. freundii*, and spontaneous chloramphenicol-resistant mutants were isolated from strain IID976. One mutant strain, C-2, was chosen for further study.

Antibacterial susceptibility was measured by an agar dilution method using Mueller-Hinton agar (Difco Laboratories). MICs were determined after incubation at 37°C for 18 h.

The A and B subunits of DNA gyrase were purified from *C. freundii* GN16598 as described previously (1). Inhibition of DNA gyrase supercoiling activity was assayed by a method described previously (1).

Outer membrane proteins of *C. freundii* strains were prepared by treatment of cell envelopes with *N*-lauroylsarcosine (Sigma Chemical Co.) (21), and analysis by urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done by the method of Uemura and Mizushima (22).

The uptake of norfloxacin by *C. freundii* cells was measured by a method described previously (1, 9).

Figure 1 shows the susceptibility distribution of the clinical isolates of *C. freundii* against quinolones. Strains were susceptible to broad concentrations of quinolones, and about 30% were resistant to ≥3.13 μg of norfloxacin and ofloxacin per ml. One isolate (GN16598) showed particularly high resistance. It has been reported that ofloxacin-resistant *C. freundii* was observed after only 5 days of ofloxacin therapy in the first patient to be treated with the quinolone (3). It is generally accepted that bacterial resistance to antimicrobial agents parallels the frequency of use of the agents. We believe this will become ever more recognizable in the future.

The susceptibilities of strains GN16598, C-2, IID976, and M2-5 are shown in Table 1. GN16598 was highly resistant to quinolones and other antimicrobial agents. M2-5 was 16 to 32 times more resistant to new quinolones than was the parent strain (IID976). However, M2-5 did not have increased resistance to nonquinolone agents. On the other hand, strain C-2 was 16 times more resistant to chloramphenicol and was also resistant to other agents.

The 50% inhibitory concentrations (IC_{50}s) of quinolones and novobiocin against the supercoiling activity of GN16598, IID976, and M2-5 DNA gyrase are shown in Table 2. Although DNA gyrase from GN16598 was resistant to quinolones, it was sensitive to novobiocin. The IC_{50} for DNA gyrase from GN16598 was the same as that for DNA gyrase from M2-5 (Nal^r). DNA gyrase from GN16598 and IID976 was defined as the mixture of Ar+Br (r, resistant) and As+Bs (s, susceptible), respectively. The IC_{50}s of norfloxacin were 18.8 and 0.65 μg/ml against Ar+Bs and As+Br, respectively. The results confirm that *C. freundii* GN16598 had an alteration in the A subunit.

The MICs of quinolones against strain GN16598 were higher than those against M2-5; however, the IC_{50}s of quinolones for DNA gyrase of the two were the same. These results suggest that other factors contribute to quinolone

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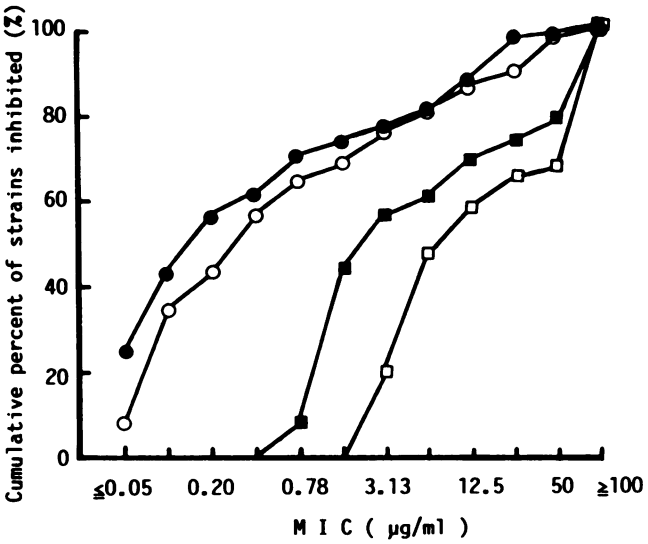

FIG. 1. Susceptibilities of 150 *C. freundii* strains to norfloxacin (●), ofloxacin (○), pipemidic acid (■), and nalidixic acid (□).

resistance in GN16598. Alteration in outer membrane permeability is another potential mechanism of resistance to quinolones (2, 6, 19). Resistance to quinolones is associated with pleiotrophic resistance to nonquinolone agents and with changes in outer membrane proteins in *Klebsiella pneumoniae*. *E. cloacae*, *Serratia marcescens*, and *Pseudomonas aeruginosa* (6, 15, 19). We therefore assessed the outer

TABLE 1. Susceptibilities of *C. freundii* GN16598, IID976, M2-5, and C-2 to quinolones and other antibacterial agents

Antimicrobial agent	MIC (μg/ml)[a] for *C. freundii*:			
	GN16598	IID976	M2-5	C-2
Norfloxacin	100	0.05	0.78	0.78
Ofloxacin	100	0.05	0.78	0.78
Ciprofloxacin	25	0.006	0.10	0.10
AM-833	50	0.05	0.78	0.78
Pipemidic acid	400	0.78	12.5	6.25
Nalidixic acid	>1,600	3.13	>1,600	50
Novobiocin	>1,600	1,600	1,600	>1,600
Chloramphenicol	>400	6.25	6.25	100
Tetracycline	400	0.78	0.78	12.5
Cefoxitin	800	100	100	400
Piperacillin	>400	3.13	3.13	400

[a] MIC determined by the agar dilution method.

TABLE 2. Inhibitory effects of quinolones and novobiocin on supercoiling activity of *C. freundii* DNA gyrase

Antimicrobial agent	IC_{50}[a] (μg/ml) for DNA gyrase from:		
	GN16598	IID976	M2-5
Norfloxacin	18.0	0.50	20.1
Ofloxacin	17.1	0.52	16.9
Ciprofloxacin	4.9	0.29	5.2
AM-833	21.0	0.55	21.2
Pipemidic acid	>800	8.9	>800
Nalixidic acid	>800	14.2	>800
Novobiocin	1.1	1.2	1.1

[a] The IC_{50} was obtained by quantitative measurement of the supercoiled pBR322 DNA peak in an agarose gel by densitometric assay.

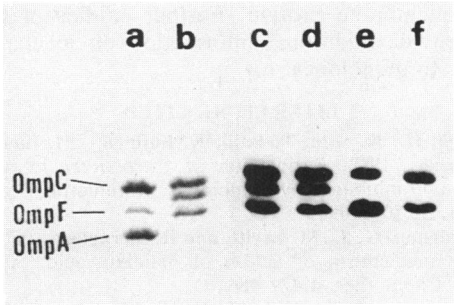

FIG. 2. Urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins of *C. freundii* IID976 (lane b or c), M2-5 (lane d), C-2 (lane e), GN16598 (lane f), and *E. coli* KL-16 (lane a). Approximately 20 μg of protein was loaded onto the gel.

membrane proteins of *C. freundii* strains and found that they were of a relatively simple composition. Three major outer membrane proteins were found in IID976 and M2-5 (Fig. 2, lanes b or c and d, respectively). However, one of the major outer membrane proteins was diminished in GN16598 and C-2 (Fig. 2, lanes f and e, respectively). The electrophoretic mobilities of these proteins differed from those of *E. coli* KL-16 (Fig. 2, lane a) outer membrane proteins, such as OmpF, OmpC, and OmpA.

We compared the uptake rate of norfloxacin by GN16598 cells with the uptake by IID976, M2-5, and C-2 cells (Fig. 3). Strain GN16598 showed about threefold-lower norfloxacin uptake than IID976 and M2-5, and uptake was almost equal to that of C-2. These findings suggested that the protein plays a very important role in susceptibility to quinolones.

It was reported that the *nfxB* and *norB* norfloxacin resistance mutation in *E. coli* was associated with additional resistances to nonquinolone agents and with decreased OmpF porin protein (9, 10). The MICs of norfloxacin against these mutants, 0.32 (*nfxB*) and 0.20 (*norB*) μg/ml, were only fourfold higher than that against the parent strain. However, the MIC of norfloxacin against the C-2 mutant (0.78 μg/ml) was 16 times higher than that against IID976 (parent). Quinolones might be more strongly affected in antibacterial activity by the lack of the outer membrane protein of *C. freundii* than by the lack of the OmpF of *E. coli*. These results suggested that quinolone resistance in GN16598 was associated with alterations of DNA gyrase and that decreased norfloxacin uptake was accompanied by the change

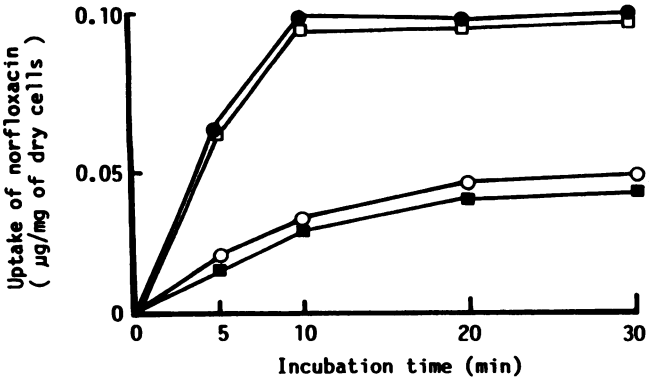

FIG. 3. Uptake of norfloxacin by *C. freundii* strains. Symbols: ■, GN16598 (clinical isolate); ●, IID976 (wild type); □, M2-5 (Nal^r); ○, C-2 (Chl^r).

of outer membrane protein. Further studies of GN16598 should provide additional information on mechanisms of resistance to quinolones.

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