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Quinolone Resistance in Clinical Isolates of *Serratia marcescens*

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The uptakes of norfloxacin by quinolone-resistant and -susceptible strains of *Serratia marcescens* were almost the same and 50% inhibitory concentrations for DNA gyrase and the MICs of quinolones were correlated, suggesting that DNA gyrase alterations are the basis of quinolone resistance.

Quinolones are having increasing clinical use because of their potent and broad antimicrobial activity. Accompanying this usage, the number of quinolone-resistant strains has increased (4, 13, 14, 21). Especially in *Serratia marcescens*, there are many strains resistant to quinolones (6). Moreover, this organism has easily acquired and showed resistance to many antibiotics (10, 15) and has been recognized as an important nosocomial pathogen. However, only preliminary investigation (5) has been reported concerning DNA gyrase (8) in this organism. We report here the purification of DNA gyrase from *S. marcescens*, the inhibitory effects of quinolones on DNA gyrase supercoiling activity, and norfloxacin uptake into bacterial cells.

S. marcescens IAM1184 was a stock collection strain of the Institute of Applied Microbiology, University of Tokyo. *S. marcescens* GN16707 and GN16720 were isolated from urinary tract infections.

T-3262, ofloxacin, norfloxacin, and pipemidic acid were commercially available materials. Susceptibility to quinolones was measured by agar dilution using Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.) with an inoculum of approximately 10^4 CFU per spot. Crude extract of DNA gyrase was prepared as previously described (1), applied to a novobiocin-Sepharose (19) column (1.0 by 10 cm), and eluted in steps with 0.1 M KCl, 2 M KCl, and 5 M urea in TED (10 mM Tris hydrochloride [pH 7.5], 1 mM EDTA [pH 7.5], 0.5 mM dithiothreitol) buffer. Active fractions eluting with 2 M KCl were loaded onto a heparin-Sepharose CL-6B column (1.0 by 15 cm), and the activity was eluted with 0.5 M KCl. The fraction eluting with 5 M urea was again purified on novobiocin-Sepharose. Active fractions were pooled and dialyzed against 50% glycerol in TED buffer. The assay of DNA gyrase was slightly modified from previous reports (7). Reaction mixtures for DNA supercoiling activity contained 20 mM Tris hydrochloride (pH 7.5), 40 mM KCl, 1 mM $MgCl_2$, 1 mM spermidine, 1 mM dithiothreitol, 10 μg of bovine serum albumin per ml, 30 μg of tRNA per ml, 1.5 mM ATP, 0.1 μg of pBR322 DNA which had been relaxed by topoisomerase I, two active proteins (1 U each), and drug solution. One unit of DNA gyrase was defined as the activity that converted 50% of relaxed pBR322 DNA to the supercoiled form. We measured 50% inhibitory doses (IC_{50}s) as previously described (17). The uptake of norfloxacin by *S. marcescens* was measured by bioassay as described previously (11). Outer membrane proteins were prepared by the method of Sawai et al. (18) and analyzed on a urea-sodium dodecyl sulfate-polyacrylamide gel as described by Uemura and Mizushima (20).

To determine whether the two proteins were actually components of *S. marcescens* DNA gyrase, we assayed their supercoiling activities. Neither protein by itself had any activity. However, supercoiling activity was observed when the two proteins were combined. Moreover, we tested the supercoiling activities of intergeneric hybrids prepared from the two isolated proteins of *S. marcescens* and *Escherichia coli* DNA gyrase. Gyrase activity was observed by combining protein that was eluted by 2 M KCl from *S. marcescens* with the B subunit of *E. coli* Hfr strain KL 16 (3, 9) and by combining protein that was eluted by 5 M urea from *S. marcescens* with the A subunit of *E. coli* KL 16 (data not shown). This interspecies complementation provided evidence for the functional equivalence of *S. marcescens* and *E. coli* subunits. The proteins that were eluted with 2 M KCl and 5 M urea are designated subunits A and B of *S. marcescens*, respectively.

The susceptibilities of *S. marcescens* strains and IC_{50}s are shown in Table 1. *S. marcescens* IAM1184 was susceptible, GN16707 was moderately resistant, and GN16720 was highly resistant to the quinolones. The MIC of norfloxacin for strain GN16707 was 16 times higher than that for IAM1184, and for strain GN16720 it was 500 times higher. The IC_{50}s for DNA gyrase from GN16707 were 10 times higher than the IC_{50}s for DNA gyrase from IAM1184, and for DNA gyrase from GN16720 they were about 150 times higher. Thus, the inhibitory effects of norfloxacin on the supercoiling activities of DNA gyrase from these three strains paralleled the antibacterial activities. With T-3262 and ofloxacin, the same parallelism was observed. However, the degrees of binding of norfloxacin by the three strains (Fig. 1) and the electrophoretic patterns of outer membrane proteins in the cell envelopes of these three strains (Fig. 2) were almost the

TABLE 1. Antibacterial activities and IC_{50}s of quinolones for DNA gyrase supercoiling activities

Quinolone	Concn of quinolone (μg/ml) for *S. marcescens*:					
	IAM1184		GN16707		GN16720	
	MIC	IC_{50}	MIC	IC_{50}	MIC	IC_{50}
T-3262	0.1	0.38	1.56	1.70	25	15.0
Ofloxacin	0.39	0.56	3.13	3.5	50	42.0
Norfloxacin	0.2	0.41	3.13	4.3	100	61.0
Pipemidic acid	1.56	9.5	50	74.0	>200	>200

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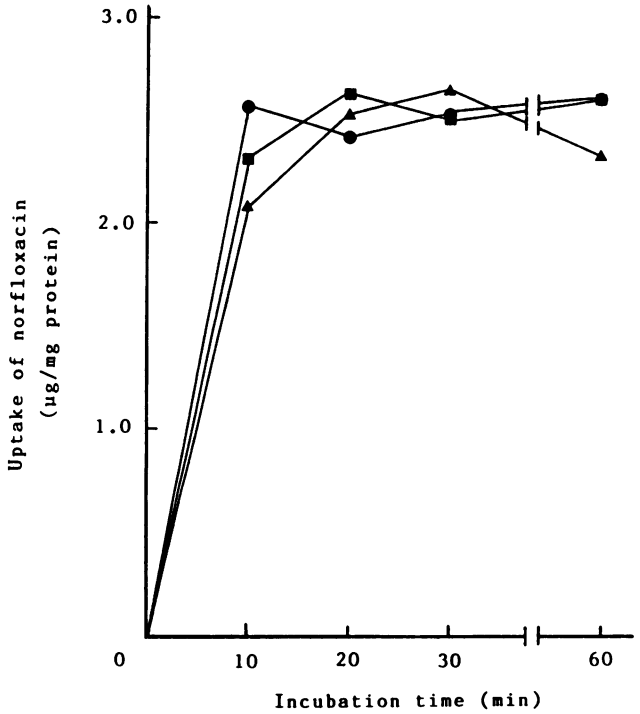

FIG. 1. Uptake of norfloxacin by *S. marcescens*. Symbols: ●, strain IAM1184; ■, strain GN16707; ▲, strain GN16720.

same. These data suggest that resistance to quinolones in GN16707 and GN16720 was caused by modification of DNA gyrase and not by decreased uptake.

To determine the subunit responsible for gyrase resistance, mixing experiments were done with heterologous DNA gyrase preparations derived from IAM1184 (AsBs), GN16720 (Ar-1Br-1), and GN16707 (Ar-2Br-2). The results presented in Table 2 indicate that IC_{50}s of norfloxacin for Ar-1Bs or Ar-2Bs were almost the same as those for Ar-1Br or Ar-2Br, while IC_{50}s for AsBr-1 or AsBr-2 were almost the same as those for AsBs. These data suggest that modification of subunit A conferred quinolone resistance in these strains.

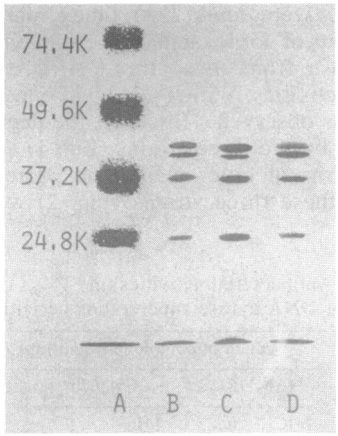

FIG. 2. Urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane proteins. Lanes: A, standard molecular weight proteins (dimer [24,800], trimer [37,200], tetramer [49,600], and hexamer [74,400] of cytochrome *c*); B, strain IAM1184; C, strain GN16720; D, strain GN16707.

TABLE 2. IC_{50}s of norfloxacin inhibiting reconstituted DNA gyrase from *S. marcescens* IAM1184, GN16720, and GN16707

DNA gyrase subunit[a]	IC_{50} (μg/ml) of norfloxacin
AsBs	0.41
AsBr-1	0.49
Ar-1Bs	70.2
Ar-1Br-1	61.0
AsBr-2	0.51
Ar-2Bs	3.9
Ar-2Br-2	4.3

[a] As and Bs, A and B subunits from strain IAM1184; Ar-1 and Br-1, A and B subunits from strain GN16720; Ar-2 and Br-2, A and B subunits from strain GN16707.

Similar results were reported in *E. coli* (17), *Pseudomonas aeruginosa* (12), and *Citrobacter freundii* (1).

We could not select highly resistant strains as one-step quinolone-resistant mutants from strain IAM1184 (data not shown). However, we could obtain spontaneous mutants (MIC of norfloxacin, 50 μg/ml) from the moderately quinolone-resistant strain. Therefore, we speculate that one-step mutants from quinolone-susceptible strains correspond to GN16707 and further mutations are required to produce GN16720.

Sanders and Watanakunakorn (16) reported that alteration of outer membrane proteins contributed to resistance to quinolones, β-lactams, and aminoglycosides in *S. marcescens*. Aoyama et al. (2) reported that highly quinolone-resistant *E. coli* mutants had alterations in both DNA gyrase and the cell membrane. Considering the increase in resistant strains of *S. marcescens*, we plan to check the possibility that quinolone-resistant mutants of *S. marcescens* have alterations of the outer membrane in addition to modification of DNA gyrase or that they have other mutations.

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