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Ciprofloxacin Resistance by Altered Gyrase and Drug Efflux System in *Pseudomonas aeruginosa*

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Ciprofloxacin resistance mechanisms were studied by investigating the inhibitory effect of ciprofloxacin on the gyrase-mediated DNA supercoiling and the intracellular accumulation of ciprofloxacin in clinical isolates of *Pseudomonas aeruginosa.* A higher amount of ciprofloxacin was required to inhibit the gyrases purified from the ciprofloxacin-resistant strains than that from the sensitive strain. Reconstitution of heterologous gyrase subunits from different strains revealed alterations in the A and/or the B subunits of gyrase in these strains. In addition, the resistant strains accumulated approximately a half amount of ciprofloxacin inside the cells, compared to the sensitive strain. However, when the active efflux was blocked by carbonyl cyanide m-chlorophenyl hydrazone treatment, intracellular concentration of ciprofloxacin was elevated about 4-7 fold in these strains, while the sensitive strain was not significantly affected by this treatment, indicating that the ciprofloxacin-resistant strains developed a drug efflux system. Interestingly, these resistant strains expressed an envelope protein of approximately 51 kD. These studies suggest that alterations in the gyrase as well as the active drug-efflux system conferred dual ciprofloxacin resistance mechanisms to these clinical isolates of *P. aeruginosa.*

Key words : Ciprofloxacin, Resistance, Gyrase, Efflux, *Pseudomonas aeruginosa*

INTRODUCTION

Since nalidixic acid, the first quinolone, was introduced in 1960's, a number of structurally modified compounds have been developed that have improved antibacterial efficacy. Especially, fluoroquinolones such as ciprofloxacin and norfloxacin showed excellent *in vitro* antimicrobial activities against a broad range of pathogens and clinical uses of these compounds showed favourable pharmacologic properties. However, their clinical use has been limited due to the rapid emergence of quinolone-resistant strains.

Quinolone inhibits gyrase, a bacterial tetrameric enzyme consisting of two A and B subunits, and thereby possesses the antibacterial action at effective concentrations. The amount of antibiotics at the target site is determined by two processes, drug uptake and efflux. Alteration in the target, uptake or efflux system can result in the development of bacterial resistance to quinolone antibiotics. Indeed, in some quinolone-resistant *Escherichia coli,* mutations of the A subunit (Cullen *et al.,* 1989; Heisig *et al.,* 1993) and the B subunit (Yoshida *et al.,* 1991; Yamagishi *et al.,* 1986) as well as decreased drug uptake (Hooper *et al.,* 1986) have been identified. Similar mutations in the A and the B subunits of gyrase (Ito *et al.,* 1994) and the development of active efflux system (Kaatz *et al.,* 1993) have been observed in *Staphylococcus aureus.* On the other hand, in Pseudomonas aeruginosa, alterations only in the A subunit of gyrase (Masecar *et al.,* 1990) and drug uptake system (Fukuda *et al,* 1990) have been described.

Here in this report, we present a new finding in *P. aeruginosa* that alterations in the B subunit of gyrase can also play a major role in rendering quinolone resistance to some clinical isolates of *P. aeruginosa.* All of the ciprofloxacin-resistant *P. aeruginosa* strains tested had the B subunits that were drastically altered. These strains also gained an active drug-efflux system which appeared to confer a low level of resistance to ciprofloxacin.

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MATERIALS AND METHODS

Bacterial strains and growth conditions

Clinical isolates of *Pseudomonas aeruginosa* used in this study were provided by Hoechst Company in Germany. Cells were grown in FEB medium (bactopeptone 10 g, beef extract 10 g, NaCl 5 g and sodi-

um diphosphate 0.8 g per liter) at 37°C unless specified otherwise.

Antibacterial susceptibility test

Antibacterial susceptibility of the bacterial strains was determined by an agar dilution method. Briefly, cells of 10^7 c.f.u./ml were inoculated on Muller Hinton agar (Difco) containing serially diluted antibiotics. The minimal inhibitory concentrations (MICs) were read as the lowest concentrations completely inhibiting visible bacterial growth after incubation at 37°C for 18 h. Ciprofloxacin was synthesized at Korea Research Institute of Chemical Technology and others were purchased from Sigma.

Purification of gyrase

Gyrase was partially purified in a similar manner as for the gyrase from *Citrobacter freundii* (Aoyama et al., 1988) with some modifications. A eight liter culture at late log phase was harvested by centrifugation at 7,500 x g for 20 min. Pellets were washed once with TGED buffer (50 mM Tris-Cl, pH. 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol) and stored at -70°C until use. All the purification steps were carried out at 4°C. Cells were lysed in the presence of 7.5 mM DTT, 18.75 mM EDTA, 0.375% Brij 58 (Sigma), 0.015% lysozyme and 1mM PMSF in TGED buffer for 20 minutes. Incubation was continued for another 20 minutes after KCl was added to the final concentration of 0.4 M. The lysates were subjected to centrifugation at 135,000 x g for 1 hr and chromosomal DNA was excluded by addition of 4% streptomycin sulfate to the supernatant. Crude protein fraction obtained by 65% ammonium sulfate precipitation followed by dialysis against TGED buffer was loaded onto a novobiocin-Sepharose column (bed volume of 20ml) which was prepared as described by Staudenbauer and Orr (1981). Proteins were first eluted with 1 M KCl in TGED buffer, and subsequently by 5 M urea in TGED buffer. The A or the B subunit present in the 1 M KCl and the 5 M urea eluates was identified by assaying the gyrase activities of each eluate alone and the mixture of each eluate with the pure A or B subunit of *Citrobacter freundii*. Active fractions containing each subunit were pooled separately and dialyzed twice against TGED buffer. The pooled fractions were concentrated by dialysis against 50% glycerol, 50 mM Tris-Cl, pH 7.5, divided into small aliquots and stored at -70°C until assay.

DNA supercoiling assay

The susceptibility of gyrase to the inhibitory action of ciprofloxacin was tested by measuring the ability of the purified gyrase to catalyze the DNA supercoiling reaction in the absence or presence of ciprofloxacin. Relaxed form of pBR322 DNA used as a substrate in this reaction was prepared by treating the supercoiled DNA with calf thymus topoisomerase I (BRL) as recommended by the manufacturer. The reaction mixture contained 0.25 μg of relaxed DNA, 1 unit of gyrase, and various concentrations of ciprofloxacin in 20 mM Tris-Cl, pH. 8.0, 20 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, and 2 mM spermidine-HCl. One unit of gyrase activity was determined as described by Okuda *et al.* (1991). The mixtures were incubated at 37°C for 1 hr and the reaction was stopped by adding DNA loading buffer containing 100 mM EDTA. Samples were subjected to 0.8% agarose gel electrophoresis and DNA was visualized on the UV illuminator. The minimal concentration of ciprofloxacin at which the supercoiling activity of gyrase was completely inhibited was defined as IC_{100}.

Determination of intracellular concentration of ciprofloxacin

Cells at late log phase were resuspended in M9 media to give an OD_{595} of 1.0. Ciprofloxacin and carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma) dissolved in 100% DMSO were added to cells at final concentrations of 100 μg/ml and 1 mM, respectively. Cells that were not treated with CCCP were supplemented with appropriate amount of DMSO. After incubation at 37°C for 15 minutes, cells were washed three times with saline and resuspended with 100 μl of saline. Cells were then lysed by boiling for 10 minutes. After centrifugation at 12,000 x g for 2 min, the supernatant was taken and the concentration of ciprofloxacin was determined by bioassay with *E. coli* 078 as a test microorganism and normalized by the amount of protein in the supernatant.

Preparation of envelope proteins

Outer membrane proteins were prepared as described by Poxton *et al.* (1985). Cells cultured to the late log phase were harvested and washed once with 0.01 M Hepes, pH. 7.4 and broken by sonication (5 times for 30 seconds with 10 seconds of intervals). Unbroken cells were removed by two runs of centrifugation at 5,000 x g for 5 minutes and envelope fractions were collected by centrifugation at 50,000 x g for 1 hr. Pellets were washed with water once, resuspended in 0.01 M Hepes and about 40 μg of protein was analyzed by SDS/10% polyacrylamide gel electrophoresis.

RESULTS

In order to study the quinolone resistance mechanisms in *Pseudomonas aeruginosa,* several strains were

Table I. Antimicrobial susceptibilities of *Pseudomonas aeruginosa* clinical isolates to ciprofloxacin

Strains	MIC (μg/ml)
1771	0.156
55	6.25
94	6.25
30973	50

The minimal inhibitory concentrations (MIC_s) of ciprofloxacin against P. aeruginosa strains were determined as described in Materials and Methods.

Table II. Susceptibilities of the reconstituted *Pseudomonas aeruginosa* gyrases to the anti-DNA supercoiling action of ciprofloxacin

Gyrase purified from strain	IC_{100} (μg/ml)
1771	1.56
55	100
94	50
30973	50

IC_{100} of ciprofoxacin was defined as the minimal concentration of ciprofloxacin at which the gyrase-catalyzed DNA supercoiling reaction was completely inhibited.

chosen that are highly resistant to the antimicrobial action of ciprofloxacin as shown in Table I. The MIC (minimal inhibitory concentration) values of ciprofloxacin against strains 55, 94, 30973 are 30- to 200-fold higher than the MIC against strain 1771.

Susceptibilities of purified gyrases to ciprofloxacin

Gyrases were partially purified from all of the above strains including the ciprofloxacin-sensitive strain and their susceptibilities to the inhibitory effect of ciprofloxacin were compared with each other as described in Methods and Materials. Since the crude preparations of the gyrase A and B subunits were obtained from separate fractions (data not shown), the holoenzyme was reconstituted by mixing the two subunits together. The activity of this reconstituted enzyme was assayed by measuring the conversion of a relaxed form of plasmid DNA into a supercoiled one. As shown in Table II, the 100% inhibitory concentrations (IC_{100}) of ciprofloxacin for the gyrases from strains 55, 94, and 30973 were 32- to 64-fold higher than that for strain 1771. This result suggests that the three resistant strains expressed gyrases that were insensitive to the inhibitory effect of ciprofloxacin and that this might be the basis for the resistance of these strains to the antimicrobial action of ciprofloxacin. It was also noted that although strain 30973 was more resistant to the antimicrobial action of ciprofloxacin than the other two resistant strains, its gyrase was almost the same as those of the two strains in terms of the sensitivity to the inhibition by ciprofloxacin. This might be explained by a possibility that a second resistance mechanism should also reside in this strain.

Table III. Susceptibilities of the heterologously reconstituted pseudomonas aeruginosa gyrases to anti-DNA supercoiling action of ciprofloxacin

Heterologous Reconstitution of			IC_{100} (μg/ml)
the A subunit from strain	with	the B subunit from strain	
55		1771	100
94		1771	1.56
30973		1771	25
1771		55	50
1771		94	50
1771		30973	50

In order to further determine which subunit of gyrase is responsible for ciprofloxacin resistance in these strains, gyrases purified from different strains were cross-reconstituted. The IC_{100} values of ciprofloxacin against the supercoiling activities of various combinations of gyrases are given in Table 3. When the B subunit of strain 1771 was mixed with the A subunit of strain 55 or 30973, the susceptibilities of these heterologously reconstituted gyrases to ciprofloxacin became as low as those of the gyrases formed from their own subunits of strain 55 or 30973. These results indicate that alterations in the A subunits of strains 55 and 30973 are playing a key role for ciprofloxacin resistance in these two strains. However, the A subunit of strain 94 did not have any effect when the B subunit of strain 1771 was combined with the A subunit of the strain 94. Since the gyrase activity of strain 94 was fairly resistant to ciprofloxacin (Table II), this result suggests that the low susceptibility of gyrase of strain 94 might have been derived from the altered B subunit. This possibility was confirmed by the fact that cross-reaction between the A subunit of strain 1771 and the B subunit of strain 94 resulted in a reconstituted gyrase that was quite insensitive to ciprofloxacin. When the B subunits from strain 55 or 30973 were cross-reacted with the A subunit of strain 1771, the IC_{100} values of ciprofloxacin were also moderately increased, indicating that strains 55 and 30973 might have had some mutations in the B as well as the A subunits of their gyrases. These are rather new findings, since mutations in the B subunit of gyrase have not yet been reported in *P. aeruginosa.* However, nucleotide sequence analysis needs to be performed to identify the mutation(s) in the B subunit.

Intracellular accumulation of ciprofloxacin

Other resistance mechanism was also investigated by measuring the amount of ciprofloxacin accumulated in the cell. As shown in Fig.1, the amounts of

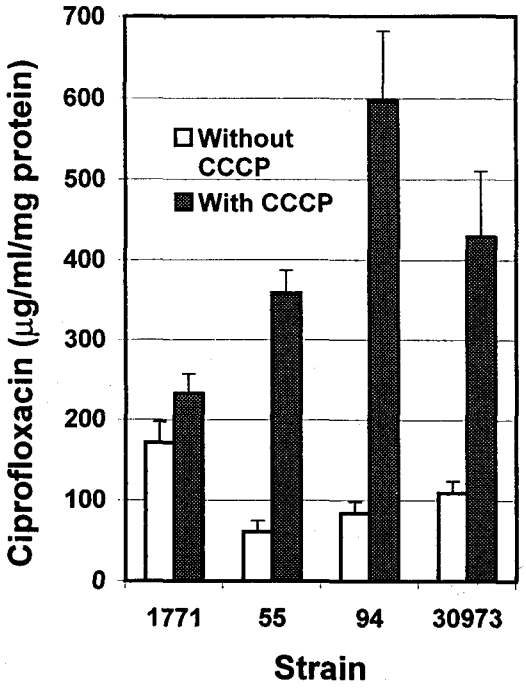

Fig. 1. Intracellular accumulation of ciprofloxacin and effects of CCCP in *P. aeruginosa* stains. After each strain was incubated for 15 min with ciprofloxacin in the absence or presence of CCCP, cells were washed and the amount of ciprofloxacin inside the cells were determined by bioassay as described in Materials and Methods.

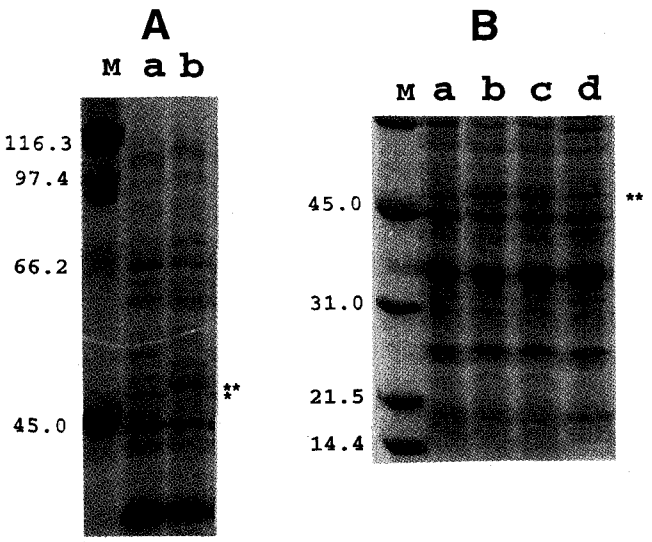

Fig. 2. Analyses by SDS-PAGE and coomassie blue staining of envelope proteins from *P. aeruginosa strains.* envelope proteins from the ciprofloxacin-sensitive strain 1771 and the ciprofloxacin-resistant strain 55 were loaded onto lanes a and b, respectively in both panels A and B, and samples from the other ciprofloxacin-resistant strains 94 and 30973 were loaded onto lanes c and d in panel B. A new protein of about 51 kD marked with "asterisks" (**) consistently appears only in the ciprofloxacin-resistant strains, while a protein band indicated by an "asterisk" (*) is often absent in these strains. Lane M in each panel shows the molecular weight markers whose sizes are shown at the left side of each panel.

ciprofloxacin accumulated inside the cells of all the three resistant strains, 55, 94, and 30973 were only one half of that in the sensitive strain, 1771. This indicates that the decreased intracellular accumulation of ciprofloxacin in these resistant strains might also have contributed to their resistance to the antimicrobial action of ciprofloxacin.

Decrease in the intracellular accumulation of ciprofloxacin can be acquired by either less uptake or more active efflux. Thus, we explored the possibility of increased efflux of ciprofloxacin in these ciprofloxacin-resistant strains. Intracellular accumulation of ciprofloxacin was assayed similarly, after blocking the active efflux system by CCCP, a protonophore which desensitizes the proton gradient of the membrane. If the resistant strains have developed an active efflux system to pump out the drug, blocking the efflux by CCCP would result in the increase in the amount of ciprofloxacin accumulated in the cell. In fact, as shown in Fig. 1, the amount of ciprofloxacin accumulated in the three resistant strains has increased 4-7 fold by CCCP treatment, while no significant effect of CCCP was noted in the sensitive strain 1771. These results suggest that the resistant strains somehow acquired an active drug efflux system and thereby gained some resistance to the antimicrobial action of ciprofloxacin.

Profiles of envelope proteins

Development of active efflux in ciprofloxacin-resistant strains suggested that these strains might express altered envelope proteins. This possibility was tested by comparing the envelope protein profiles of the ciprofloxacin-sensitive and ciprofloxacin-resistant strains. Analysis by SDS polyacrylamide gel electrophoresis indicated that a new protein with an apparent molecular weight of 51 kD was present in all of the three ciprofloxacin-resistant strains but not in the sensitive strain (Fig. 2). In the ciprofloxacin-resistant strains, this new protein was seen consistently in several repeated experiments, however occasionally accompanied by the disappearance of a protein of a slightly lower molecular weight.

Since cross-resistance to imipenem and chloramphenicol but hypersusceptibility to β-lactams and aminoglycosides are some of the characteristics of quinolone-resistant *P. aeruginosa* with altered quinolone uptake (Fukuda *et al.,* 1990; Lei *et al.,* 1991), we were also interested in investigating whether the strains used in the present study exhibit similar phenomenon to these non-quinolone antibiotics. As shown in Table 4, the ciprofloxacin-resistant strains 55, 94 and 30973 were somewhat resistant to most of the

Table IV. MIC_s of non-quinolone antibiotics against *Pseudomonas aeruginosa* clinical isolates

Strains	MIC (μg/ml)					
	CEF	IMP	RFP	GEN	TET	CM
1771	1.563	0.781	25	1.563	25	50
55	25	0.781	50	6.25	50	100
94	25	0.781	50	6.25	50	100
30973	12.5	1.563	100	6.25	50	100

CEF, IMP, RFP, GEN, TET, and CM stand for cefpirome, imipenem, rifampicin, gentamicin, tetracycline, and chloramphenicol, respectively.

antibiotics tested including cefpirome, a β-lactam compound, suggesting that these three resistant strains are different from those bacteria having altered drug uptake system.

Taken together, these results suggest that the altered expression of envelope proteins in the ciprofloxacin-resistant *P. aeruginosa* might be responsible for the elevated drug-efflux, which then contributes to the resistance to antimicrobial agents such as ciprofloxacin and cefpirome.

DISCUSSION

In this study, we demonstrated that ciprofloxacin-resistant clinical isolates of *P. aeruginosa* employed two resistance mechanisms. First, the resistant strains had altered A and/or B subunits of gyrase. Second, they accumulated a less amount of ciprofloxacin inside the cell than the ciprofloxacin-sensitive strain. Alteration in the A and/or the B subunit of gyrase resulted in a gyrase 32- to 64-fold less sensitive to the inhibitory action of ciprofloxacin, while gaining the drug efflux system had only a two-fold effect on the intracellular concentration of ciprofloxacin. Thus, alteration in the A and/or the B subunit of gyrase appears to be the major mechanism for ciprofloxacin resistance in these strains of *P. aeruginosa.* While alteration in the B subunit of gyrase has been reported in *E. coli* (Yoshida *et al.,* 1991; Yamagishi *et al.,* 1986) and *S. aureus* (Ito *et al.,* 1994), this is the first report showing an alteration of the B subunit in *P. aeruginosa.*

Among the A and the B subunits of gyrase, the A subunit is the target that quinolones interact with. Thus, alteration in this target can affect the susceptibility of its gyrase to the inhibitory effect of ciprofloxacin. However, studies by us in *P. aeruginosa* and others in other bacteria (Yoshida *et al.,* 1991; Ito *et al.,* 1994) indicate that alteration in the B subunit can also produce similar results. A recent report (Yoshida *et al.,* 1993) suggests that the affinity of DNA/gyrase complex for quinolone is determined by the concerted interaction of the A and the B subunits. Therefore, some alterations in the B subunits would result in a gyrase that has a lower affinity for ciprofloxacin and thus becomes less susceptible to the inhibitory action of ciprofloxacin.

In ciprofloxacin-sensitive strain 1771, an active drug efflux system does not appear to be present, because CCCP, an inhibitor of active efflux did not have a significant effect on the amount of ciprofloxacin accumulated in this strain. In contrast, the ciprofloxacin-resistant strains 55, 94, 30973 exploited a drug-efflux system to lower the amount of ciprofloxacin in their cells.

Proteins mediating the quinolone efflux have not yet been identified in *P. aeruginosa.* We showed in this study that an envelope protein of about 51 kD was present in ciprofloxacin-resistant strains of *P. aeruginosa* but not in the sensitive one. This protein might be either the efflux transporter or one of those proteins expressed in some quinolone-resistant *P. aeruginosa* strains that have decreased uptake of quinolone due to the mutations in *nfxB* or *nfxC* gene (Hirai *et al.,* 1987; Fukuda *et al,* 1990). However, the ciprofloxacin-resistant strains used in this study differ from the *nfxB* and *nfxC* mutants. The strains used in this study were resistant to a β-lactam compound, while the *nfxB* and *nfxC* mutants are characteristically hypersusceptible to β-lactams. Furthermore, the efflux but not the uptake system appeared to be altered in the strains used in this study. Thus, the 51 kD protein expressed in ciprofloxacin-resistant clinical isolates of *P. aeruginosa* studied here might be a new entity associated with the drug-efflux in these bacteria. Confirmation of this possibility will await the genetic and molecular biological analyses on this protein.

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