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Resistance Mechanism of *Acinetobacter* spp. Strains Resistant to DW-116, a New Quinolone

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DW-116 is a new fluoroquinolone antimicrobial agent with a broad spectrum. In order to elucidate the resistance mechanism to DW-116 in *Acinetobacter* spp. bacteria, total chromosomal DNA was isolated from 10 strains of *Acinetobacter* spp. resistant to DW-116. Quinolone resistance determinant region (QRDR) of DNA gyrase gene was amplified by PCR. The 345 bp nucleotide fragment yielded was inserted into pKF 3 which was used as the vector. Comparisons of the DNA sequences of 8 strains with that of the wild type strain revealed a Ser-83 to Leu mutation in mutants and all ten strains contained one silent mutation(T→G) in QRDR. From *Acinetobacter* MB4-8 strain, DNA gyrase was isolated and purified, through novobiocin-sepharose, heparin-sepharose affinity column chromatography. The enzyme was composed of two subunits and the molecular mass of subunits A and B were 75.6 and 51.9 kDa, respectively. The supercoiling activity of the reconstituted DNA gyrase composed of subunit A from *Acinetobacter* MB4-8 and subunit B from *E. coli* was not inhibited by 128 μg/ml of ciprofloxacin. It might be said that one of the resistance mechanisms to DW-116 in *Acinetobacter* MB4-8 was subunit A alteration of DNA gyrase.

Key words : DW-116, Fluoroquinolone, PCR, DNA gyrase, QRDR, *Acinetobacter*, Ciprofloxacin

INTRODUCTION

The quinolones are clinically useful group of antibacterial agents. Among the post potent of quinolones are the fluoroquinolones such as ciprofloxacin, ofloxacin, which are established therapeutic agents (Neu, 1987; Mueller *et al.*, 1994; Aldridge, 1994; Piddock,1993). The DNA gyrases are type II topoisomerase which catalyze the supercoiling of relaxed covalently closed circular DNA which is known to be coupled with the hydrolyzed of ATP. The enzyme from *E. coli* (topoisomerase II) is composed of two different subunits A and B, which are encoded by the genes of *gyrA* and *gyrB*, respectively (Gellert *et al.*, 1977; Liu and Wang, 1983; Miller and Seurlock, 1983) The *gyrA* protein constitutes the target for nalidixic acid and oxolinic acid, and the *gyr B* protein is the target for novobiocin and coumermycin A1. The target of new quinolones, i.e., norfloxacin, ofloxacin, ciprofloxacin and enoxacin, is subunit A; and these drugs exhibit marked inhibitory effects. Evidence has been provided that other gram-negative bacteria possess DNA gyrase and that it is inhibited by quinolones (Sato *et al.*, 1983; Shen *et al.*, 1989). Many studies have been carried out with quinolone-resistant gram-negative bacteria. Two main mechanisms of resistance which may occur either alone or combined have been found: (i) modification of the DNA gyrase in either subunit A or B, which can give rise to low-levels or high levels of resistance (Heisig and Wiedeman, 1991; Sreedharan *et al.*, 1991; Yoshida *et al.*, 1991) and (ii) decreased uptake of quinolones, which correlates with a decrease in the quantity of porins (Bryan and Bedard, 1991; Hooper *et al.*, 1989; Li *et al.*, 1995).

DW-116 is a new fluoroquinolone with the formula {1-(5-fluoro-2-pyridyl)-6-fluoro-7-(4-methyl-1-piperazynyl)-1,4-dihydro-4-oxoquinoline-3-carboxylic acid hydrochloride} ($C_{20}H_{18}F_2N_4O_3 \cdot HCl$, m.w. 436.84) which was synthesised by the R & D Center, Dong- Wha Pharmaceutical Company in Korea (Yoon *et al.*, 1996). In preliminary study, DW-116 showed relatively poor *in vitro* activity against control strains, but good pharmacokinetic profiles similar to rufloxacin (Choi *et al.*, 1997; Yoon *et al.*, 1996). The reference antibiotic, rufloxacin had relatively poor *in vitro* activity but it showed good pharmacokinetic profiles, potent therapeutic effects and stimulation of immune system, etc. (Aoki, 1994). In this report, we describe the resistance mechanism of DW-116 observed in clinical isolates of *Acinetobacter* spp.

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MATERIALS AND METHODS

Bacterial strains

Clinical isolates were obtained from hospitals in Seoul between 1990 and 1994. Among 500 clinical isolates of *Acinetobacter* spp., 10 strains were selected by susceptibility test. The bacterial strains can grow under above 2 μg/ml of ciprofloxacin and DW-116 and other fluoroquinolones.

Antimicrobial susceptibility

MICs were determined by the agar dilution method with Mueller-Hinton agar (Difco) (NCCLS, 1993). The MIC was defined as the lowest concentration of antimicrobial agent that inhibited visible growth of bacteria after 18 h at 37°C.

Chemical agents

Novobiocin, dithiothreitol (DTT), tRNA of *E. coli*, spermidine, proteinase K, lysozyme, Brij 58, streptomycin sulfate, and ATP were purchased from Sigma Chemical Co; Topoisomerase I was from Bethesda Research Laboratories. Subunits A and B of DNA gyrase of *E. coli* KL-16 were purified as described (Staudenbauer and Orr, 1981). Epoxy activated Sepharose 6B and heparin-Sepharose CL-6B were purchased from Pharmacia Fine Chemicals. DW-116, rufloxacin and ciprofloxacin were provided by the R & D Center, Dong-Wha Pharmaceutical Company, Korea. Sparfloxacin was provided by the Rhone-Poulenc Rorer Company, France. Ofloxacin was provided by the Daiichi Seiyaku Company, Japan.

PCR

Chromosomal DNA were prepared from each strain using Genomic DNA purification kit (Promega). To identify *gyrA* mutations in resistant isolates, PCR of QRDR of *gyrA* and cloning of QRDR and DNA sequencing were used (Heisig and Weidemann, 1991). It was utilized conserved amino acid sequence motif found in diverse *gyrA* gene. Two primers, 5'-GCCATACCTACG-GCGATACC-3' and 5'-AAATTCTGCCCGTGTCGTTGGT-3' from Amitof, USA, were used. The reactions were performed in 10 mM Tris·Cl (pH 8.3)- 50 mM KCl-1.8 mM MgCl2-0.01% gelatin containing 200 μM (each) the four deoxynucleoside triphosphate, 2.5 U of *Vent* polymerase, 100 pmoles (each) primer, and 100 ng of chromosomal DNA in a total volume of 100 μl. Thirty cycles were used for each reaction with one cycle consisting 1 min at 94°C, 1 min at 57°C and 1 min 20 sec at 72°C. Reactions were performed in a Progene (Techne) as described above.

Nucleotide sequencing

The PCR products containing QRDR of *gyrA* gene from clinical isolates were cloned in *E. coli* TH2 (pKF 3 plasmid) and nucleotide sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977).

Enzyme purification

The cells of *Acinetobacter* spp. grown in Mueller-Hinton medium were collected by centrifugation at 7000×g for 15 min at 4°C. The frozen cell suspension was thawed in a 37°C water bath, chilled to 4°C (twice), and cell lysis was done. Brij 58 (0.5%) was added, then solid ammonium sulfate was added (30~60%), was loaded on novobiocin-Sepharose column (bed volume, 20 ml). Novobiocin-Sepharose was prepared by coupling of novobiocin to epoxy-activated Sepharose 6B as described by Staudenbauer and Orr (1981). The column was washed with 100 ml of the same buffer and eluted stepwise by 0.2 M KCl-2 M KCl-5M urea in TED buffer. The active fractions of subunits A and B were pooled separately and dialysate of subunit A was loaded onto a column of heparin-Sepharose CL-6B (bed volume, 20 ml). The dialysate of subunit B fractions was further purified by chromatography on novobiocin-Sepharose again.

Enzyme assay

The assay of DNA gyrase subunit was done by the method of Aoyama *et al.* (1988). The assay of the purified gyrase measures supercoiling of DNA as analyzed by agarose gel electrophoresis. The supercoiling reaction is unique to gyrase. Plasmid pBR 322 DNA, used as the substrate in the assay of DNA gyrase was relaxed by topoisomerase I.

Inhibition of DNA gyrase supercoiling activity

Inhibition of DNA gyrase supercoiling activity was assayed in a manner similar to that described previously (Aoyama *et al.*, 1988). One unit of enzyme activity was defined as the amount that brought 50% of relaxed pBR 322 to the supercoiled form in agarose gel electrophoresis as described by Gellert *et al.* (1977). The standard reaction mixture contained 40 mM Tris·Cl (pH 7.5), 20 mM KCl, 4 mM $MgCl_2$, 2 mM spermidine, 1 mM ATP, 2 mM DTT, 20 μg of tRNA per ml, 0.1 μg of relaxed pBR 322 DNA, drug solution, and DNA gyrase subunits A and B. After incubation at 37°C for 1 h, the reaction was stopped by the addition of 3 μl of proteinase K (1 mg/ml). The reaction mixture was subjected to agarose gel electrophoresis (0.8% agarose in 40 mM Tris acetate, 2 mM EDTA). The inhibition of supercoiling activity of intergeneric hybrids reconstituted from subunit A of *Acinetobacter* sp. MB4-8 and subunit B from *E. coli* KL-16 was tested with various concentration of cipro-

floxacin.

RESULTS AND DISCUSSION

Quinolone-resistant strains were selected from clinical isolates. The antimicrobial susceptibility of *Acinetobacter* spp. strains is shown in Table I. Ten strains of *Acinetobacter* spp. were resistant to all quinolones tested. These strains were 10 to 100 times more resistant than susceptible strains to quinolones. Especially, two strains of *Acinetobacter* MB4-8 and MB4-10 showed high resistance to quinolones.

By using the PCR protocols described above, a 345 bp DNA fragment from *Acinetobacter* spp. chromosomal DNA was amplified (Fig. 1). To confirm that the amplification product was from the *gyrA* gene, it was sequenced and compared with the known *gyrA* sequences from other bacteria. As shown in Fig. 2, all

Table I. The susceptibility of *Acinetobacter* spp. to quinolones

Strain	MIC (μg/ml)*				
	SPFX	CPFX	OFLX	RFLX	DW-116
MB4- 6	0.25	4	4	16	8
MB4- 8	8	32	128	256	128
MB4-12	0.5	4	4	16	8
MB4-14	0.5	4	4	32	16
MB4-15	0.5	4	4	16	8
MB4-16	0.5	4	4	16	8
MB4-21	1	4	4	64	32
MB4-24	0.5	8	8	32	32
MB4-98	0.25	4	8	8	8
MB4-10	8	32	64	64	32
MB4-53	0.008	0.015	0.12	0.25	0.12

*inoculum size, 10^6 cfu, Mueller-Hinton broth
SPFX: Sparfloxacin, CPFX: Ciprofloxacin, OFLX: ofloxacin, RFLX: rufloxacin

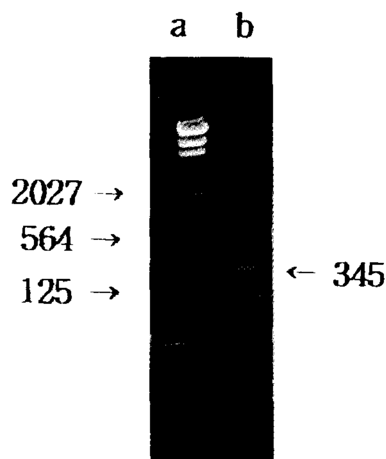

Fig. 1. PCR product of QRDR of the *gyr* A gene of clinical isolates, *Acinetobacter* spp. Agarose gel (2%) electrophoresis of PCR product. Lane a, DNA size marker (λ, *Hind*III); lane b, PCR product.

wild type -GTTGGTGACGTAATCGGTAAATATCACCGCATGGTGACT-

	(Gly)	(Ser)
	silent mutation	
MB4-6	G (Gly)	T (Leu)
MB4-8	G	T (Leu)
MB4-12	G	T (Leu)
MB4-14	G	C (Ser)
MB4-15	G	T (Leu)
MB4-16	G	T (Leu)
MB4-21	G	T (Leu)
MB4-24	G	C (Ser)
MB4-98	G	T (Leu)
MB4-10	G	T (Leu)

Fig. 2. Nucleotide changes and amino acid substitutions in the DNA gyrase protein (*gyrA*) of the clinical isolates of *Acinetobacter* spp.

of ten clinical isolates were characterized by a T- to G- mutation at codon 77 of gyrA gene, resulting in no amino acid change. Eight strains were characterized by additional C-to T- mutations at codon 83 leading to Ser-to Leu- substitutions, and two other strains were characterized by no mutation at codon 83.

DNA gyrase has been purified from *Acinetobacter* MB4-8, a highly resistant strain to ciprofloxacin, by affinity column chromatography on novobiocin-Sepharose. The active fractions of subunit A and B were further purified by chromatography on heparin-Sepharose and novobiocin-Sepharose, respectively. The molecular masses of subunits A and B were 75.6 and 51.9 kDa, respectively, on SDS-PAGE (Fig. 3). *Acinetobacter* DNA gyrase was similar to *E. coli* DNA gyrase in its cofactor requirements. The enzyme catalyzed supercoling of the relaxed pBR 322 DNA. In the absence

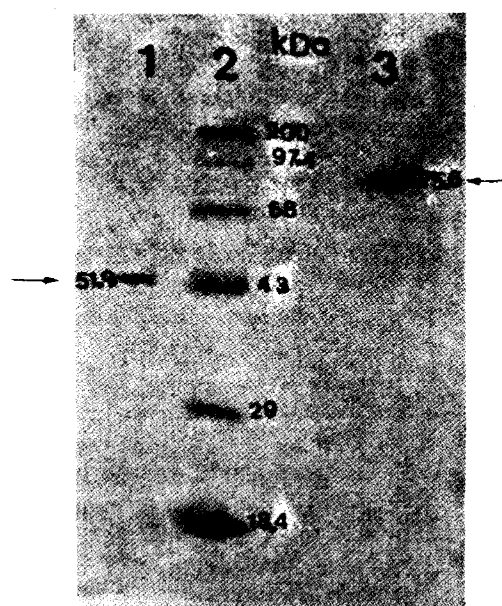

Fig. 3. SDS-PAGE (12%) of subunits of *Acinetobacter* MB4-8 DNA gyrase lane 1, subunit B, lane 2, molecular mass standards, lane 3, subunit A.

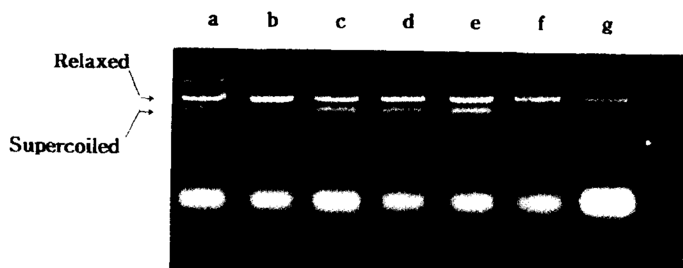

Fig. 4. Supercoiling activity of DNA gyrase prepared from *Acinetobacter* MB4-8 under different conditions. DNA gyrase reactions were performed as described in the text. lane a, 1.5 mM $MnCl_2$; lane b, relaxed pBR 322 DNA; lane c, 1.5 mM $CaCl_2$; lane d, 2 mM $FeCl_2$; lane e, $MgCl_2$; lane f, 1 mM $CuCl_2$; lane g, ATP omitted.

of ATP, no reaction took place (Fig. 4). In addition to ATP, the DNA supercoiling reaction required Mg^{2+} (Fig. 4), and Ca^{2+}, Fe^{2+} and Mn^{2+} gave slightly lower levels of activity. The supercoiling activity was inhibited by Cu^{2+}, and Hg^{2+} at a concentration of 1 or 2 mM (Fig. 4).

The supercoiling activity of the reconstituted DNA gyrase composed of subunit A from *Acinetobacter* MB4-8 and subunit B from *E. coli* KL-16, and that of subunit A from *E. coli* KL-16 and subunit B from Acinetobacter MB4-8 was tested (Fig. 5). An active enzyme was obtained by combining the subunit A of *Acinetobacter* MB4-8 and the subunit B of *E. coli* Kl-16. In this case, the supercoiling activity was not inhibited at a concentration 128 μg/ml of ciprofloxacin. The reconstituted DNA gyrase composed of the subunit B of *Acinetobacter* MB4-8 and the subunit A of *E. coli* Kl-16 also showed the good activity. However, the supercoiling activity was inhibited by ciprofloxacin, and completely inhibited at a concentration 4 μg/ml of ciprofloxacin in this case.

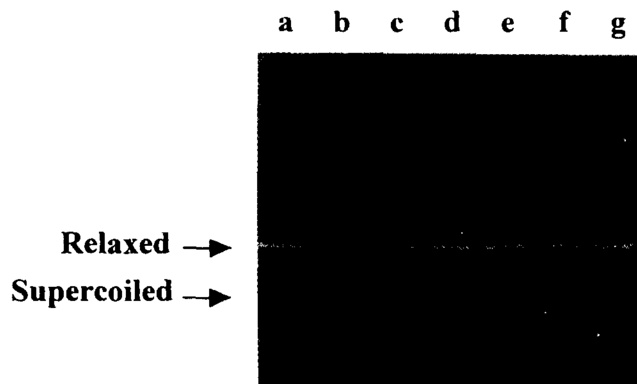

Fig. 5. The inhibition of supercoiling activity of the reconstituted DNA gyrase composed of subunit A from *Acinetobacter* MB4-8 and subunit B from *E. coli* MB4-01 by ciprofloxacin. Supercoiling activity was not inhibited by ciprofloxacin. The reaction mixture for lane a contained no drug. The reaction mixtures for lanes b-f contained ciprofloxacin of the following concentration (μg/ml), lane b, 8; lane c, 16; lane d, 32; lane e, 64; lane f, 128; lane g, relaxed pBR 322 alone.

From the results the resistance to ciprofloxacin can be thought to be due to the alteration of subunit A of DNA gyrase in *Acinetobacter* MB4-8 strain. The mechanism of resistance to quinolone antibacterial agents have mainly been studied in *E. coli.* (Hooper *et al.*, 1989; Sato *et al.*, 1983; Yoshida *et al.*, 1991). Two main mechanisms of resistance which may occur alone or combined have been found. The modification of the DNA gyrase in either subunit A or B can give rise to low levels or high levels of resistance (Heisig and Wiedeman, 1991; Sreedharan *et al.*, 1991; Yoshida *et al.*, 1991). The decreased uptake of quinolones, which correlates with a decrease in the quantity of porins is another reason for the quinolone resistance (Bryan and Bedard, 1991; Hooper *et al.*, 1989; Li *et al.*, 1995). Among 10 clinical isolates of *Acinetobacter* spp. which were resistant to quinolones, eight strains was found to have the altered base sequences in QRDR of subunit A of DNA gyrase. The base alterations were resulted in the Ser-83 to Leu mutations in mutants. This results support that the alteration of subunit A of DNA gyrase is one of the main reason for the quinolone resistance.

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