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Involvement of an Efflux System in High-Level Fluoroquinolone Resistance of *Shigella dysenteriae*

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Shigella dysenteriae **represent one of the growing list of antibiotic-resistant bacteria. Quinolones are widely employed to treat shigellosis. However, quinolone resistance has already been reported, necessitating an understanding of the mechanisms of development of resistance. We demonstrate that high-level fluoroquinolone resistance of *S. dysenteriae* exposed to these antibiotics may occur in the absence of *gyrA* mutations and involve a proton motive force(pmf)-dependent efflux system.** © 1998 Academic Press

S. dysenteriae has been identified by the World Health Organization as one of the growing antibiotic-resistant bacteria posing a major threat to mankind (1). Nalidixic acid was used for the treatment of children with shigellosis caused by strains of *S. dysenteriae* that are resistant to ampicillin and cotrimoxazole (2,3). However, in 1990, 58% of 585 *S. dysenteriae* type I isolates in Bangladesh were resistant to nalidixic acid (3). Although fluoroquinolones remain widely used in the therapy of shigellosis, in China, upto 50% of *S. dysenteriae, S. sonnei* and *S. boydii* are resistant to these drugs (4). It is therefore imperative to understand the mechanisms associated with quinolone resistance of *S. dysenteriae.* In the present study we demonstrate the involvement of an energy-dependent efflux system in high-level fluoroquinolone resistance of *S. dysenteriae* exposed to fluoroquinonoles and lacking any mutation in the quinolone resistance determining region (QRDR) of the *gyrA* gene.

MATERIALS AND METHODS

Bacterial strains and chemicals. *S. dysenteriae* C152, a clinical isolate was obtained from the School of Tropical Medicine, Calcutta and grown routinely in Tryptic Soy Broth. Norfloxacin, ofloxacin, ethidium bromide, benzylpenicillin, cefoxitin, cephaloridine, piperacillin and nalidixic acid were purchased from Sigma (U.S.A.), chloramphenicol and tetracycline were purchased from Boehringer Mannheim (Germany), ciprofloxacin was a gift from Ranbaxy Pharmaceuticals (India) and gentamycin was a product of Nicholas Piramal India Limited.

Isolation of norfloxacin-resistant mutants. Spontaneous norfloxacin-resistant mutants were isolated from the sensitive strain C152 by plating 10^{10} CFU of an exponentially growing culture of C152 on MacConkey agar plates containing 1, 2 or 4 μg/ml norfloxacin. Mutants showing resistance to norfloxacin could be obtained at frequencies of 10^{-7} to 10^{-8}. MICs were determined on Mueller-Hinton agar plates containing serial dilutions of antibiotics by inoculating with 10^8 CFU and reading after 18h of growth at 37°C.

Preparation of outer membranes. Membranes prepared as described by Mahapatra *et al.* (5) were treated with 2% Sarkosyl at a final concentration of 2% at 30°C for 1 h with vigorous shaking and centrifuged at 48,000 × g for 30 min at 4°C. The pellet constituting the outer membranes (OMs) was washed twice with 100 μl of 0.5% Sarkosyl and stored at −20°C. OMs were run on SDS/polyacrylamide (12.5%)/urea(8 M) gels.

Liposome swelling assay. This was carried out as described by Nikaido and Rosenberg (6). Briefly, 2.5 μmol of egg phosphatidylcholine and 0.1 μmol of dicetyl phosphate were dried as a thin film. The film was suspended in 0.2 ml of buffer in which crude OM (100 μg protein) was added. The mixture was sonicated and dried under vacuum. The film was reconstituted with 0.4 ml of a solution containing 20 mM (isotonic concentration) stachyose in 10 mMTris-HCl, pH 8 and diluted in isotonic solutions of the test solute. Liposome swelling was measured as the initial decrease in absorbance at 400 nm. Isotonic concentrations were determined as described by Yoshimura and Nikaido (7).

Accumulation of antibiotics in sensitive and resistant cells. Accumulation of norfloxacin and ciprofloxacin was studied as described by Charvalos *et al.* (8) with some modifications. Cells were grown upto an OD_{600} of 0.4, harvested and washed with 50 mM phosphate buffer, pH 7.2 (PB). Cells were resuspended in the same buffer maintaining an OD_{600} of 20 per ml, and energized with 0.2% glucose for 20 min at 30°C. Norfloxacin or ciprofloxacin was added at a concentration of 10 μg/ml. Aliquots of 0.5 ml were withdrawn at different time intervals and dispensed into tubes containing 1.5 ml PB. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) (100 μM) was added at 20 min and further aliquots were withdrawn. Cells were immediately harvested, washed with 2 ml ice-cold PB and finally resuspended in 2 ml of 0.1(M) glycine hydrochloride, pH 3. After incubation for 60 min at 37°C, the suspension was centrifuged and the fluorescence of the supernatant was monitored in a spectrofluorimeter (for norfloxacin : excitation wavelength 282 nm, emission wavelength 448 nm; for ciprofloxacin: excitation wavelength 275 nm, emission wavelength 440 nm). To study the accumulation of chloramphenicol

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and tetracycline, cells energized with glucose were incubated with 1μM [^{3}H] chloramphenicol (10 Ci/mmol) or 5 μM [^{3}H]tetracycline (0.5 Ci/mmol). Aliquots were removed at different time intervals before and after addition of CCCP as described above and filtered through Whatman GF/C filters (0.22 μ). Filters were washed, dried and counted in a liquid scintillation counter.

DNA manipulations, PCR amplification of 5′end region of gyrA of S. dysenteriae, and nucleotide sequencing. Molecular biological reagents/enzymes were obtained from Qiagen (Germany), Boehringer Mannheim (Germany), Life Technologies (U.S.A.) and Amersham Life Sciences (U.K.) Routine molecular biological techniques were carried out according to standard protocols (9). Two primers from the conserved regions of *gyrA* of *Escherichia coli* were selected for PCR amplification of the 5′ end coding region comprising the QRDR of *gyrA* of *S. dysenteriae.* The two primers were A: 5′-TACACCGGTCAACATTGAGG-3′ and B: 5′-TTAATGATTGCCGCCGTCGG-3′, which were identical in sequence to nucleotide positions 24 to 43 and complementary in sequence to positions 652 to 671 of *E. coli* K-12 *gyrA* (10). Chromosomal DNA was prepared from each strain by boiling one colony in 1 ml of water for 8 min followed by centrifugation in an Eppendorf centrifuge for 10 s. 1μl of the supernatant DNA was used for PCR amplification in 100 μl reaction mixtures containing 1 μM of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTP and 2.5 U of Taq DNA polymerase (Perkin-Elmer Cetus). 30 cycles were performed in a Perkin-Elmer GeneAmp 2000 Thermal Cycler with the following temperatures: 90°C, 1 min; 64°C, 1 min; 72°C, 2 min; a final cycle of 10 min at 70°C was performed. The PCR products were gel purified, digested with SacI and SmaI, generating 582 bp *gyrA* fragments and ligated into a SacI-SmaI digested pK19 vector. After transformation and purification of recombinant plasmid DNA, double-stranded DNA sequencing was performed using [^{35}S]dATP and Thermosequenase cycle sequencing kit (Amersham Life Sciences).

RESULTS AND DISCUSSION

MICs against different antibiotics. The mutant NRM16 which was 40-fold more resistant to norfloxacin (NFX) than the parent C152 (Table 1), will be discussed here. The pattern of susceptibility towards two other fluoroquinolones, ciprofloxacin(CIP) and ofloxacin(OFX) was similar to that observed for norfloxacin. Testing of the MICs of several structurally unrelated antibiotics showed a 2-fold increase in MIC of tetracycline and a slight but consistent increase in the MIC of chloramphenicol in the case of NRM16. No difference in MIC was observed against gentamycin, ethidium bromide (a basic dye), benzylpenicillin and other beta-lactams.

TABLE 1

MICs of *S. dysenteriae* C152 and NRM16

	MIC(μg/ml)	
	C152	NRM16
Norfloxacin	0.5	20
Ciprofloxacin	0.4	20
Ofloxacin	0.4	20
Tetracycline	80	160
Chloramphenicol	100	150
Ethidium bromide	125	125
Gentamicin	12	12
Benzyl penicillin	8	8
Cephaloridine	4	4
Cefoxitin	5	5
Piperacillin	8	8

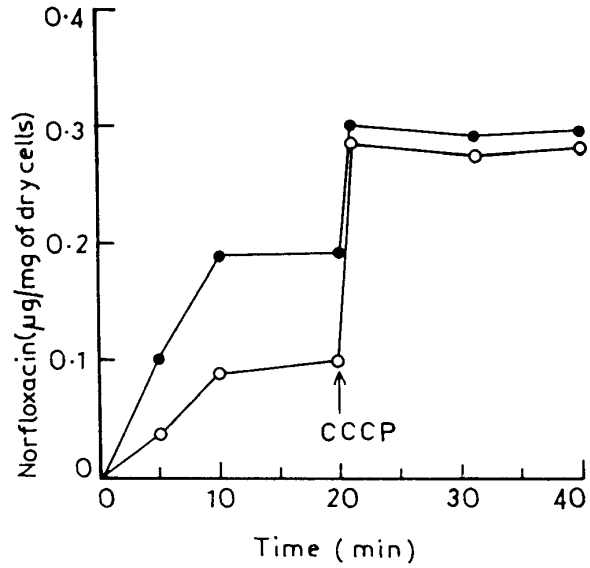

FIG. 1. Accumulation of norfloxacin in *S. dysenteriae* C152 and NRM16. The accumulation of norfloxacin was studied as described in the Methods section. ●, C152; ○, NRM16

Nucleotide sequencing of the quinolone resistance-determining region (QRDR). The DNA gyrase of *E. coli* is the primary target of the quinolones. It consists of subunits A and B which are products of the *gyrA* and *gyrB* genes respectively, and mutations in *gyrA* represent one of the most common mechanisms of quinolone resistance in Gram-negative bacteria (10-12). These mutations are located within a region termed the quinolone resistance determining region (QRDR). The QRDR of the *gyrA* gene of *S. dysenteriae* strain C152 exhibited 99% homology with the *E. coli gyrA* sequence. The nucleotide sequences of strain C152 and NRM16 were identical in the QRDR. The mutant NRM16 therefore presents a case of fluoroquinolone resistance without the involvement of a *gyrA* mutation, the most frequently observed mechanism of resistance in Gram-negative bacteria.

The absence of any mutation in the *gyrA* gene and the slight but consistent increase in MICs of structurally unrelated antibiotics such as chloramphenicol and tetracycline, suggested that access of drugs to their targets might be affected in NRM16. This could be a result of decreased outer membrane permeability and/or enchanced efflux of drugs from the cells of NRM16, since both permeability barriers and active efflux systems have been implicated in resistance to antibiotics including fluoroquinolones in Gram-negative as well as Gram-positive bacteria (12-15).

TABLE 2

Accumulation of Ciprofloxacin, Chloramphenicol, and Tetracycline in NRM16

	Percent accumulation relative to C152 (100)		
CCCP	Ciprofloxacin	Chloramphenicol	Tetracycline
−	35 (32–37)	85 (85–87)	68 (65–70)
+	95 (91–97)	99 (93–105)	93 (90–95)

Data represent the means of five separate experiments. The ranges of values are listed in parentheses.

Outer membrane protein profiles and outer membrane permeability. The outer membrane protein (OMP) profiles of C152 and NRM16 were identical with that reported previously (16) with two major OMPs of M_r 43,000 and 38,000. Outer membrane permeabilities were assessed by testing the uptake of arabinose and glucose in the two strains by using the liposome swelling technique. No differences in the rates of permeation of these molecules were observed (data not shown). The outer membrane permeability barrier appeared unlikely to be a factor contributing to the resistance of NRM16 to NFX and other fluoroquinolones.

Accumulation of quinolones by the strains C152 and NRM16. The accumulation of NFX at steady state was less in NRM16 compared to that in the susceptible parent strain C152 (Fig. 1). The level of accumulation was increased upon the addition of the protonophore CCCP. This suggested that a proton motive force (pmf)-dependent efflux pump was involved in efflux of NFX. Addition of CCCP increased NFX accumulation in NRM16 to a level comparable to that of C152. Similar results were obtained with CIP (Table 2).

Accumulation of tetracycline and chloramphenicol. Similar experiments with radiolabeled tetracycline or chloramphenicol reflected small but consistent differences in their uptake between the sensitive and resistant strains in the absence of CCCP (Table 2), which was abolished on addition of CCCP. This was in harmony with the fact that the increase in MICs of these antibiotics in NRM16 compared to C152, was also not large.

SUMMARY AND CONCLUSIONS

Rahaman *et al.* have demonstrated that the mutation Ser-83 to Leu in the gyrase A subunit results in a 8-fold increase in the MIC of CIP in *S. dysenteriae* (18). The results obtained in this study indicate that high-level quinolone resistance arising from exposure of *S. dysenteriae* to NFX may involve a mechanism other than a mutation in the *gyrA* gene. The decreased accumulation of quinolones in the mutant compared to the parent strain and the effect of CCCP in bringing accumulation to similar levels in C152 and NRM16, obviously suggest a role of a pmf-dependent efflux pump in high-level quinolone resistance of NRM16. Since efflux pumps confer resistance to a structurally diverse array of antibacterials simultaneously, this aspect was also investigated. Among other drugs tested, there was a 2-fold increase in MIC of tetracycline and a small but consistent increase in MIC of chloramphenicol in NRM16. This is, to our knowledge, the first report of quinolone resistance in *S. dysenteriae* involving a pmf-dependent efflux system and in the absence of any mutation in the *gyrA* gene.

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