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Ofloxacin Resistance Mechanism in PA150 and PA300-Clinical Isolates of *Pseudomonas aeruginosa* in Korea

Soondeuk Lee and Yeonhee Lee*

Department of Biology, Seoul Women's University, Seoul 139-774, Korea

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Five hundred and seventy clinical strains of *Pseudomonas aeruginosa* were isolated from August 1993 to August 1994 in Korea and screened for their resistance to ciprofloxacin, norfloxacin, and ofloxacin. Among these, two *P. aeruginosa* strains (PA150 and PA300) were selected based on their strong resistance (MICs > 50 μg/ml) to all three quinolones. The susceptible strain as well as two resistant strains had proton gradient-dependent efflux system. Efflux system in PA300 showed different specificities to ofloxacin and ciprofloxacin while PA 150 had less permeability for ofloxacin. Ofloxacin had a less inhibitory action on DNA synthesis in permeabilized cells of PA150 and PA300 than 1771M. When quinolone resistance determining region (QRDR) in *gyrA* was sequenced, PA300 had one missense mutation, Asn 116Tyr, which was newly reported in this work. The results showed that PA150 became ofloxacin resistant by reduced ofloxacin accumulation due to the existence of efflux system and low permeability, while resistance of PA300 was due to the efflux system and a mutation in QRDR of *gyrA* -the target site of quinolone.

Key words : Fluoroquinolone, Ofloxacin, Resistance mechanism, Permeability, Efflux system, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is a common nosocomial pathogen and its resistance to antibacterial agents has been increasing (Masecar *et al.*, 1990). According to WHO focal point in the surveillance of antimicrobial resistance in Korea, 41% of *P. aeruginosa* isolated in 1994 had quinolone resistance. This number was extremely high compared to the numbers of resistant bacteria in other Eastern countries including China (14%), Malaysia (16%), Philippines (31%), Australia (11%), Singapore (10%) and those of other gram negative bacilli (0~25%). In Korea, there has been a great concern about the occurrence of resistant strains, since antibacterial agents are sold over the counter.

Since the quinolone resistance mechanism of *P. aeruginosa* in Korea has not been extensively studied, we do not know whether it is similar to those found in other countries (Chamberland *et al.*, 1989; Kureishi *et al.*, 1994; Legakis *et al.*, 1989; Masecar *et al.*, 1990; Robillard and Scarpa, 1988; Yonezawa *et al.*, 1995). To find out this, we screened 570 clinical strains and selected two *P. aeruginosa* strains (PA150 and PA300) whose MICs to ciprofloxacin, norfloxacin, and ofloxacin were higher than 50 μg/ml. We compared these two quinolone resistant strains with the susceptible strain (1771M) in followings; ofloxacin permeability, an efflux system for ofloxacin, and a mutation in quinolone-resistance determining region (QRDR) in DNA gyrase subunit A.

Correspondence to: Yeonhee Lee, Department of Biology, Seoul Women's University, 126 Kongnungdong, Nowongu, Seoul 139-774, Korea

MATERIALS AND METHODS

Media, chemicals and antibiotics

Mueller-Hinton and LB were purchased from Difco (Detroit, U.S.A.). [^{3}H]dTTP (specific activity: 30 Ci/mM) was purchased from Amersham (Buckinghamshire, England). GF/C filter was purchased from Whatman (Springfield, England). Quinolones and other reagents were purchased from Sigma Chemical Co. (St. Louis, U.S.A.).

Bacterial strains

From August 1993 to August 1994, 570 strains were isolated from patients in Je-il Hospital and Korea Cancer Center Hospital in Seoul, Korea. These strains were identified as *P. aeruginosa* by various biochemical tests. Each isolate was inoculated into LB containing each of 10 μg/ml ciprofloxacin, norfloxacin, or ofloxacin.

A susceptible strain, *P. aeruginosa* 1771M was provided from Korea Research Institute of Chemical Technology.

MIC determination

MICs (Minimum Inhibitory Concentration) were determined as recommended by National Committee for Clinical Laboratory Standards (1990).

Determination of ofloxacin concentration inside cells

Ofloxacin concentration inside cells was measured by a method described in a previous paper (Kim *et al.*, 1996). Bacterial cells at log phase were collected with centrifugation, resuspended in new LB making A_{530} to 10, and further incubated for 30 min at 37°C. Ofloxacin was added to the cell suspension (final conc., 50 μg/ml) and the reaction mixture (total volume, 300 μl) was incubated for 2 min at 37°C. Then carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to this (final conc., 200 μM) and the reaction was continued for another 8 min. The reaction was stopped with centrifugation after 200 μl of the reaction mixture was loaded on 1 ml of cold silicon oil in a 1.5 ml microcentrifuge tube. The bottom layer which containing cells was cut with a cutter, transferred into a new tube, and cells were suspended in 10 mM phosphate buffer (pH 7.0) with vigorous skaking. Ofloxacin was extracted from cells with boiling in a boiling water bath for 10 min and assayed with a fluoresence spectrophotometer (Kontrons SFM25, Switzland) after cells were removed with centrifugation at 10,000×g for 10 min.

Effect of ciprofloxacin on ofloxacin concentration inside cells

Cells were incubated in the presence of various concentrations of ofloxacin and 50 μg/ml of ciprofloxacin for 10 min at 37°C and fluoroquinolones were extracted from cells following a method described above. The concentrations of ciprofloxacin and ofloxacin inside cells were measured at 254 nm using high performance liquid chromatography (Young-in Co., Korea). Twenty μl of the extract was injected into C_{18} column (3.9× 150 mm) and eluted with 25% methanol in 0.1 M phosphate (pH 2.3) at a flow rate of 0.8 ml/min.

Assay of DNA synthesis in permeabilized cells

Permeabilization of bacterial cells and *in vitro* DNA synthesis were performed following a method described in a previous paper (Kim *et al.*, 1996). Bacterial cells at log phase were harvested with centrifugation at 10,000×g for 10 min and suspended in one tenth volume of cells with buffer A [10 mM Tris-HCl (pH 7.8), 50 mM NH_4Cl, 10 mM $(CH_3COO)_2Mg \cdot 4H_2O$, 7 mM β-mercaptoethanol]. Cells were treated with toluene (final conc., 2%) and shaken for 10 min in an ice bath. The cell suspension was diluted with three volumes of buffer B [10 mM Tris-HCl (pH 7.4), 62 mM NH_4Cl, 19 mM $(CH_3COO)_2Mg \cdot 4H_2O$]. Permeabilized cells were added to the reaction mixture containing 40 μM of dNTP, 0.2 mM of NADH, and ofloxacin (final conc., 100 μg/ml). After 5 min incubation at 37°C, 0.1 μCi of [^{3}H]dTTP was added to the reaction mixture (total volume, 200 μl) and incubated for 10 min. The reaction was stopped by adding 1 ml of cold 10% TCA. The acid insoluble precipitates were collected on a GF/C filter (Sigma Chemical Co., MO) using a vacuum manifold (Hoefer, U.S.A.). Filters were presoaked in 0.05% thymidine solution to prevent nonspecific binding of [^{3}H]thymidine. After filters were washed with 4 ml each of 5% TCA, 0.1 N HCl, and 95% ethanol and dried in the air. The amount of radioactivity remaining on a filter was measured in a liquid scintillation counter (Packard TRI-CARB 4530, U.S.A.).

PCR of QRDR in *gyrA*

Chromosomal DNA was isolated using G Nome kit (Bio101, U.S.A.). PCR of QRDR was performed in a thermal cycler (Equibio, Belgium) with 1 μg of chromosomal DNA in 100 μl of a reaction mixture containing 100 pmole each of primer 1 and 2, 0.2 mM dNTP, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, and 5 U of Taq DNA polymerase. After chromosomal DNA was denatured at 94°C for 4 min, PCR was done by 25 cycles of incubation for 1 min at 94°C, 2 min at 58°C, and 2.5 min at 72°C. The reaction mixture was incubated for additional 7 min at 72°C to complete the reaction. Primers were the ones designed by Kureish *et al.* (1994) and synthesized at Ransom Hill Bioscience Inc. (Ramona, CA). PCR products were checked with electrophoresis in an 1% agarose gel.

DNA sequencing

PCR amplified *gyrA* fragments from three strains were isolated from a gel using Wizard PCR Preps. (Promega, WI) and nucleotide sequences were determined using ABI PRISM Dye Terminator Cycle Sequencing Core Kit (Perkin-Elmer, U.S.A.) in an automatic DNA sequencer (Perkin-Elmer, U.S.A.).

RESULTS AND DISCUSSION

Quinolone resistant strains among clinical isolates

Among 570 clinical isolates of *P. aeruginosa* which were collected during one year period (from August, 1993 to August, 1994), 201 isolates showed quinolone resistance. The numbers of resistant strains to ciprofloxacin, norfloxacin, and ofloxacin were 21, 17, and

Table I. MICs of *P. aeruginosa* 1771M, PA150, and PA300

strains	antibiotics (μg/ml)		
	ciprofloxacin	norfloxacin	ofloxacin
P. aeruginosa 1771M	0.5	1.56	3.12
PA150	50	100	50
PA300	50	100	50

26, respectively. Seven isolates had resistance to both ciprofloxacin and ofloxacin, 10 strains to ciprofloxacin and norfloxacin, 18 strains to ofloxacin and norfloxacin, and 102 strains showed resistance to all three quinolones. Two *P. aeruginosa* strains (PA150 and PA300) were selected based on their strong resistance to all three quinolones (Table I).

Ofloxacin concentration inside cells

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) had been reported to be an inhibitor of an efflux system in *P. aeruginosa* (Chamerland *et al.*, 1989) as well as in *Escherichia coli* (Cohen *et al.*, 1988; Lee *et al.*, 1993) and *Staphylococcus aureus* (Kaatz *et al.*, 1991, 1993; Neyfakh *et al.*, 1993). If there were proton-gradient dependent efflux system for ofloxacin, it would be inhibited by the addition of CCCP and so the ofloxacin concentration inside cells would increase. As soon as CCCP was added to cells, ofloxacin concentration inside cells was increased in all three strains (Fig. 1). Ofloxacin concentration in both PA150 and PA300 increased 200% while it increased 120% in the susceptible strain. This suggested that PA150 and PA300 obtained their resistance to quinolone via efflux system. PA150 had low ofloxacin concentration inside cells in the presence of CCCP as well as in the absence of CCCP and this must be due to the low ofloxacin permeability.

Effect of ciprofloxacin on ofloxacin concentration inside cells

Three efflux systems have been found in *P. aeruginosa*, MexAB-OprM, MexCD-OprJ, and MexEF-OprN (Bianco *et al.*, 1997; Li *et al.*, 1995; Schweizer *et al.*, 1988). It was recently suggested that each quinolone is effluxed via specific active efflux pump (Kohler *et al.*, 1997). As it was shown in Fig. 1, 1771M, PA150, and PA300 had proton gradient-dependent efflux pumps. Since ciprofloxacin and ofloxacin are both hydrophilic, we wondered whether they were exported by the same efflux pump. If both quinolones were effluxed via same efflux pump, ofloxacin will be effluxed less in the presence of ciprofloxacin. As shown in Table II and Fig. 2, ofloxacin concentrations in all three strains decreased in the presence of ciprofloxacin. Ofloxacin consisted 40% of total quinolone concentration in

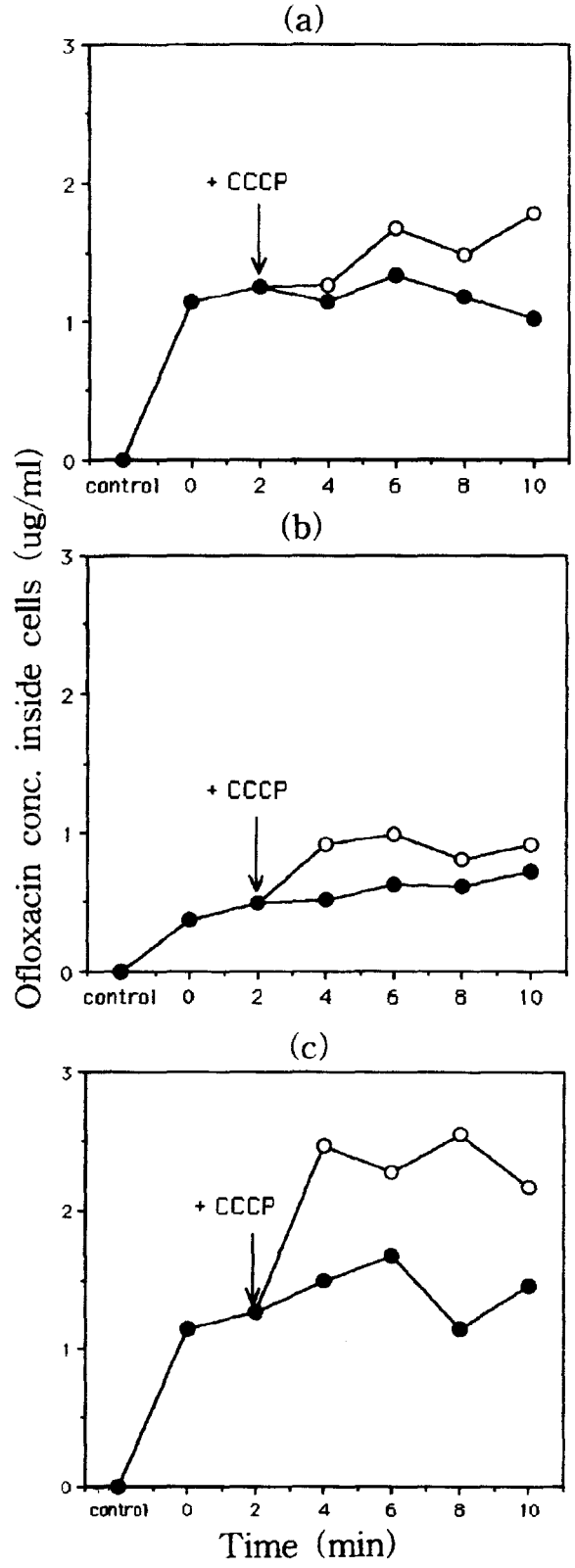

Fig. 1. Ofloxacin concentration inside cells in the presence of CCCP. Bacterial cells in log phage were incubated in the presence of 50 μg/ml ofloxacin. CCCP was added at the time indicated by an arrow. (a), 1771M; (b), PA150; (c), PA 300; ●, control; ○, CCCP

Table II. Ciprofloxacin and ofloxacin concentrations inside cells[a]

CCCP	quinolone	1771M	PA150	PA300
		%	%	%
-[b]	ofloxacin	42	40	59
	ciprofloxacin	58	60	41
	total quinolones	100	100	100
+[c]	ofloxacin	42	42	33
	ciprofloxacin	58	58	66
	total quinolones	100	100	100

[a]Cells were incubated with 50 μg/ml of ciprofloxacin and 50 g/ml of ofloxacin. Quinolones were extracted from cells by boiling, separated through C_{18} in HPLC, and detected at 254 nm.
[b]Cells were incubated with quinolones in the absence of CCCP.
[c]Cells were incubated with quinolones in the presence of CCCP.

1771M and PA150 and 60% in PA300 (Table II). When efflux pump was blocked with CCCP, the total amount of quinolones increased in all three strains with the same ratio (ofloxacin:ciprofloxacin=40:60) in the susceptible strain and PA150. However, the ratio of ofloxacin to ciprofloxacin was reversed from 60:40 to 40:60 in PA300. This showed that the efflux system in PA300 had a higher affinity to ciprofloxacin. Recently, Kohler *et al.* (1997) suggested that ofloxacin and ciprofloxacin select the MexCD-OprJ efflux system and a protonable C-7 substituent in combination with a C-6 fluorine atom is a structural dominant of quinolones involved in efflux pump substrate specificity. If this hypothesis were right, MexCD-OprJ efflux system may have stronger affinity for ciprofloxacin than ofloxacin. In our experiment, the extent of efflux of ofloxacin and ciprofloxacin were different although they carried the same charge not only at C-7 substituent but also at the other substituents. Perhaps the affinity for the efflux pump is determined by the charge of compound and the affinity is determined by the size or shape of compound.

Effect of ofloxacin on DNA synthesis in permeabilized cells

Alteration in DNA gyrase were checked with two methods-alteration at gene level with direct DNA sequencing and alteration at protein level with assay of *in vitro* DNA synthesis in permeabilized cells. The direct DNA sequencing took lots of time and labor even though it is the best way to detect a change at gene level, while *in vitro* DNA synthesis in permeabilized cells is easy to perform to detect a change in DNA gyrase. In permeabilized cells, quinolone could directly reach to DNA gyrase and exert a direct inhibitory action avoiding uptake barrier and efflux pump. As shown in Fig. 3, 100 μg/ml ofloxacin decreased DNA synthesis to 11% in 1771M, 19% in PA150, and

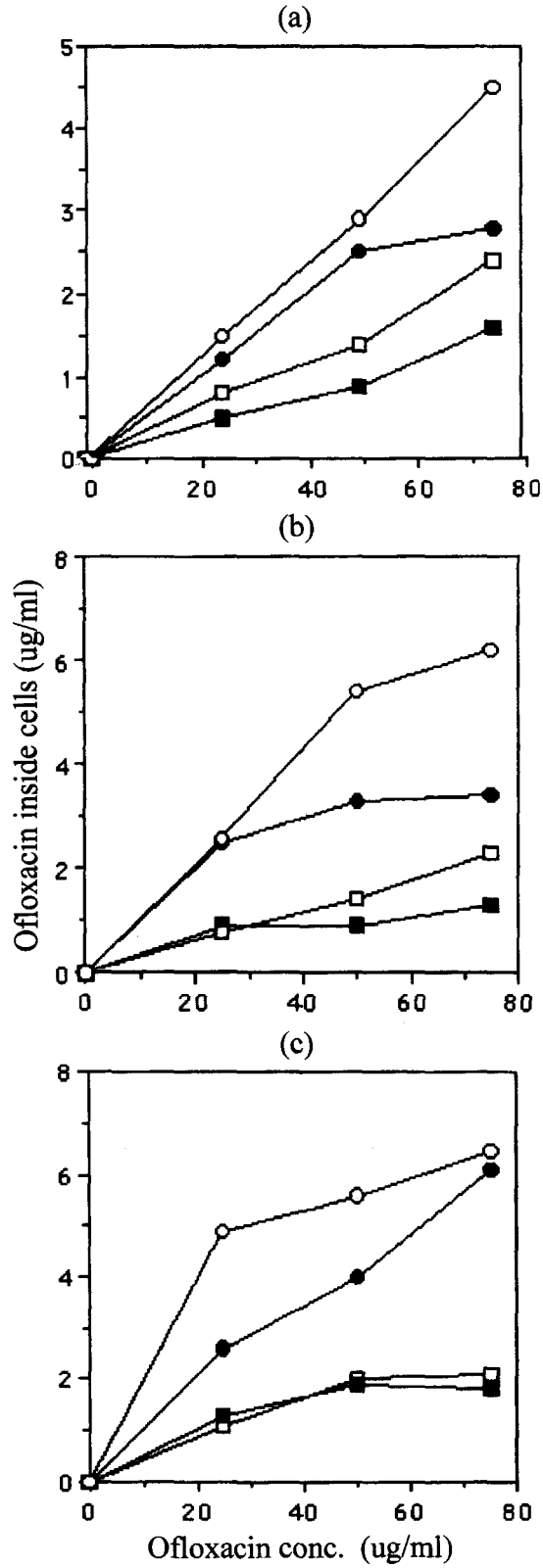

Fig. 2. Effect of ciprofloxacin on ofloxacin concentration inside cells. Cells were exposed to ciprofoxacin (50 μg/ml) and ofloxacin (0, 25, 50, 75 μg/ml). Quinolones were analysed with HPLC. (a), 1771M; (b), PA150; (c), PA300; ●, ofoxacin; ○, ofloxacin and CCCP; ■, ofloxacin and ciprofloxacin; □, ofloxacin, ciprofloxacin, and CCCP

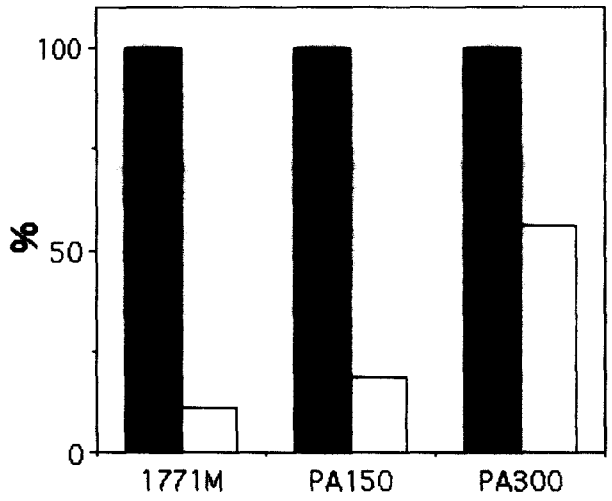

Fig. 3. DNA synthesis in permeabilized cells. Bacterial cells were permeabilized and incubated in the presence of [^{3}H] TTP and ofloxacin (100 μg/ml) for 10 min. The amount of DNA synthesized in the absence of ofloxacin was considered as 100%. ■, control; □, ofloxacin

56% in PA300 suggesting a change in DNA gyrase of PA300.

DNA sequence in QRDR of *gyrA*

Changes in QRDR in *gyrA* were known to cause the quinolone-resistance in *E. coli* (Cambau *et al.*, 1993), *Enterococcus faecalis* (Korten *et al.*, 1994), *S. aureus* (Hopewell *et al.*, 1990), *Salmonella typhymurium* (Reyna *et al.*, 1995), and *P. aeruginosa* (Robillard and Scarpa, 1988). In the *gyrB* gene, two quinolone resistance determining sites (amino acids 426 and 447) have been found (Yoshida *et al.*, 1991). However, the level of resistance attributable to the mutation in *gyrB* was much lower than that in *gyrA*. In *P. aeruginosa*, the substitution of Thr83Ile, Asp87Asn, Asp87Gly, and Asp87Tyr in *gyrA* had been reported to be responsible for the quinolone resistance (Kureishi *et al.*, 1994; Robillard and Scarpa, 1988). When QRDRs from +147 nt (61 residue) to +511 nt (160 residue) were sequenced, PA150 had one silence mutation at Val85 (GTC→GTT) and PA300 had 11 silence mutations and 1 missense mutation-Asn116Tyr (AAC→TAC), which was newly reported in this work. It is possible that mutations occur outside of the QRDR; however, the similarity in the clustering of mutations in QRDR of *E. coli* and *S. aureus* strongly suggests that this region is especially important in intermolecular interaction in the quinolone-gyrase-DNA complex.

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