

## Accumulation of ofloxacin and tosufloxacin in fluoroquinolone-resistant *E coli*<sup>1</sup>

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**KEY WORDS** microbial drug resistance; ofloxacin; tosufloxacin; fluoroquinolone; *Escherichia coli*; cell membrane permeability

### ABSTRACT

**AIM:** To make sure whether there is a difference in mechanism existed in the resistant *E coli* strains accumulated hydrophilic fluoroquinolone ofloxacin and hydrophobic fluoroquinolone tosufloxacin. **METHODS:** Fluoroquinolone accumulation in bacteria and effect of active efflux were measured by fluorescence method. Analysis of outer membrane proteins was made by SDS-PAGE. *E coli* strains included JF701 and JF703 that are OmpC- or OmpF-deficient mutants of *E coli* K-12 respectively, and the susceptible strain Ecs and its *in-vitro* selected resistant strains R2, R256, and clinical resistant isolates R5, R6. **RESULTS:** Ecs accumulated ofloxacin almost at the same concentration as JF701, but JF703 did about 1/2 of that lower than JF701. However, four resistant strains accumulated ofloxacin about 5 to 7-fold lower than those susceptible strains. On the other hand, there was no significant difference for the accumulation of tosufloxacin between fluoroquinolone-resistant and -susceptible strains. After addition of proton ionophore DNP for 5 min and 10 min, the accumulation of tosufloxacin slowly decreased in *E coli* strains, whereas the accumulation of ofloxacin was increased, especially in the resistant strains. A good relevance exists between the accumulation increment of ofloxacin and its MIC for each *E coli* strain after addition

of DNP for 5 min and 10 min ( $r = 0.9623$  and  $0.8006$  respectively). Furthermore, both OmpF and OmpC in Ecs, OmpF-deficiency in R2, R256 and OmpC-deficiency in R5, R6 were observed. **CONCLUSION:** The accumulation of ofloxacin other than tosufloxacin could be reduced by OmpF-deficiency and active efflux, and the latter may be an important factor in the development of resistance to hydrophilic fluoroquinolone in *E coli*.

### INTRODUCTION

Fluoroquinolone resistance is mediated by target changes<sup>(1)</sup> and/or decreased intracellular accumulation<sup>(2,3)</sup>. The latter resulted from an efflux mechanism which has been described in several Gram positive and Gram negative bacteria<sup>(4)</sup>. In such a situation, drug is pushed out of the bacteria before it reaches the target.

In *E coli*, reduced accumulation of fluoroquinolone seems not only involved endogenous active efflux system<sup>(5)</sup> or multiple-drug efflux pumps<sup>(4)</sup>, but also involves the loss of some outer membrane proteins (OMP), especially porin OmpF<sup>(6)</sup>. However, OmpF-deficiency seems not to affect the accumulation of hydrophobic fluoroquinolones such as sparfloxacin (SPLX) and pefloxacin (PFLX)<sup>(7)</sup>. The impact of these mechanisms on the bacterial resistance to fluoroquinolones is still unclear.

The goal of the present study was to investigate the possible role of OmpF-deficiency and active efflux in the development of resistance to hydrophilic or hydrophobic fluoroquinolones by *E coli*.

### MATERIALS AND METHODS

**Fluoroquinolones** Hydrophilic fluoroquinolone ciprofloxacin (CPLX) and ofloxacin (OFLX) were supplied generously by Haimen Pharmaceutical Co (Zhejiang, China). Hydrophobic fluoroquinolone tosufloxacin (TSLX) was procured from Sichuan Industrial

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Institute of Antibiotics (Chengdu, Sichuan, China). The net contents of these drugs are all above 99 %.

**Bacterial strains** JF701 and JF703 are OmpC- and OmpF-deficient mutants of *E coli* K-12, respectively<sup>[8]</sup>. The other *E coli* strains in the study were isolated by this laboratory. Among them, Ecs was a sensitive strain isolated from a patient with urinary infection and its *in vitro* selected fluoroquinolone-resistant mutants R2, R256 were created by serially passaging Ecs through Mueller-Hinton (MH) agar containing increasing concentrations of CPLX, and CPLX was started at one-half the MIC and was doubled thereafter. R2 and R256 demonstrated the target CPLX MIC of 8 and 256 mg/L respectively. CPLX-resistat clinical strains R5, and R6 (having CPLX MIC of 128 mg/L) were isolated respectively from patients with pulmonary and urinary infections. All strains were plasmid-free and DNA sequence of *gyrA* gene showed that in R256, R5 and R6 point mutation happened at C-248 and G-259.

**Chemicals, biochemicals, and media** Protein molecular weight markers ( $M_r = 14.3 - 200$ ); *N, N*-methylenebisacrylamide and acrylamide were purchased from Life Technologies (Eggenstein, Germany); Sarkosyl and sodium dodecyl sulfate-slab were purchased from Sigma. Tryptone and yeast extract were from Unipath Ltd (England); All other chemicals and biochemicals were at least of analytical grade.

**Determination of MIC** MIC were determined by the standard method of two-fold antibiotic dilution in agar. Inoculation of 10<sup>4</sup> - 10<sup>5</sup> CFU were spotted onto MH agar plates containing fluoroquinolone. MIC were read after 18 h of incubation at 37 °C. Fluoroquinolone-sensitivity was identified by breakpoint (CPLX ≥ 4 mg/L, OFLX ≥ 8 mg/L, TSLX ≥ 2 mg/L indicating resistance) according to National Committee of Laboratory Standards (NCCLS), 1997.

**Fluoroquinolone accumulation and active efflux** Drug accumulation was measured as described by Mortimer and Piddock<sup>[9]</sup>; Bacteria were grown in LB broth to an  $A_{600}$  of 0.6 - 0.8 and harvested by centrifugation. Bacteria were washed once with 50 nmol/L sodium phosphate buffer (pH 7.0) at 4 °C, and resuspended in the same buffer at about 40 g (dry weight) of cells per liter. Fluoroquinolone was added to a final concentration of 20 mg/L thereafter. Samples (1.5 mL) were removed at 10, 20, 40 s, 1, 2, 3, 4, 5, 15, and 30 min. Then proton-energy inhibitor DNP, which was prepared as a 20 mmol/L stock solution in 50 % ethanol, was added into the bacterial suspension to a final concentration

of 2 mmol/L and the same volume samples were removed at 5 and 10 min after cells were treated.

Samples were diluted immediately into 1.5 mL chilled sodium phosphate buffer (pH 7.0), and then centrifuged in a microcentrifuge at 12 000 × *g*, 4 °C, for 5 min. Bacteria were washed again in the same chilled buffer and recentrifuged for 5 min. The cell pellet was then suspended in 3.0 mL glycine hydrochloride (0.1 mol/L, pH 3.0) for 2 h at 26 °C, and centrifuged at 12 000 × *g*, for 10 min. The decanted supernatant was centrifuged for another 5 min. Fluorescence of each drug in the final supernatant was then determined at the relevant excitation and emission spectra by a fluorescence spectrophotometer (RF-5000, Shimadzu, Japan). In order to quantify the accumulation of the drug in bacteria, a standard curve of 0.01, 0.05, 0.10, 0.50, 1.00, 1.50, 2.00, 4.00, and 8.00 mg/L of each fluoroquinolone in 3.0 mL glycine hydrochloride (0.1 mol/L, pH 3.0) was prepared and examined simultaneously. The excitation and emission wavelengths for OFLX and TSLX were 296 nm/500 nm and 273 nm/432 nm, respectively.

**Analysis of outer membrane proteins (OMP)** OMP were prepared by sonic disruption followed by differential extraction with sarkosyl<sup>[10]</sup>. The protein composition was analyzed by sodium dodecyl sulfate-slab polyacrylamide (12 %) gel containing 6 mol/L of urea. Each sample contained 50 μg - 80 μg of protein and was heated at 100 °C for 10 min in sample buffer before analysis.

## RESULTS

**Susceptibility** As shown in Tab 1 wild strain Ecs and OmpC-deficient strain JF701 (OmpF<sup>+</sup>) had almost the same sensitivity to fluoroquinolones. Although OmpF-deficient strain JF703 (OmpC<sup>+</sup>) was less susceptible than JF701 and Ecs, according to the NCCL standard

Tab 1. MIC (mg/L) of fluoroquinolones for *E coli* strains.

<i>E coli</i> strains	CPLX	OFLX	TSLX
Ecs	< 0.06	< 0.06	< 0.06
JF701	< 0.06	< 0.06	< 0.06
JF703	0.25	0.5	2
R2	8	16	32
R256	256	64	64
R5	128	64	128
R6	128	32	128

(1997), JF703 was still susceptible to fluoroquinolones. On the contrary, the laboratory-derived resistant isolates R2, R256, as well as clinical resistant isolates R5, R6 were resistant to 3 kinds of fluoroquinolones. Furthermore, cross-resistance of fluoroquinolones was observed in this study, since strains R2 and R256 were only obtained by stepwise exposure of its parent strain Ecs to increasing concentrations of CPLX.

**Fluoroquinolone accumulation and effect of DNP** A biphasic pattern of accumulation of both drugs was observed in all 7 strains with an initial rapid phase of accumulation seen within the first 2 min (Tab 2, Fig 1),

Tab 2. Parameters of OFLX accumulation in *E coli* strains.  $\bar{x} \pm s$ .

<i>E coli</i> strains	Duration of rapid increase/s	Steady-state accumulation concentration/ $\mu\text{g} \cdot \text{g}^{-1}$ cells*	Increase in accumulation after addition of DNP/%	
			5 min	10 min
Ecs	120	29.88 $\pm$ 1.27	64.85	92.19
JF701	60	38.69 $\pm$ 0.75	9.33	16.18
JF703	60	18.73 $\pm$ 1.53	16.98	21.68
R2	60	5.60 $\pm$ 0.35	79.29	271.07
R256	240	3.27 $\pm$ 0.30	185.93	334.25
R5	120	4.97 $\pm$ 0.14	13.08	225.35
R6	60	5.73 $\pm$ 0.17	142.76	189.18

\* The mean of concentration accumulated at 900, 1200, and 1800 s.

followed by a steady-state phase. During the latter phase, the level of accumulation of OFLX in wild strain Ecs appeared to be the same as in *OmpC*-deficient K-12 strain JF701, but about 1/2 to 1/3 reduction in accumulation was observed in *OmpF*-deficient strain JF703 than in JF701 or Ecs (Tab 2). However, accumulation of OFLX in the resistant strains (R2, R256, R5, and R6) was about 7-fold lower than that in the susceptible strains (Ecs, JF701, and JF703) (Tab 3). There was no significant difference in accumulation of TSLX between fluoroquinolone-resistant and -susceptible strains (Fig 1, Tab 3).

Tab 3. Comparison of steady-state concentration of fluoroquinolones accumulated into *E coli* strains.  $\bar{x} \pm s$ .

Drugs	Sensitive <sup>a</sup>	Resistant <sup>b</sup>	<i>t</i>	<i>P</i>
Ofloxacin	29 $\pm$ 10	4 $\pm$ 1	4.9626	0.0042
Tosufloxacin	158 $\pm$ 35	124 $\pm$ 13	1.8270	0.1271

<sup>a</sup> The mean of concentration accumulated in Ecs, JF701, and JF703.

<sup>b</sup> The mean of concentration accumulated in R2, R256, R5, and R6.

After addition of the proton ionophore DNP at 2 mmol/L for 5 to 10 min, the steady-state level of OFLX was obviously increased, especially in the resistant

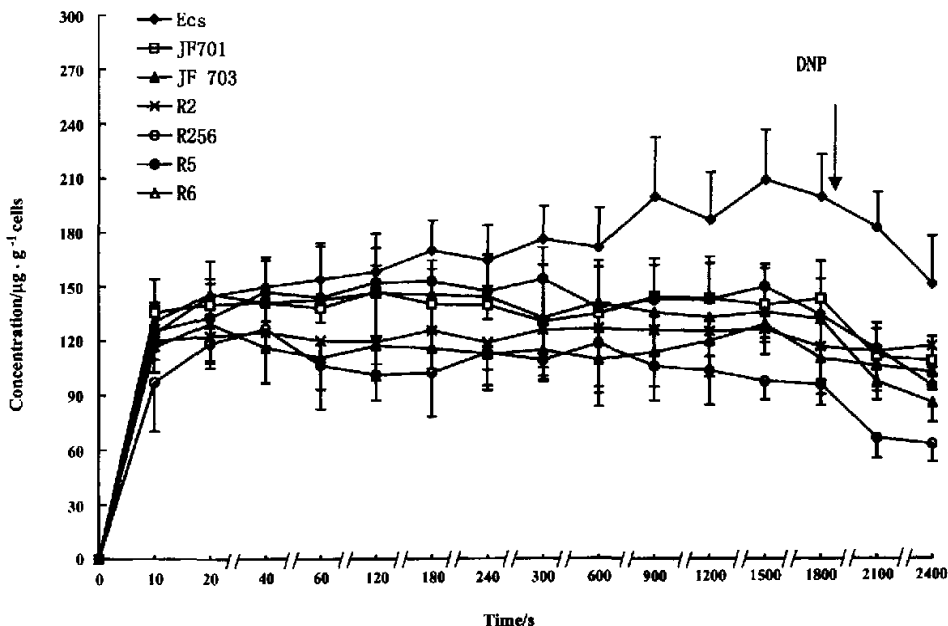


Fig 1. Accumulation of tosylfloxacin in *E coli*.

strains, whereas the steady-state level of TSLX showed a little decrease in all 7 strains (Fig 1). A good relevance between the increment of OFLX accumulation and MIC for the susceptible and resistant strains was obtained after addition of DNP for 5 min and 10 min (Relative coefficient  $r$  were 0.9623 and 0.8006, respectively).

**Outer membrane proteins** As shown in Fig 2, wild strain Ecs had both porins OmpF and OmpC, but porin OmpF-deficiency in R2, R256 (Fig 2, lane c and lane d) and porin OmpC-deficiency in R5, R6 (Fig 2, lane a and lane b) were observed.

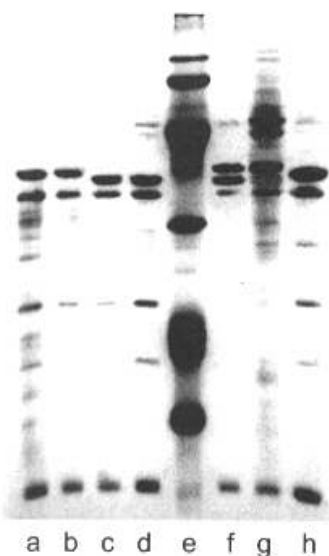


Fig 2. SDS-PAGE electrophoresis of outer membrane proteins (Omp). Each lane indicated Omp from one strain; a: R6; b: R5; c: R256; d: R2; e: MW marker; f: Ecs; g: JF701; h: JF703.

## DISCUSSION

**Absence of porin OmpF in fluoroquinolone-resistance** The high percentage of the maximum concentration of both fluoroquinolones accumulated by the end of the initial rapid phase indicates that fluoroquinolones accumulation into *E. coli* is a rapid process (Tab 2, Fig 1) and this results have also been previously observed in a number of quinolones in *E. coli*<sup>(11)</sup>. Some studies have found that fluoroquinolones accumulation in laboratory-derived porin OmpF-deficient strains decreases 2- to 5-fold compared to that before the OmpF-deficiency and the cause of this decrease can be attributed only to the OmpF-deficiency<sup>(11)</sup>.

In this study, accumulation of the relative hydrophobic TSLX in OmpF-deficient strain JF703 was almost the same as that in OmpC-deficient strain JF701 (OmpF<sup>+</sup>) or wild strain Ecs (OmpF<sup>+</sup>OmpC<sup>+</sup>) (Fig 1). No significant difference between the accumulation of TSLX in the resistant strains (R2, R256, R5, and R6) and susceptible strains (Ecs, JF701 and JF703) was found (Tab 3). On the contrary, the accumulation of OFLX in the susceptible strains was 5- to 7-fold more than that in the resistant strains (Tab 2), and the accumulation in OmpF-deficient strain JF703 was only 1/2 to 1/3 less than that in JF701 and Ecs, respectively (Tab 2). These results suggested that the OmpF-deficiency could decrease the accumulation of hydrophilic fluoroquinolones, but had little effect on the accumulation of hydrophobic fluoroquinolones.

The analysis of OMP of all 5 isolates of *E. coli* in the present study only showed that the *in-vitro* selected resistant strains R2, R256 were deficient in porin OmpF (Fig 2). Considering that well characterized OmpF- or OmpC-deficient mutants (JF703 and JF701) were all susceptible to fluoroquinolones (Tab 1), we postulate that the absence of porin OmpF may not be an important factor in the *E. coli* resistance to fluoroquinolone.

### Efflux of drug in fluoroquinolone-resistance

Active efflux is proposed as a major mechanism of resistance in antibiotics<sup>(2,4)</sup>, but its significance in the fluoroquinolone-resistant *E. coli* remains to be determined, especially for the hydrophobic fluoroquinolone. DNP, a protonophore destroying proton motive force, frequently used in the examination of drug active efflux, could increase intracellular accumulation of fluoroquinolone by inhibiting the action of active efflux system.

Upon the addition of DNP, it was surprising that a slow decrease in accumulation of TSLX was observed in both susceptible and resistant strains, indicating that active efflux mechanism has little influence on its intracellular accumulation. In contrast, almost 7 strains in the study have shown active efflux of OFLX (Tab 2). The phenomenon of active efflux of OFLX in strain Ecs indicated that active efflux system might be a normal structural feature of *E. coli*. Furthermore, we also observed that the accumulation of OFLX increased dramatically in the resistant strains (Tab 2). The increment in OFLX accumulation was significantly relative to its MIC for both susceptible and resistant strains. These data showed that the enhancement of active efflux system was consistent with the increase of fluoroquinolone-resistant levels of bacteria, since the external concentration of OFLX in this

study was constant (20 mg/L, see methods). Thus, the efflux mechanism may play an important role in *E coli* resistance to the hydrophilic fluoroquinolone other than resistance to the hydrophobic fluoroquinolone.

It appears very likely that active efflux system observed in this study is encoded by chromosomal genes because all strains we used were plasmid-free. Such chromosomally encoded fluoroquinolone efflux process may be widespread in the bacterial world<sup>[4]</sup>. Although our data demonstrates that an active efflux process exists for the hydrophilic fluoroquinolone, the identity of this putative system remains to be established.

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氧氟沙星和托氟沙星在氟喹诺酮耐药大肠杆菌中的蓄积<sup>1</sup>

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**关键词** 微生物抗药性; 氧氟沙星; 托氟沙星; 氟喹诺酮; 大肠杆菌; 细胞膜渗透性

**目的:** 研究大肠杆菌耐药菌株蓄积亲水性氟喹诺酮药物氧氟沙星和疏水性药物托氟沙星的机制有无差异. **方法:** 荧光测定法检测菌体内的药物蓄积量和主动外排; SDS-PAGE 法分析细菌外膜蛋白. **结果:** 氧氟沙星在 Ecs 与 JF701 的蓄积量基本一致, 在 JF703 中较 JF701 低 1/2, 但在 4 株耐药菌的蓄积量较这些敏感菌低 5-7 倍; 托氟沙星在氟喹诺酮耐药和敏感菌株中的蓄积则未见显著差异. 加入质子载体 DNP 后 5, 10 min, 细菌中托氟沙星的蓄积缓慢下降, 氧氟沙星的蓄积增加, 尤其在耐药菌株明显. 氧氟沙星的蓄积增加与其对细菌的 MIC 呈正相关 ( $r$  分别为 0.9623 和 0.8006). 此外, Ecs 中 OmpF 和 OmpC 完整; R2、R256 和 R5、R6 分别发生 OmpF 和 OmpC 缺失. **结论:** OmpF 缺失和药物主动外排可导致氧氟沙星, 而不是托氟沙星在菌体内的蓄积减少.

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