ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1996, p. 2380–2386 Vol. 40, No. 10
0066-4804/96/$04.00+0

Contributions of Individual Mechanisms to Fluoroquinolone Resistance in 36 *Escherichia coli* Strains Isolated from Humans and Animals

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Received 26 March 1996/Returned for modification 30 May 1996/Accepted 22 July 1996

Twenty-eight human isolates of *Escherichia coli* from Argentina and Spain and eight veterinary isolates received from the Ministry of Agriculture Fisheries and Foods in the United Kingdom required 2 to >128 μg of ciprofloxacin per ml for inhibition. Fragments of *gyrA* and *parC* encompassing the quinolone resistance-determining region were amplified by PCR, and the DNA sequences of the fragments were determined. All isolates contained a mutation in *gyrA* of a serine at position 83 (Ser83) to an Leu, and 26 isolates also contained a mutation of Asp87 to one of four amino acids: Asn ($n = 14$), Tyr ($n = 6$), Gly ($n = 5$), or His ($n = 1$). Twenty-four isolates contained a single mutation in *parC*, either a Ser80 to Ile ($n = 17$) or Arg ($n = 2$) or a Glu84 to Lys ($n = 3$). The role of a mutation in *gyrB* was investigated by introducing wild-type *gyrB* (pBP548) into all isolates; for three transformants MICs of ciprofloxacin were reduced; however, sequencing of PCR-derived fragments containing the *gyrB* quinolone resistance-determining region revealed no changes. The analogous region of *parE* was analyzed in 34 of 36 isolates by single-strand conformational polymorphism analysis and sequencing; however, no amino acid substitutions were discovered. The outer membrane protein and lipopolysaccharide profiles of all isolates were compared with those of reference strains, and the concentration of ciprofloxacin accumulated (with or without 100 μM carbony cyanide *m*-chlorophenylhydrazone [CCCP]) was determined. Twenty-two isolates accumulated significantly lower concentrations of ciprofloxacin than the wild-type *E. coli* isolate; nine isolates accumulated less then half the concentration. The addition of CCCP increased the concentration of ciprofloxacin accumulated, and in all but one isolate the percent increase was greater than that in the control strains. The data indicate that high-level fluoroquinolone resistance in *E. coli* involves the acquisition of mutations at multiple loci.

Despite initial optimism, resistance to fluoroquinolone antibiotics among bacteria has increased significantly since their introduction into medicine and agriculture in the late 1980s and early 1990s. Consequently, the mechanism of resistance in fluoroquinolone-resistant bacteria has been the subject of intense research, and in recent years dramatic advances have been made both in our understanding of these mechanisms and in the range of species that have been characterized. It is now clear that a number of factors can contribute to fluoroquinolone resistance in both gram-negative and gram-positive bacteria. In gram-negative organisms, such as *Escherichia coli*, the primary target of fluoroquinolone antibiotics is the topoisomerase II enzyme, DNA gyrase, which is essential for DNA synthesis (7). DNA gyrase consists of two A and two B subunits, encoded by the *gyrA* and *gyrB* genes, respectively (25). Alterations in DNA gyrase have been shown to reduce the affinity of the enzyme for fluoroquinolones and decrease the susceptibility of the organism to the antibiotics. Most mutations have been shown to reside in a small region near the start of the *gyrA* gene, termed the quinolone resistance-determining region (QRDR), although mutations have also been reported in *gyrB* (for a recent review, see reference 27). Recently, topoisomerase IV has also been shown to be inhibited by fluoroquinolones, and it has been suggested that in *E. coli* this enzyme may be a secondary target for fluoroquinolone action in the absence of a susceptible DNA gyrase (12). Decreased susceptibility has also been associated with reduced intracellular accumulation of fluoroquinolones because of changes in the cell envelope (27). In gram-negative bacteria these may include the loss of outer membrane porins or alterations to the lipopolysaccharide (LPS) of the outer membrane, or both (11). In certain organisms, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, reduced accumulation has also been associated with enhanced efflux of fluoroquinolones from the cell (13, 29). Mechanisms of resistance involving the bacterial cell envelope frequently confer multiple drug resistance to several chemically unrelated but clinically important drugs (19, 23). In *E. coli*, mutations in *marR* of the *marRAB* operon result in overproduction of the MarA activator, which controls the expression of a number of loci thought to be involved in multiple-antibiotic resistance (3). The most important of these has recently been demonstrated to be *acrAB*, which encodes a multiple-antibiotic efflux pump (24).

Despite much research effort into the detailed characterization of resistance mechanisms in specific laboratory-derived mutants, the prevalence and relative contribution of individual mechanisms with regard to fluoroquinolone resistance in human and veterinary isolates is far from clear. In order to determine the role of each known resistance mechanism in the overall phenotype, we characterized 36 fluoroquinolone-resistant isolates of *E. coli* obtained from humans in Spain and Argentina and from veterinary sources in the United Kingdom. For comparison, two National Culture Collection fluoroquinolone-susceptible control strains were also analyzed. We have shown that high levels of resistance are associated with multiple changes affecting the cell envelope and both of the target enzymes. The likelihood of such multiple mutants arising spon-

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taneously within an infecting population are extremely slim, suggesting that individual mutations are accumulated by strains either within one host during prolonged antibiotic exposure or as they are spread from host to host or that such mutants arise subsequent to the antibiotic challenge, perhaps because of adaptive mutagenesis (8).

MATERIALS AND METHODS

Bacteria. Thirty-eight strains of *E. coli* were examined throughout the study: 19 clinical isolates from Hospital de La Princesa, Madrid, Spain (isolates I236 to I254), previously described by Alarcon et al. (2); 9 clinical isolates from Sonia Arduino, Clinica Independecia, Buenos Aires, Argentina (isolates I275 to I283); 8 veterinary isolates (calves and chickens) from the Central Veterinary Laboratory, Ministry of Agriculture Fisheries and Food in the United Kingdom (isolates I87 to I94); and 2 control strains, NCTC 10418 (isolate I113) and NCTC 10538 (isolate I114). Strain I152 (PC2909; PhoE$^+$) was used as a positive control strain for Western blotting (immunoblotting) of PhoE (16). The identities of all isolates were checked by API 20E strips (Bio-Merieux), and all strains were stored at 4°C on infusion agar slopes (Southern, Corby, United Kingdom) or at −70°C on Protect tubes (Technical Services Consultants, United Kingdom).

Media and antibiotics. All strains were cultured in Iso-Sensitest medium (Unipath, Basingstoke, United Kingdom). Luria-Bertani (LB) medium containing 300 mM NaCl (high salt) or no added NaCl (low salt) was used to regulate the expression of OmpC and OmpF outer membrane proteins (OMPs), respectively. All chemicals and antibiotics except ciprofloxacin (Bayer AG), nalidixic acid (Sterling Winthrop), cefoxitin (Merck Sharpe & Dohme), and amikacin (Bristol-Myers Squibb), were purchased from Sigma Chemicals (Poole, United Kingdom).

Determination of antibiotic susceptibility. The MIC of each agent for each strain was determined by a routine agar plate dilution method with Iso-Sensitest medium and a final inoculum of 10^6 CFU. All plates were incubated aerobically at 37°C overnight. The MIC of the antibiotic was defined as that concentration (in micrograms per milliliter of agar) at which no more than six colonies were detected; a slight haze of growth was ignored. In addition, the MIC of ciprofloxacin was determined on low-salt (no added NaCl) and high-salt (300 mM NaCl) LB medium by using inocula grown overnight in either high- or low-salt LB broth, as appropriate.

Measurement of ciprofloxacin accumulation. The concentration of ciprofloxacin that accumulated after 5 min of exposure to drug was measured as described by Mortimer and Piddock (21). Accumulation was also measured in the presence of 100 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which disrupts the proton motive force and which has been shown to inhibit active efflux across the cell envelope of a number of bacteria. CCCP was added after the bacteria had been exposed to drug for 5 min, and then the concentration that accumulated was determined after sampling at 10 and 15 min.

Analysis of OMPs. *E. coli* isolates were grown at 37°C in high- or low-salt LB broth. Outer membranes were prepared by differential centrifugation, sonication, and Sarkosyl extraction as described by Piddock et al. (28). The protein concentration was determined by the method of Lowry et al. (20), after which the membrane suspension was adjusted to a final concentration of 1 μg/μl with sodium dodecyl sulfate (SDS) loading buffer and boiled for 10 min. Five microliters of each sample was electrophoresed by SDS-polyacrylamide gel electrophoresis (PAGE) (17) at 200 V in a minigel apparatus (Atto) and stained with Coomassie Blue (BDH). The presence of PhoE in the outer membranes was determined by Western blotting the SDS-PAGE minigels and immunostaining with anti-PhoE monoclonal antibody (J. Tomassen, Utrecht, The Netherlands). OmpF and OmpC were identified by expression in high- and low-salt media and by determining the susceptibility of each strain to the OMP-specific phages SS4 (OmpC) and K2O (OmpF).

Analysis of LPS content. The LPSs of all strains were extracted by digesting a washed cell suspension with proteinase K at 60°C for 2 h. LPS was identified by the method of Lesse et al. (18), and gels were silver stained by the method of Tsai and Frasch (32).

Preparation and amplification of chromosomal DNA. Chromosomal DNA was prepared and purified by cetyltrimethylammonium bromide (CTAB)-chloroform extraction (4). Amplification of the QRDR of *gyrA* (36) (nucleotides 166 to 355) was achieved by PCR with the following primers: 5′-ACGTACTAGGCAATG ACTGG-3′ and 5′-AGAAGTCGCCGTCGATAGAAC-3′, the latter of which was biotinylated at the 5′ end and purified on a cartridge oligonucleotide purification column (Cruachem) prior to use. One microliter (approximately 0.5 mg) of DNA was added to a total volume of 50 μl of the PCR mixture containing 250 nM (each) primer, 0.2 mM (each) deoxynucleoside triphosphates 3 mM Mg^{2+}, and 1 U of *Taq* polymerase by using the reaction buffer supplied (Boehringer), and the mixture was overlaid with paraffin oil. PCR was carried out with a Hybaid thermal reactor with an initial denaturing step at 94°C (5 min); this was followed by 94°C (1 min), 55°C (1 min), and 72°C (1 min) for 30 cycles and a final extension step at 72°C (10 min).

Amplification of the analogous region from *parC* (nucleotides 137 to 401) (15, 26) was achieved as described above, but the following primers were used: 5′-TGTATGCGATGTCTGAACTG-3′ and 5′-biotin-CTCAATAGCAGCTC GGAATA-3′. Likewise, amplification of *gyrB* (nucleotides 1223 to 1426) was achieved with primers 5′-CAGACTGCCAGGAACGCGAT-3′ and 5′-biotin-A GCCAAGTGCGGTGATAAGA-3′, and amplification of *parE* (nucleotides 1498 to 1232) was achieved with primers 5′-TACCGAGCTGTTCCTTGTGG-3′ and 5′-biotin-GGCAATGTGCAGACCATCAG-3′, based on the published *gyrB* and *parE* sequences, respectively (1, 14).

SSCP analysis. Single-strand conformational polymorphism (SSCP) analysis of the PCR products was performed on a 0.5× mutation detection endpoint (MDE) gel (FMC Bioproducts) and in 0.6× TBE (Tris-borate-EDTA) gel at 10 W for 15 h in a thermostable electrophoresis apparatus (Appligene) at 18°C. Samples were prepared by heating 2 μl of the PCR products with 4 μl of formamide loading buffer at 95°C for 2 min prior to loading on the gel. The SSCP patterns were visualized by silver staining (Promega).

Solid-phase sequencing of PCR-amplified DNA. The PCR products were purified by binding to streptavidin-coated magnetic particles Dyna (M-280; Dynal, Bromborough, United Kingdom), and the unbound strand was eluted with 0.1 M NaOH according to the manufacturer's instructions. Purified bound single-stranded DNA from a single PCR was resuspended in 15 μl of distilled water. Sequence determination was performed on 7.5 μl of bound DNA with 250 nM the unbiotinylated primer and Sequenase 2.0 (United States Biochemicals) according to the manufacturer's protocol, but with the modifications recommended by Dynal.

Complementation with plasmid pBP548. Plasmid pBP548 encodes the wild-type *gyrB* gene from *E. coli* cloned into a low-copy-number vector which also carries resistance markers for kanamycin, amikacin, and gentamicin (9). pBP548 was introduced by electrotransformation, and transformants were selected on the basis of their ability to grow on medium containing either kanamycin (50 μg/ml) or amikacin (25 μg/ml), depending on the resistance phenotype of the host strains. The presence of pBP548 in the transformants was confirmed by extraction of plasmid DNA by alkaline lysis and agarose gel electrophoresis.

TABLE 1. Antibiotic susceptibilities of *E. coli* isolates investigated in the study

Antibiotic	MIC (μg/ml)[a]		
	Range	50%	90%
Nalidixic acid (2)[b]	256–256	256	256
Ciprofloxacin (0.015)	2–128	8	32
Gentamicin (0.06)	1–128	8	128
Kanamycin (2)	4–>256	4	>256
Amikacin (2)	2–4	2	4
Cefoxitin (4)	4–32	8	32
Tetracycline (2)	2–>256	128	>256
Chloramphenicol (4)	8–256	128	256

[a] 50% and 90%, MICs at which 50 and 90% of isolates are inhibited, respectively.

[b] Values in parentheses represent the MICs for the susceptible strain I113.

RESULTS

Susceptibilities to antibiotics. The susceptibilities of all isolates to several antibiotics were determined (Table 1). All isolates were very resistant to nalidixic acid (MIC, 256 μg/ml) compared with susceptible control strain I113 (MIC, 2 μg/ml). All isolates were also resistant to ciprofloxacin, although the MICs varied from 2 to 128 μg/ml. Many isolates were also resistant to some of the other antibiotics tested, particularly tetracycline and chloramphenicol. At least 27 of the 36 isolates could be classified as multiply drug resistant (i.e., resistant to at least two other classes of antibiotic). Nineteen isolates required a higher concentration of ciprofloxacin for inhibition in high-salt medium (i.e., under conditions which repress OmpF) (Table 2); however, this phenomenon was restricted to those isolates for which MICs in Iso-Sensitest media were ≥4 μg/ml.

Genetic analysis of *gyrA*. A 190-bp biotinylated PCR product, covering the entire QRDR of *gyrA*, was obtained from all *E. coli* isolates. Solid-phase sequencing of the amplified DNA revealed the presence of a mutation at codon 83 in all isolates; furthermore, in every case this was found to be due to the substitution of leucine for serine (Table 2). Many of the isolates (26 of 36) also possessed mutations at codon 87, resulting

TABLE 2. Results of biochemical and genetic characterizations of *E. coli* strains investigated in the study

Strain	Ciprofloxacin MIC (μg/ml)[a]			Substitution in GyrA	Complementation of GyrB[b]	Substitution in:		OMP[c]	LPS[d]	Uptake (SSC [ng/mg of cells])[e]		% Increase in uptake
	Iso	Low	High			ParC	ParE			Without CCCP	With CCCP	
I113	0.015	<0.1	<0.1	wt[f]	−	wt	wt	F+ C+	R	41.9	48.2	15
I114	0.06	<0.1	<0.1	wt	−	wt	wt	F+ C+	ND	44.8	56.4	26
I87	2	2	2	S83L[g]	NT	wt	wt	F^C C−	S	13.6	45.8	236
I88	2	2	2	S83L	−	wt	wt	F^C C−	S	16.2	52.2	222
I89	2	2	2	S83L	−	wt	wt	F+ C+*	S	28.6	50.5	77
I90	2	2	2	S83L	−	wt	wt	F+ C+*	R	19.6	38.4	96
I94	2	2	2	S83L	NT	wt	wt	F^C C−	S	12.8	54.2	323
I239	2	2	2	S83L	−	wt	wt	F+ C+	S	25.6	60.1	135
I241	2	2	4	S83L D87G	−	wt	wt	F+ C+	S	34.7	88.0	154
I238	4	4	4	S83L	−	wt	wt	F^C C−	S	11.3	53.2	371
I93	4	8	8	S83L	NT	S80I	wt	F− C−*	R	19.8	33.9	71
I242	4	8	16	S83L D87N	+	E84K	wt	F+ C+	S	51.4	95.0	85
I250	8	4	8	S83L D87G	−	S80R	wt	F− C+	S	40.8	76.6	88
I92	8	8	16	S83L D87Y	−	S80I	ND	F+ C+*	S	45.3	62.3	38
I248	8	4	32	S83L D87N	−	S80I	wt	F+ C+*	S	43.0	55.9	30
I91	8	8	32	S83L D87Y	−	S80I	wt	F+ C+	S	32.7	46.2	41
I240	8	8	32	S83L	+	E84K	wt	F^C C−	S	25.4	41.1	62
I243	8	8	32	S83L D87N	+	S80I	wt	F+ C+*	S	56.5	97.4	72
I245	8	8	32	S83L D87N	−	S80I	wt	F− C+*	S	20.5	44.9	119
I246	8	8	32	S83L D87N	−	S80I	wt	F+ C+*	S	20.5	37.1	81
I251	8	8	32	S83L D87N	−	S80I	wt	F+ C+	S	50.9	72.8	43
I277	8	8	32	S83L D87N	−	S80I	wt	F+ C+	S	35.7	42.0	18
I278	8	8	32	S83L D87Y	−	S80I	wt	F+ C+*	S	33.9	57.0	68
I279	8	8	32	S83L D87N	−	wt	wt	F− C+*	R	26.6	60.2	126
I281	8	8	32	S83L D87Y	−	S80I	wt	F+ C+*	S	23.9	38.2	60
I282	8	8	32	S83L D87G	−	S80I	wt	F+ C+*	S	49.7	73.6	48
I237	16	16	32	S83L	−	wt	wt	F^C C−	S	46.0	75.3	64
I236	32	32	32	S83L D87N	NT	E84K	wt	F+ C+*	S	38.4	73.9	92
I244	32	32	32	S83L D87Y	+	S80I	wt	F+ C+*	S	57.2	119.3	109
I247	32	32	32	S83L D87N	−	S80I	wt	F+ C+*	S	37.5	59.0	57
I249	32	32	32	S83L D87G	NT	S80I	wt	F+ C+*	S	42.1	69.3	65
I276	32	32	32	S83L D87H	−	S80I	wt	F+ C+*	S	45.8	64.8	41
I252	32	32	64	S83L D87N	−	S80I	wt	F+ C+*	S	56.5	89.0	58
I280	32	32	64	S83L D87N	−	S80I	wt	F+ C+*	S	39.0	48.1	23
I283	32	32	64	S83L D87N	−	wt	wt	F+ C+*	R	42.7	72.9	71
I253	32	32	128	S83L D87N	−	S80R	wt	F^C C−	S	26.3	85.2	224
I275	32	32	128	S83L D87N	−	S80I	wt	F− C+	S	26.7	35.7	34
I254	128	128	128	S83L D87N	−	wt	nd	F+ C+	S	19.1	82.3	333

[a] Determined in low-salt LB (no added NaCl), high-salt LB (300 mM NaCl), or Iso-Sensitest (Iso) medium.
[b] Complementation with pBP548 (*gyrB*). NT, not transformed.
[c] Presence or absence of OmpF (F) and OmpC (C) as determined by SDS-PAGE. *, expression of unidentified 40-kDa protein. F^C, consitutive expession of OmpF.
[d] Rough (R) or smooth (S) LPS. ND, not determined.
[e] SSC, steady-state concentration after 5 min of incubation with 10 μg of ciprofloxacin per ml with or without 100 μM CCCP.
[f] wt, wild type.
[g] The designations indicate the substituted amino acids and the position number; e.g., S83L indicates substitution of a serine for a leucine at position 83.

in substitution of asparagine ($n = 14$), tyrosine ($n = 6$), glycine ($n = 5$), or histidine ($n = 1$) for aspartate at this residue. Interestingly, 30 of the 36 isolates exhibited *gyrA* sequences which also differed from the published *E. coli gyrA* sequence (36) by three nucleotide changes at nucleotides 255, 273, and 300, none of which result in amino acid substitutions. Susceptible strain I113 also possessed this alternative sequence, but strain I114 did not.

Genetic analysis of *parC*. Mutations within the A subunit of topisomerase IV have been shown to increase fluoroquinolone resistance in a number of organisms, including *E. coli* (6, 15). A 265-bp product covering the region of *parC* analogous to the QRDR of *gyrA* was amplified and analyzed by direct solid-phase sequencing (Table 2). Twenty-one isolates were shown to contain a mutation resulting in a change at the serine at nucleotide 80, which is analogous to the serine at nucleotide 83 in *gyrA*. For most isolates (19 of 21) this resulted in the substitution of isoleucine for serine; however, for two isolates arginine was substituted. Three isolates showed a change at glutamate 84 (analogous to aspartate 87 in *gyrA* mutants), resulting in replacement with lysine. No double *parC* mutations were found in any isolates.

Genetic analysis of *gyrB*. Alterations in the B subunit of DNA gyrase, although less common than those in the A subunit, have previously been shown to confer decreased fluoroquinolone susceptibility (35). Provision of wild-type *gyrB* via introduction of plasmid pBP548 has been shown to result in the reestablishment of fluoroquinolone susceptibility, because the wild-type allele is dominant. In contrast, introduction of the control vector pBP507 has no effect (9). In order to identify potential *gyrB* mutants, isolates were transformed with plasmid pBP548, which expresses the wild-type *E. coli gyrB* gene. Despite repeated attempts five isolates could not be transformed; however, for 3 of 31 transformants selected, a greater than twofold decrease in the MICs of ciprofloxacin were found (Table 2), suggesting that these isolates may carry in *gyrB*

mutations which contribute to the fluoroquinolone resistance phenotype. A 204-bp fragment covering the QRDR of *gyrB* was amplified from the three suspected *gyrB* mutants as well as from the five nontransformable isolates; however, direct sequencing revealed no codon changes in any of the isolates. PCR and sequencing were repeated on DNA and whole-cell samples, but with the same results.

Genetic analysis of *parE*. Recently, mutations in the topisomerase IV B subunit gene, *parE*, have been implicated in resistance to fluoroquinolone antibiotics in *E. coli* (30) at a position analogous to mutations in *gyrB*. A 265-bp PCR product encompassing the region analogous to the QRDR of *gyrB* was amplified from 34 isolates and was analyzed by SSCP analysis to detect nucleotide changes compared with the DNA sequences of the two control strains. Despite repeated attempts, no PCR products could be obtained for two isolates (isolates I254 and I282). The two control strains gave different patterns; sequencing revealed that this was due to three nonsubstituting nucleotide changes in I113 compared with the nucleotides in I114, which agreed with the published sequence (14). All but seven isolates showed SSCP patterns identical to those of either I113 or I114. Sequencing of the PCR products from these isolates revealed additional nucleotide changes; however, none of these resulted in an amino acid substitution. These results suggest that mutations in *parE* are not common in ciprofloxacin-resistant clinical isolates.

Accumulation of ciprofloxacin. Fluoroquinolones are not subject to enzymatic degradation, so the accumulation of antibiotics within cells is determined by the relative rates of influx and efflux across the cell envelope. The steady-state concentration of ciprofloxacin taken up by the isolates was measured after 5 min of exposure to determine whether decreased accumulation may contribute to the fluoroquinolone resistance phenotype. Uptake measurements were repeated on at least two separate occasions with duplicate samples, and values were within the 10% variation consistently observed by this method (21). The results from one set of experiments are presented in Table 2. The average concentration of ciprofloxacin accumulated by the wild-type control strains was 43.5 ng/mg (dry weight) of cells; allowing for a typical experimental error rate of up to 10%, the range of concentrations of accumulation of ciprofloxacin in wild-type strains was 39 to 48 ng/mg of cells. Fourteen isolates accumulated ciprofloxacin to a concentration of ≥39 ng/mg of cells. Twenty-two isolates accumulated less ciprofloxacin than wild-type strains; five accumulated 33 to 39 ng of ciprofloxacin per mg of cells (>75%), eight accumulated 22 to 33 ng of ciprofloxacin per mg of cells (50 to 75%), and nine accumulated <22 ng of ciprofloxacin per mg of cells (<50%).

A number of factors can influence the level of cell-associated drug in such assays, including permeation, efflux, and binding of the drug to cellular components. In order to determine the role of energy-dependent efflux on ciprofloxacin accumulation in the resistant isolates, accumulation was measured after the addition of the proton gradient disrupter CCCP. The addition of CCCP increased the steady-state concentrations in all strains, including the controls, suggesting that an active efflux mechanism for fluoroquinolones is present in *E. coli*. The percent increase in accumulation after the addition of CCCP was greater than the average value for wild-type strains (21%) in all but one isolate, suggesting that resistant isolates may exhibit enhanced efflux (Table 2). This was particularly striking in 11 isolates, in which the concentration of ciprofloxacin accumulated more than doubled after the addition of CCCP.

Outer membrane analysis. Outer membranes were prepared from cells grown overnight in either low- or high-salt medium to induce expression of the outer membrane porins OmpF and OmpC, respectively. SDS-PAGE revealed several different OMP profiles among the strains (Table 2 and Fig. 1). OMPs were identified on the basis of relative mobility, phage susceptibility, and differential expression in high- and low-salt media. Twenty-one isolates showed complete repression of OmpF in high-salt medium, similar to that shown by the wild-type strains I113 and I114; however, repression of OmpC in low-salt medium varied. Eleven isolates lacked either OmpF or OmpC altogether, and one isolate (isolate I250) lacked both. Seven isolates appeared to lack OmpC completely and to overexpress a protein with a molecular mass similar to that of OmpF, irrespective of the salt concentration in the growth medium, suggesting that OmpF may be overexpressed in these strains. Twenty isolates expressed an additional OMP of approximately 40 kDa. It was hypothesized that this protein could be PhoE, an inducible phosphate transport protein; however, this was shown to be unlikely by its failure to be detected in any of the isolates by Western blotting with a monoclonal antibody specific to PhoE, even though PhoE was clearly identified in the positive control strain I152. The identity of this 40-kDa protein remains unknown.

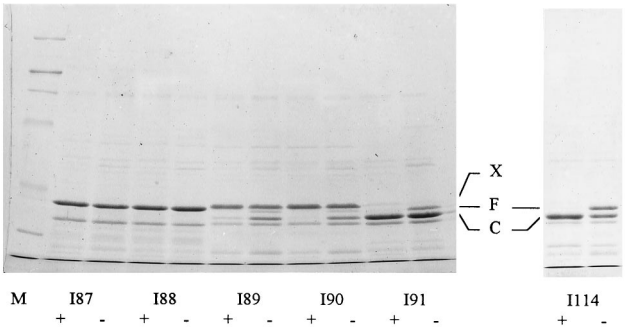

FIG. 1. Example of 10% SDS-PAGE showing OMPs of isolates grown in the presence (+) and absence (−) of 300 mM NaCl. C, OmpC; F, OmpF; X, unidentified 40-kDa protein.

The highest MICs of ciprofloxacin were usually, although not always, associated with those isolates lacking OmpF. There was no apparent relationship between MICs and expression of the 40-kDa protein or the protein with a molecular mass similar to that of OmpF. It has previously been demonstrated that changes in the LPS moiety of the outer membrane can influence the accumulation of fluoroquinolones (11); thus, the LPSs of all isolates were analyzed to look for changes in structure. The majority of isolates exhibited smooth LPS, i.e., with long side chains; three isolates had rough LPS and accumulated <50% the concentration of ciprofloxacin compared with the amount accumulated by the wild-type bacteria; two of the three isolates also lacked OmpF and had a phenotype resembling that of a *norC* mutant.

DISCUSSION

The present study represents the most exhaustive investigation to date of those factors which contribute to in vivo-selected fluoroquinolone resistance in bacteria. Previous publications have focused on *gyrA* (37), although Vila et al. (33) reanalyzed their isolates for mutations in *gyrB* and *parC*. In the present study, 36 isolates of *E. coli* from both clinical and veterinary sources were analyzed in terms of antibiotic susceptibility, genetics, cell envelope biochemistry, and drug accumulation. The resulting data suggest that the resistance phenotype, as represented by the MIC of ciprofloxacin, is determined

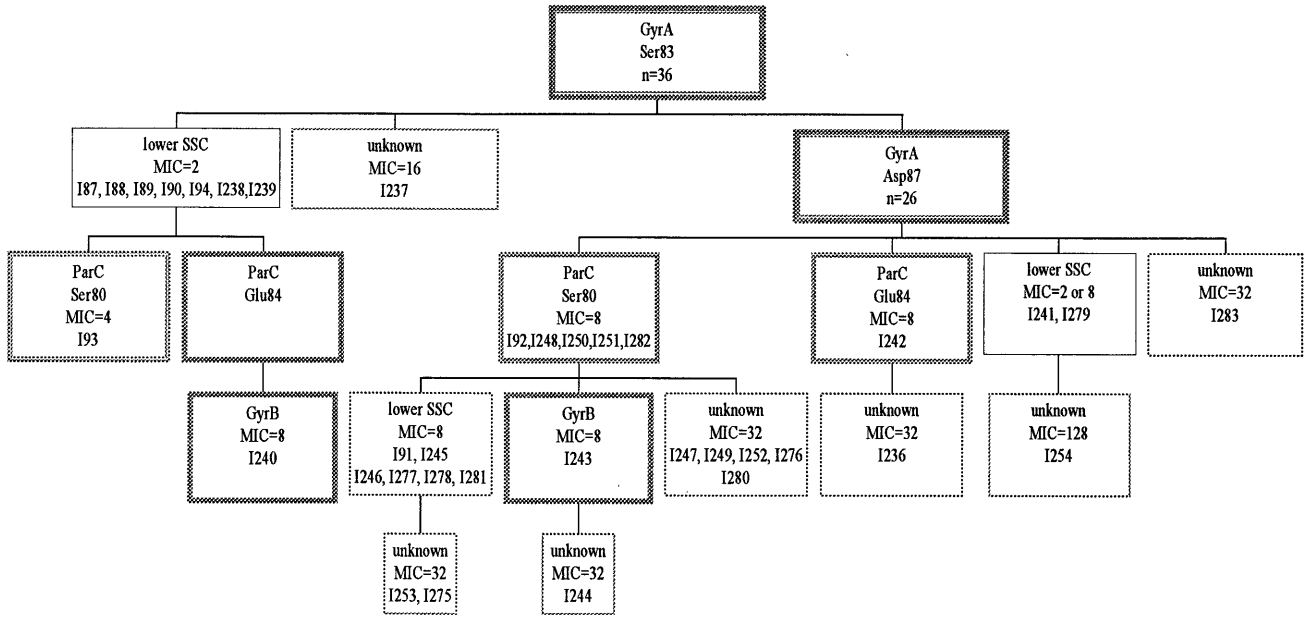

FIG. 2. Schematic diagram showing the characteristics of each isolate (this is not meant to imply the order in which individual characteristics arise). SSC, steady-state concentration.

by several different factors. Determining the contribution of each factor to the phenotype is complicated by the number of variables involved; however, certain patterns can be discerned (Fig. 2).

All 36 isolates carried a mutation in *gyrA*, resulting in a substitution of leucine for serine at codon 83; thus, this would seem to be a preliminary first step in the acquisition of fluoroquinolone resistance in vivo. Eight isolates revealed no further mutation in *gyrA*, *gyrB*, or *parC*, and seven of these isolates showed reduced ciprofloxacin accumulation and required 2 μg/ml for inhibition. Previously, a single mutation has been shown to confer an MIC of 0.25 μg/ml (34), suggesting that in these seven isolates reduced accumulation contributes to the MIC. The remaining isolate, I237, showed no reduced ciprofloxacin accumulation but required 16 μg of ciprofloxacin per ml for inhibition; the mechanism of ciprofloxacin resistance in this isolate remains unclear and warrants further investigation. Two of the isolates with a single mutation in *gyrA* also had a single mutation in *parC*; one, isolate I93, required 4 μg of ciprofloxacin per ml for inhibition, suggesting that mutation in *parC* increased the MIC of ciprofloxacin by 1 dilution. The other isolate, I240, required 8 μg of ciprofloxacin per ml for inhibition; however, transformation with pBP548 (wild-type *gyrB*) increased the isolate's susceptibility, suggesting that a mutation in *gyrB* further increased the MIC. Surprisingly, sequencing of the *gyrB* QRDR from I240 and the two other suspected *gyrB* mutants identified by complementation with pBP548 (I243 and I244) revealed no changes, suggesting either that introduction of pBP548 had an indirect effect on susceptibility or that these isolates possess a mutation(s) outside the sequenced region of *gyrB*. Work is in progress to further characterize these isolates.

Twenty-six isolates had a further mutation in *gyrA* resulting in changes at the aspartate at codon 87, most commonly to a basic amino acid such as arginine ($n = 14$). Double mutation in *gyrA* alone has previously been shown to confer a ciprofloxacin MIC of 0.5 μg/ml (31); however, for all four isolates which fell into this category, MICs were greater than 2 μg/ml, indicating further mechanisms of resistance. The remaining 22 isolates with double *gyrA* mutations also had a mutation in the topisomerase IV A subunit gene, *parC* (20 isolates in the serine at codon 80 and 2 isolates in the glutamate at codon 84); of these, 6 revealed neither further mutation in *gyrB* nor decreased accumulation and required 8 μg of ciprofloxacin per ml for inhibition, suggesting that double mutation in *gyrA* plus mutation at *parC* confers this level of resistance. No isolates contained *parC* mutations at both the serine at codon 80 and the glutamate at codon 84; this is in contrast to the work of Vila et al. (33), in which they found several such mutants among the isolates which they analyzed.

The data suggest there is little selective advantage in the clinical environment to mutations in *parC* unless the sensitivity of DNA gyrase to fluoroquinolones has been reduced to at least that of the topoisomerase IV enzyme. The minimum requirement for mutation in *parC* would appear to be a previous mutation at the serine at codon 83 of *gyrA*; however, all but one *parC* mutant also had an additional mutation in *gyrA* at the aspartate at codon 87 or in *gyrB*, suggesting that such changes usually precede a mutation in *parC*.

Twelve isolates were resistant to higher levels of ciprofloxacin (≥16 μg/ml), indicating a further mechanism(s) of resistance. These could include additional mutations in the target genes outside the QRDR which further decrease enzyme sensitivity or a mutation(s) in *parE*, the topisomerase IV B subunit gene. A recent report suggests that mutations in *parE* can indeed occur in *E. coli* and may contribute to fluoroquinolone resistance (30); however, SSCP and DNA sequence analyses of *parE* PCR products from 34 of the 36 isolates revealed no amino acid-substituting changes, suggesting that such mutations are rare in the clinical setting. Measurement of ciprofloxacin accumulation showed great variation between isolates, but in all cases, the addition of 100 μM CCCP resulted in increased concentrations of ciprofloxacin that were accumulated, supporting the hypothesis that *E. coli* possesses a mechanism(s) for effluxing fluoroquinolones which is at least partially dependent on maintenance of the proton gradient (5).

Active efflux appeared to be enhanced in virtually all isolates; however, there was no correlation between the levels of efflux (as estimated by the percent increase in accumulation after the addition of CCCP) and MIC.

A lack of OmpF because of transposon insertion in the structural gene has previously been shown to increase the MIC of ciprofloxacin from 0.015 to 0.06 μg/ml (22). Although the loss of OmpF alone is likely to be insignificant in determining the MICs for bacteria possessing mutations affecting the target proteins, it may be important when combined with an enhanced efflux pump. SDS-PAGE analysis of outer membranes revealed that four isolates lost OmpF; however, the loss of OmpF in these strains did not correlate with either increased efflux or an increased MIC. In contrast, measurement of ciprofloxacin susceptibility in high- and low-salt media revealed that the MICs for many isolates were greater under conditions which repressed OmpF. This would seem to support the role of OmpF in fluoroquinolone susceptibility; however, OmpF was either absent or expressed constitutively in many of these isolates, suggesting an alternative mechanism for the effect of osmolarity on susceptibility. This may involve a direct influence on DNA gyrase activity, because it is known that DNA supercoiling is regulated by osmolarity and that this also affects OMP expression (10).

Twenty isolates constitutively expressed an additional OMP of 40 kDa. This was shown not to be PhoE by Western blotting, as proposed previously (22), and its identity remains unknown. Expression of this protein did not correlate with either ciprofloxacin accumulation or the MIC.

The present study demonstrates that high-level fluoroquinolone resistance is a multifactorial process and that the most resistant isolates (MICs, >8 μg of ciprofloxacin per ml) usually possess at least three mutations within target genes and show enhanced efflux. What is not clear is how such strains arise. It is very unlikely that high-level resistant strains containing multiple mutations are selected in a single step within a host organism, because the chances of selecting such a mutant, carrying just three chromosomal point mutations, from a random population would be on the order of 1 in 10^{21}. Two hypotheses exist to explain the occurrence of such mutants: (i) certain bacteria within an infecting population are able to survive the lethal effects of the antibiotic, allowing advantageous mutations to accumulate via a process of adaptive mutagenesis, such as has been described for various auxotrophic mutations (8). Those which acquire the right combination of mutations before becoming nonviable are able to resume growth. Studies are in progress to examine whether antibiotic resistance mutations can arise via such a process. Alternatively, (ii) conditions of low selective pressure allow single mutants (e.g., (isolates with a change from a serine to a leucine at position 83 in *gyrA*) to divide and establish themselves as the dominant population; continued selective pressure or a new antibiotic challenge then favors those progeny containing further mutations. Such an environment could be fostered by the use of fluoroquinolone antibiotics in agriculture. The majority of mutants with single *gyrA* mutations characterized in the present study were isolated from veterinary rather than clinical sources, suggesting that the digestive tracts of animals may act as nurseries for mutants with low-level resistance. The worry is that transfer of such mutants to humans permits the subsequent selection of clinically resistant mutants during fluoroquinolone therapy. Furthermore, unlike plasmid-mediated resistance, which often disappears in the absence of continued selective pressure, chromosome-mediated resistance is usually maintained; thus, it is necessary to eradicate the resistant strain to prevent transfer between animals and humans and between humans.

ACKNOWLEDGMENTS

M.J.E. is a Lilly Industries Research Fellow.

We thank The British Society for The Study of Infection for support for this project.

REFERENCES

1. **Adachi, T., M. Mizuuchi, E. A. Robinson, E. Appella, M. H. O'Dea, M. Gellert, and K. Mizuuchi.** 1987. DNA sequence of the *E. coli gyrB* gene: application of a new sequencing strategy. Nucleic Acids Res. **15:**771–784.
2. **Alarcon, T., J. Pita, M. Lopez Brea, and L. J. V. Piddock.** 1993. High-level quinolone resistance amongst clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from Spain. J. Antimicrob. Chemother. **32:**605–609.
3. **Ariza, R. R., S. P. Cohen, N. Bachhawat, S. B. Levy, and B. Demple.** 1994. Repressor mutations in the *marRAB* operon that activate oxidative stress genes and multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **176:**143–148.
4. **Ausubel, F., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1995. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
5. **Cohen, S. P., D. C. Hooper, J. S. Wolfson, K. S. Souza, L. M. McMurray, and S. B. Levy.** 1988. Endogenous active efflux of norfloxacin in susceptible *Escherichia coli*. Antimicrob. Agents Chemother. **32:**1187–1191.
6. **Ferrero, L., B. Cameron, B. Manse, D. Lagneaux, J. Crouzet, A. Famechon, and F. Blanche.** 1994. Cloning and primary structure of *Staphylococcus aureus* DNA topoisomerase IV: a primary target of fluoroquinolones. Mol. Microbiol. **13:**641–653.
7. **Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. Tomizawa.** 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. USA **74:**4772–4776.
8. **Hall, B. G.** 1994. Selection or mutations: Which, if either, comes first? FEMS Microbiol. Lett. **117:**237–242.
9. **Heisig, P.** 1993. High-level fluoroquinolone resistance in a *Salmonella typhimurium* isolate due to alterations in both *gyrA* and *gyrB* genes. J. Antimicrob. Chemother. **32:**367–377.
10. **Higgins, C. F., C. J. Dorman, D. A. Stirling, L. Waddel, I. R. Booth, G. May, and E. Bremer.** 1988. A physiological role for DNA supercoiling in the osmotic control of gene expression in *Salmonella typhimurium* and *Escherichia coli*. Cell **52:**569–584.
11. **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. **31:**582–586.
12. **Hoshino, K., A. Kitamura, I. Morrissey, K. Sato, J. Kato, and H. Ikeda.** 1994. Comparison of inhibition of *Escherichia coli* topoisomerase IV by quinolones with DNA gyrase inhibition. Antimicrob. Agents Chemother. **38:**2623–2627.
13. **Kaatz, G. W., S. M. Seo, and C. A. Ruble.** 1993. Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **37:**1086–1094.
14. **Kato, J., Y. Nishimura, R. Imamura, H. Niki, S. Higara, and H. Suzuki.** 1990. New topoisomerase essential for chromosome segregation in *Escherichia coli*. Cell **63:**393–404.
15. **Khodursky, A. B., E. L. Zechiedrich, and N. R. Cozarelli.** 1995. Topoisomerase IV is a target of quinolones in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **92:**11801–11805.
16. **Korteland, J., J. Tommassen, and B. Lugtenberg.** 1982. PhoE protein pore of the outer membrane of *Escherichia coli* K12 is a particularly efficient channel for organic and inorganic-phosphate. Biochim. Biophys. Acta **690:** 282–289.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
18. **Lesse, A. J., A. A. Campagnari, W. E. Bittner, and M. A. Apicella.** 1990. Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Immunol. Methods **126:**109–117.
19. **Levy, S. B.** 1992. Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. **36:**695–703.
20. **Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
21. **Mortimer, P. G., and L. J. V. Piddock.** 1991. A comparison of methods used for measuring the accumulation of quinolones by Enterobacteriaceae, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. J. Antimicrob. Chemother. **28:**639–653.
22. **Mortimer, P. G., and L. J. V. Piddock.** 1993. The accumulation of five antibacterial agents in porin-deficient mutants of *Escherichia coli*. J. Antimicrob. Chemother. **32:**195–213.
23. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264:**382–388.
24. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major

role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **178:**306–308.
25. **Peebles, C. L., N. P. Higgins, K. N. Kreuzer, A. Morrison, P. O. Brown, A. Sugino, and N. R. Cozzarelli.** 1978. Structure and activities of *Escherichia coli* DNA gyrase. Cold Spring Harbor Symp. Quant. Biol. **43:**41–52.
26. **Peng, H., and K. J. Marians.** 1993. *Escherichia coli* topoisomerase IV. Purification, characterization, subunit structure, and subunit interactions. J. Biol. Chem. **268:**24481–24490.
27. **Piddock, L. J. V.** 1995. Mechanisms of resistance to fluoroquinolones: state-of-the-art 1992–1994. Drugs **49**(Suppl. 2)**:**29–35.
28. **Piddock, L. J. V., E. A. Traynor, and R. Wise.** 1990. A comparison of the mechanisms of decreased susceptibility of aztreonam-resistant and ceftazidime-resistant Enterobacteriacae. J. Antimicrob. Chemother. **26:**749–762.
29. **Poole, K., K. Krebes, C. McNally, and S. Neshat.** 1993. Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175:**7363–7372.
30. **Soussy, C. J., E. Y. Ng, S. Oubdesselam, J. Tankovic, and D. C. Hooper.** 1996. Contribution of mutation in the *parE* gene to quinolone resistance in *E. coli* harboring a mutation in the gyrA gene, abstr. A-112, p. 153. *In* Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
31. **Troung, Q. C., S. Ouabdessalam, D. C. Hooper, N. J. Moreau, and C. J. Soussy.** 1995. Sequential mutations of *gyrA* in *Escherichia coli* associated with quinolone therapy. J. Antimicrob. Chemother. **36:**1055–1059.
32. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
33. **Vila, J., J. Ruiz, P. Goni, and M. T. Jimenez de Anta.** 1996. Detection of mutations in *parC* in quinolone-resistant clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **40:**491–493.
34. **Vila, J., J. Ruiz, F. Marco, A. Barcelo, P. Goni, E. Giralt, and T. Jimenez de Anta.** 1994. Association between double mutation in *gyrA* gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. Antimicrob. Agents Chemother. **38:**2477–2479.
35. **Yamagishi, J., H. Yoshida, M. Yamyoshi, and S. Nakamura.** 1986. Nalidixic acid-resistant mutations of the *gyrB* gene of *Escherichia coli*. Mol. Gen. Genet. **204:**367–373.
36. **Yoshida, H., M. Bogaki, M. Nakamura, and S. Nakamura.** 1990. Quinolone resistance-determining region in the DNA gyrase *gyrA* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **34:**1271–1272.
37. **Yoshida, H., T. Kojima, J. Yamagishi, and S. Nakamura.** 1988. Quinolone-resistant mutations of the *gyrA* gene of *Escherichia coli*. Mol. Gen. Genet. **211:**1–7.