Low-Level Resistance to the Cephalosporin 3'-Quinolone Ester Ro 23-9424 in *Escherichia coli*

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Received 9 September 1994/Returned for modification 16 November 1994/Accepted 6 December 1994

Four spontaneous, single-step mutants of *Escherichia coli* K-12 resistant to low levels of the cephalosporin 3'-quinolone ester Ro 23-9424 were isolated at a frequency of 10^{-10} to 10^{-11} mutants per CFU plated. The mutants were cross-resistant to both cephalosporin (cefotaxime) and quinolone (floxacin) components. Accordingly, they had altered porins and replicative DNA biosynthesis resistant to floxacin. There was no increase in β -lactamase activity when tested with nitrocephin, and the penicillin-binding protein profiles were normal.

Ro 23-9424 is a synthetic, broad-spectrum antibacterial agent consisting of a cephalosporin (cefotaxime) linked through an ester bond to a fluoroquinolone (floxacin) (6). Ro 23-9424 combines the antibacterial spectrum and potency of both classes of antibiotics (1). Its mode of action in *Escherichia coli* involves initial binding to penicillin-binding protein (PBP) 3, resulting in inhibition of septation. The quinolone moiety is subsequently released, which then inhibits DNA gyrase activity (4, 6, 8).

As part of the biological characterization of Ro 23-9424, *E. coli* mutants resistant to it were isolated and the mechanism of their resistance was examined.

Bacterial strains are listed in Table 1. Organisms were routinely grown in antibiotic medium 3 or nutrient broth (Difco Laboratories, Detroit, Mich.) at 37°C. Floxacin and ceftriaxone were from Hoffmann-La Roche, Inc. (Nutley, N.J.); norfloxacin and cefoxitin were from Merck and Co. (Rahway, N.J.); ciprofloxacin was from Miles Laboratories (West Haven, Conn.); and cefotaxime was from Hoechst-Roussel (Summit, N.J.).

Antibacterial activity was determined by broth dilution with antibiotic medium 3 in 24-well plates (1 ml/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.

To isolate spontaneously resistant mutants, cultures of *E. coli* JF568, a K-12 strain (13), were grown in antibiotic medium

3 to mid-logarithmic phase (A_{660} of 0.4) or stationary phase (cultures grown overnight). Cells were harvested, washed, and concentrated to 10^{10} CFU/ml in SM buffer (0.1 M NaCl, 17 mM $MgSO_4$, 5.0 mM Tris-HCl [pH 7.5], 0.01% gelatin). Aliquots (100 μ l) were spread onto nutrient agar plates containing 0.2 or 0.4 μ g of Ro 23-9424 per ml ($4\times$ and $8\times$ MIC, respectively) and incubated for 48 h at 37°C. Resistance frequency was obtained as the number of Ro 23-9424-resistant mutants divided by the number of CFU plated. Mutants were confirmed as *E. coli* JF568 derivatives with Enterotube II kits from Roche Diagnostic Systems (Nutley, N.J.).

The susceptibilities of the mutant strains to OmpF- or OmpC-specific phages were determined as an indicator of porin expression. Mid-logarithmic-phase cells (200 μ l) 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 μ l of SM buffer or bacteriophage in SM buffer (K20, OmpF-specific; PA2, OmpC-specific) (15) were spotted directly onto the agar surface and allowed to soak into the agar. Plates were incubated at 37°C, and the number of PFU was counted after 18 h. The plaquing efficiency was calculated by dividing the number of PFU per milliliter obtained with the mutant strains by the number of PFU per milliliter obtained with strain JF568.

Outer membrane proteins were prepared as described previously (11) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 8 M urea by the method of Lundrigan and Earhart (10).

TABLE 1. *E. coli* strains used in this study

Strain	Characteristics	Source
JF568	Same as K-12 but with <i>aroA57 cyc-1 his-53 ilv-277 lacY29 metB65 purE41 rpsL77 tsx-63 xyl114</i>	J. Foulds (13)
JF701	Same as JF568 but with <i>ompC624</i> (OmpC ⁻)	J. Foulds
JF703	Same as JF568 but with <i>ompF254</i> (OmpF ⁻)	J. Foulds
JSC120	Same as JF568 but with Ro 23-9424 ^r	This work
JSC121	Same as JF568 but with Ro 23-9424 ^r	This work
JSC122	Same as JF568 but with Ro 23-9424 ^r	This work
JSC123	Same as JF568 but with Ro 23-9424 ^r	This work

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

Strain	MIC (μ g/ml) ^a						
	23-9424	FLX	NOR	CIP	FOX	CRO	CTX
JF568	0.05	0.1	0.1	0.05	2	0.05	0.05
JF701	ND ^b	0.1	0.1	0.1	8	0.1	ND
JF703	0.1	0.3	0.4	0.4	16	0.2	ND
JSC120	0.3	0.2	0.4	0.1	16	0.2	0.1
JSC121	0.3	0.2	0.4	0.05	12	0.2	0.1
JSC122	0.3	0.2	0.4	0.05	12	0.1	0.1
JSC123	0.2	0.2	0.2	0.05	12	0.1	0.1

^a FLX, floxacin; NOR, norfloxacin; CIP, ciprofloxacin; FOX, cefoxitin; CRO, ceftriaxone; CTX, cefotaxime.

^b ND, not determined.

TABLE 3. Efficiencies of plaquing by OmpF- and OmpC-specific phages on Ro 23-9424-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
JSC120	4×10^{-5}	$<1 \times 10^{-7}$
JSC121	8.3×10^{-5}	0.98
JSC122	4.7×10^{-5}	0.17
JSC123	0.33	1.8

PBPs were assayed in solubilized membranes as described previously (7). PBP binding was measured as inhibition of [14 C]penicillin G binding. Replicative (ATP-dependent) DNA biosynthesis, a convenient indicator of DNA gyrase activity (14), was carried out in toluene-permeabilized cells as described previously (12).

Single-step mutants of *E. coli* JF568 resistant to low levels of Ro 23-9424 were isolated at a frequency of 10^{-10} to 10^{-11} mutants per CFU plated. Mutants could be selected at $4 \times$ MIC, but not at $8 \times$ MIC, of Ro 23-9424. The resistance frequency is similar to those reported for fleroxacin (2) and norfloxacin (9) but substantially lower than those reported for the older quinolones nalidixic and oxolinic acid (16) and for β -lactams (3).

Strains resistant to Ro 23-9424 were cross-resistant to both quinolone and β -lactam antibiotics (Table 2). In contrast, *E. coli* mutants resistant to fleroxacin were not necessarily cross-resistant to β -lactam antibiotics (2), and *E. coli* mutants resistant to ceftriaxone, but not fleroxacin, showed little cross-resistance to Ro 23-9424 (5, 6).

All four strains had altered porins, as determined by outer membrane protein profiles (data not shown) and susceptibilities to porin-specific phages (Table 3). Three strains had altered OmpF protein, and one strain had altered OmpF and OmpC proteins. Replicative DNA biosynthesis in the four strains was resistant to fleroxacin and in two strains (JSC120 and JSC122) was resistant to norfloxacin but not to ciprofloxacin (Table 4). None of the strains had altered PBP profiles (Fig. 1) or increased β -lactamase activity when tested with the chromogenic cephalosporin nitrocephin (data not shown). Thus, low resistance to Ro 23-9424 appears to result from the combined effects of decreased outer membrane permeability and altered DNA gyrase.

TABLE 4. Inhibition of replicative DNA biosynthesis by fluoroquinolones

Strain	IC ₅₀ ^a (μ g/ml)		
	FLX	NOR	CIP
JF568	2	2	2
JF703	1	1	1
JSC120	30	11	4
JSC121	10	3	2
JSC122	8	15	2
JSC123	20	3	2

^a IC₅₀, 50% inhibitory concentration.

We thank John Foulds and Carl Schnaitman for their generous gifts of *E. coli* strains and bacteriophages and K.-K. Chan for providing Ro 23-9424.

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FIG. 1. Binding of ceftriaxone to PBPs of *E. coli* JF568, JSC130, JSC120, JSC121, JSC122, and JSC123. Lanes: C, control; 1, 0.1 μ g of ceftriaxone per ml; 2, 0.5 μ g of ceftriaxone per ml.

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