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Fleroxacin Resistance in *Escherichia coli*

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Spontaneous fleroxacin-resistant mutants of *Escherichia coli* K-12 were isolated at a frequency of 10^{-10} to 10^{-11} mutants per CFU plated. All mutants exhibited quinolone-resistant replicative DNA biosynthesis, and 4 of 11 mutants also had decreased amounts of OmpF or OmpC porin. None of the mutants had changes solely in porin proteins.

Fluoroquinolones are synthetic, broad-spectrum antibacterial agents whose primary mechanism of action is the inhibition of DNA gyrase activity (2). The frequency of resistance to fluoroquinolones in laboratory strains of *Escherichia coli* is much lower than frequencies for the older related compounds nalidixic and oxolinic acids (13). Genetic and biochemical analyses of *E. coli* mutants resistant to norfloxacin have indicated that low-level resistance (4× MIC) is associated with either decreased outer-membrane permeability or altered DNA gyrase (4); high-level resistance is associated with mutations in both permeability and gyrase (5). The gyrase mutations have been mapped at the *gyrA* gene, which encodes the A subunit of DNA gyrase, while the permeability mutations have been mapped to loci that affect expression of the OmpF porin (13).

Fleroxacin (Ro 23-6240; AM 833) is a trifluorinated quinolone with an antimicrobial spectrum and potency similar to those of norfloxacin (12). In the present study, low-level resistance to fleroxacin was examined in a laboratory strain of *E. coli* K-12 to determine resistance frequency and nature.

Bacterial strains used are listed in Table 1. They were routinely grown in Antibiotic Medium 3 (Difco Laboratories, Detroit, Mich.) or nutrient broth (Difco) at 37°C. Fleroxacin, ceftriaxone, and coumermycin were from Hoffmann-La Roche Inc. (Nutley, N.J.); norfloxacin and cefoxitin were from Merck & Co. (Rahway, N.J.); ciprofloxacin was from Miles Laboratories, Inc. (West Haven, Conn.); and novobiocin was from Sigma Chemical Co. (St. Louis, Mo.).

To isolate mutants spontaneously resistant to fleroxacin (Flx^r), *E. coli* JF568 was grown in Antibiotic Medium 3 to either mid-logarithmic phase (A_{660}, 0.4) or stationary phase (overnight culture). Cells were harvested by centrifugation, washed, and concentrated to 10^{10} CFU/ml in SM buffer (0.1 M NaCl, 17 mM $MgSO_4$, 5.0 mM Tris hydrochloride [pH 7.5], 0.01% gelatin). Aliquots (100 μl) were spread onto nutrient agar plates containing 0.4 or 0.8 μg of fleroxacin per ml (4× or 8× MIC, respectively) and incubated for 48 h at 37°C. For viable counts, dilutions of the bacterial suspension were made in SM buffer and plated onto nutrient agar. The frequency of resistance was obtained by dividing the number of fleroxacin-resistant mutants by the number of CFU plated. The identity of the mutants as *E. coli* JF568 derivatives was confirmed by the use of Enterotube II kits from Roche Diagnostic Systems (Nutley, N.J.). Mutants resistant to four times the MIC of fleroxacin were isolated at a frequency of 10^{-10} to 10^{-11} mutants per CFU. A similar frequency was obtained with mid-logarithmic-phase and stationary-phase cultures. Single-step mutants resistant to higher levels of fleroxacin could not be isolated after several attempts.

Antibacterial activity was determined by broth dilution with Antibiotic Medium 3 in 24-well plates (1 ml per well). An inoculum of 5×10^4 logarithmic-phase cells was used. The MIC was the concentration that inhibited visible growth after 18 h of incubation at 37°C. All Flx^r strains exhibited MICs of fleroxacin that were 8- to 10-fold higher than those for the wild-type strain, JF568 (Table 2). MICs of norfloxacin and ciprofloxacin were also increased for the mutant strains. MIC increases of ciprofloxacin (4× to 8× MIC for wild type) were slightly less than those of fleroxacin, except for strain JSC101. MICs of the β-lactams cefoxitin and ceftriaxone for the mutant strains were similar to those for JF568, except for strains JSC102, JSC103, JSC104, and JSC106. All strains retained the susceptibility of the parent, JF568, to novobiocin and coumermycin, which are inhibitors of the gyrase B subunit (3).

Susceptibility of the mutant strains of OmpF- or OmpC-specific bacteriophages was determined as an indicator of porin expression. Mid-logarithmic-phase cells (200 μl) were mixed with 2.0 ml of molten soft agar (0.5% agar) and poured onto the surface of a nutrient agar (1.5% agar) plate. After hardening, 5-μl aliquots of SM or dilutions of bacteriophage in SM (OmpF-specific K20, OmpC-specific PA2) were spotted directly onto the agar surface and allowed to soak into the agar. The plates were incubated at 37°C, and the PFU

TABLE 1. *E. coli* strains

Strain	Characteristics	Source
JF568	K12 *aroA357 cyc-1 his-53 ilv-277 lacY29 metB65 purE41 rpsL77 tsx-63 xyl14*	J. Foulds (9)
JF701	JF568 *ompC264* (OmpC$^-$)	J. Foulds
JF703	JF568 *ompF254* (OmpF$^-$)	J. Foulds
JSC100	JF568 Flx^r	This work
JSC101	JF568 Flx^r	This work
JSC102	JF568 Flx^r	This work
JSC103	JF568 Flx^r	This work
JSC104	JF568 Flx^r	This work
JSC106	JF568 Flx^r	This work
JSC107	JF568 Flx^r	This work
JSC108	JF568 Flx^r	This work
JSC109	JF568 Flx^r	This work
JSC110	JF568 Flx^r	This work
JSC111	JF568 Flx^r	This work

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TABLE 2. Susceptibility to various antibiotics of strains used in this study

Strain	MIC (μg/ml)[a]						
	FLX	NOR	CIP	FOX	CRO	NB	COU
JF568	0.1	0.1	0.05	2	0.05	200	8
JF701	0.1	0.1	0.1	8	0.1	ND	ND
JF703	0.3	0.4	0.4	16	0.2	200	16
JSC100	1.0	0.8	0.4	2	0.05	200	8
JSC101	1.5	1.5	0.6	4	0.1	200	ND
JSC102	1.0	1.5	0.4	8	0.2	200	8
JSC103	1.0	1.5	0.4	8	0.2	200	8
JSC104	0.7	1.5	0.2	8	0.1	200	ND
JSC106	0.8	1.5	0.4	8	0.2	200	ND
JSC107	0.8	1.5	0.2	4	0.1	200	8
JSC108	1.0	0.8	0.2	4	ND	200	ND
JSC109	1.0	1.0	0.2	4	ND	200	ND
JSC110	1.0	1.0	0.2	2	ND	200	ND
JSC111	1.0	1.0	0.2	2	ND	200	ND

[a] FLX, Fleroxacin; NOR, norfloxacin; CIP, ciprofloxacin; FOX, cefoxitin; CRO, ceftriaxone; NB, novobiocin; COU, coumermycin; ND, not determined.

were counted after 18 h. The plaquing efficiency was calculated by dividing the number of PFU/ml obtained with the mutant strains by the number of PFU/ml obtained with strain JF568.

Outer-membrane proteins were prepared as described elsewhere (7). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by using a modification of the procedure of Lundrigan and Earhart (6). The separating gel contained 10% polyacrylamide, 8 M urea, 0.1% SDS, and 0.375 M Tris buffer (pH 8.8). The stacking gel contained 5% acrylamide, 8 M urea, 0.1% SDS, and 0.125 M Tris buffer (pH 6.8). The running buffer contained 12 mM Tris, 0.05% SDS, and 1.0 M glycine (pH 8.3).

Four of the fleroxacin-resistant strains showed reduced plaquing efficiencies in the plaquing assay (Table 3). Plaquing by K20 (11) was reduced in OmpF-deficient strain JF703 and in strains JSC102, JSC103, JSC104, and JSC106. These strains produced no OmpF protein detectable by SDS-polyacrylamide gel electrophoresis (Fig. 1). Because plaquing by K20 was only slightly reduced in JSC104, it is possible that this strain has an OmpF protein with altered electrophoretic mobility but little alteration in the K20 attachment site.

TABLE 3. Efficiency of plaquing by OmpF- and OmpC-specific phage on fleroxacin-resistant mutants

Strain	Efficiency of plaquing	
	K20 (OmpF)	PA2 (OmpC)
JF568	1	1
JF701	1.2	6.1×10^{-6}
JF703	1.6×10^{-6}	1.1
JSC100	1.1	1.0
JSC101	1.5	1.2
JSC102	1.7×10^{-6}	1.3
JSC103	7.8×10^{-5}	2.9×10^{-5}
JSC104	0.85	1.4×10^{-5}
JSC106	1.7×10^{-6}	1.5
JSC107	1.6	0.73
JSC108	0.94	0.74
JSC109	1.1	1.2
JSC110	1.2	1.0
JSC111	1.14	0.9

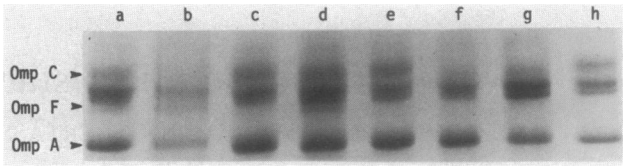

FIG. 1. Outer membrane protein profiles of *E. coli* strains. Lanes: a, JF568; b, JF701 (OmpC$^-$); c, JF703 (OmpF$^-$); d, JSC100; e, JSC102; f, JSC103; g, JSC104; h, JSC106. Lanes b and h contained 10 μg of protein; all other lanes contained 20 μg of protein.

The OmpC-specific phage PA2 (1) showed reduced plaquing efficiency on control strain JF701 and fleroxacin-resistant strains JSC103 and JSC104; the reportedly OmpC-specific bacteriophage SS4 (11) showed reduced plaquing efficiency only on strain JSC103 (data not shown). Strains JF701, JSC103, and JSC104 lacked or produced only small amounts of the OmpC porin (Fig. 1). As expected, the four fleroxacin-resistant strains with altered outer-membrane properties also showed increased MICs of the β-lactams cefoxitin and ceftriaxone (Table 2).

In vitro inhibition of replicative DNA biosynthesis was used as a convenient indicator of DNA gyrase activity (10). Replicative DNA biosynthesis was measured as ATP-dependent incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material in toluene-permeabilized cells of the wild-type and mutant strains (8) (Table 4). The 50% incorporation concentrations (IC_{50}s) (concentrations required to reduce ATP-dependent label incorporation by 50%) of quinolones for JF568 and JF703 were similar, indicating that reduced outer-membrane permeability by itself does not affect the assay. All strains isolated as fleroxacin-resistant had IC_{50}s of fleroxacin 3- to 10-fold greater than JF568 had. IC_{50}s of norfloxacin and ciprofloxacin were also greater for these strains, although the size of the increase was generally smaller with ciprofloxacin.

In summary, single-step mutants of *E. coli* JF568 resistant to low levels of fleroxacin were isolated at a frequency similar to that reported for norfloxacin (10^{-10} to 10^{-11}) (4, 5). This frequency is 300-fold lower than that reported for the older quinolones, nalidixic and oxolinic acid (13). In all fleroxacin-resistant strains, replicative DNA biosynthesis, and therefore DNA gyrase activity, was resistant to fluoroquinolones. By phage typing, outer-membrane protein pro-

TABLE 4. Inhibition of replicative DNA biosynthesis by fluoroquinolones

Strain	IC_{50} (μg/ml)		
	Fleroxacin	Norfloxacin	Ciprofloxacin
JF568	2	2	2
JF703	1	1	1
JSC100	18	43	18
JSC101	20	17	18
JSC102	16	15	3
JSC103	14	13	5
JSC104	15	13	3
JSC106	7	12	6
JSC107	19	20	11
JSC108	19	20	12
JSC109	16	18	6
JSC110	17	17	6
JSC111	18	18	9

files, and antibiotic susceptibilities, some strains (JSC102, JSC103, JSC104, and JSC106) also had diminished expression of porins or had expression of porins with altered properties. In contrast to strains with low-level resistance to norfloxacin (4), none of the fleroxacin-resistant mutants had changes solely in porin proteins.

Thus, it appears that reduced outer-membrane permeability is not sufficient to produce resistance to fleroxacin. However, reduced outer-membrane permeability does contribute to the development of resistance. The average IC_{50}s for the inhibition of gyrase activity by quinolones were lower in fleroxacin-resistant, porin-deficient strains than in resistant strains with a normal complement of porins (e.g., fleroxacin, 18.3 versus 13.0 μg/ml; norfloxacin, 17.8 versus 13.3 μg/ml; and ciprofloxacin, 11.5 versus 4.3 μg/ml). Reduced outer-membrane permeability may allow the survival of strains with gyrase mutations that confer a lower level of resistance to fluoroquinolones than do gyrase mutations in strains with a normal complement of porins.

The frequency and nature of resistance to fleroxacin observed for laboratory strains of *E. coli* may have clinical implications. The low frequency of resistant mutants is in itself an attractive characteristic of fluoroquinolones. The fact that loss of functional porins alone apparently does not result in fleroxacin resistance suggests that fleroxacin could be an effective antibiotic when altered outer-membrane permeability has rendered other antibiotics ineffective.

LITERATURE CITED

1. **Bassford, P. J., Jr., D. L. Diedrich, C. A. Schnaitman, and P. Reeves.** 1977. Outer membrane proteins of *Escherichia coli*. VI. Protein alteration in bacteriophage-resistant mutants. J. Bacteriol. **131**:608–622.
2. **Gellert, M.** 1981. DNA topoisomerases. Annu. Rev. Biochem. **50**:879–910.
3. **Gellert, M., M. H. O'Dea, T. Itoh, and J.-I. Tomizawa.** 1976. Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. USA **73**:4474–4478.
4. **Hirai, K., H. Aoyama, S. Suzue, T. Irikura, S. Iyobe, and S. Mitsuhashi.** 1986. Isolation and characterization of norfloxacin-resistant mutants of *Escherichia coli* K-12. Antimicrob. Agents Chemother. **30**:248–253.
5. **Hooper, D. C., J. S. Wolfson, K. S. Souza, C. Tung, G. L. McHugh, and M. N. Swartz.** 1986. Genetic and biochemical characterization of norfloxacin resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **29**:639–644.
6. **Lundrigan, M. D., and C. F. Earhart.** 1984. Gene *envY* of *Escherichia coli* K-12 affects thermoregulation of major porin expression. J. Bacteriol. **157**:262–268.
7. **Matsuyama, S.-I., K. Inokuchi, and S. Mizushima.** 1984. Promoter exchange between *ompF* and *ompC*, genes for osmoregulated major outer membrane proteins of *Escherichia coli* K-12. J. Bacteriol. **158**:1041–1047.
8. **Moses, R. E., and C. C. Richardson.** 1970. Replication and repair of DNA in cells of *Escherichia coli* treated with toluene. Proc. Natl. Acad. Sci. USA **67**:674–681.
9. **Nikaido, H., E. Y. Rosenberg, and J. Foulds.** 1983. Porin channels in *Escherichia coli*: studies with β-lactams in intact cells. J. Bacteriol. **153**:232–240.
10. **Pedrini, A. M., D. Geroldi, A. Siccardi, and A. Falaschi.** 1972. Studies on the mode of action of nalidixic acid. Eur. J. Biochem. **25**:359–365.
11. **Schnaitman, C. A., and G. A. McDonald.** 1984. Regulation of outer membrane protein synthesis in *Escherichia coli* K-12: deletion of *ompC* affects expression of the OmpF protein. J. Bacteriol. **159**:555–563.
12. **Verbist, L.** 1987. Comparative *in vitro* activity of Ro 23-6240, a new trifluorinated quinolone. J. Antimicrob. Chemother. **20**: 363–372.
13. **Wolfson, J. S., and D. C. Hooper.** 1985. The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity in vitro. Antimicrob. Agents Chemother. **28**:581–586.