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Genetic Characterization of Highly Fluoroquinolone-Resistant Clinical *Escherichia coli* Strains from China: Role of *acrR* Mutations

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The genetic basis for fluoroquinolone resistance was examined in 30 high-level fluoroquinolone-resistant *Escherichia coli* clinical isolates from Beijing, China. Each strain also demonstrated resistance to a variety of other antibiotics. PCR sequence analysis of the quinolone resistance-determining region of the topoisomerase genes (*gyrA*/*B*, *parC*) revealed three to five mutations known to be associated with fluoroquinolone resistance. Western blot analysis failed to demonstrate overexpression of MarA, and Northern blot analysis did not detect overexpression of *soxS* RNA in any of the clinical strains. The AcrA protein of the AcrAB multidrug efflux pump was overexpressed in 19 of 30 strains of *E. coli* tested, and all 19 strains were tolerant to organic solvents. PCR amplification of the complete *acrR* (regulator/repressor) gene of eight isolates revealed amino acid changes in four isolates, a 9-bp deletion in another, and a 22-bp duplication in a sixth strain. Complementation with a plasmid-borne wild-type *acrR* gene reduced the level of AcrA in the mutants and partially restored antibiotic susceptibility 1.5- to 6-fold. This study shows that mutations in *acrR* are an additional genetic basis for fluoroquinolone resistance.

Fluoroquinolones are powerful broad-spectrum antimicrobial agents used for the treatment of a wide variety of community-acquired and nosocomial infections (35, 45). However, resistance to fluoroquinolones has increased markedly since their introduction in the late 1980s (1, 7, 26, 32, 39, 44, 49). In Beijing from 1997 to 1999, approximately 60% of *Escherichia coli* strains isolated from hospital-acquired infections and 50% of the *E. coli* strains isolated from the community were resistant to ciprofloxacin. Of those fluoroquinolone-resistant strains, 80% exhibited ciprofloxacin MICs of >32 μg/ml (references 54 and 61 and unpublished data). These findings contrast with much lower frequencies in other parts of the world.

Mechanisms of fluoroquinolone resistance fall into two principal categories: alterations in drug targets (e.g., DNA gyrase or topoisomerase IV) (12, 19, 34, 52) and decreased cellular accumulation of quinolones involving the major multidrug efflux pump, AcrAB (23, 37). Mutations causing quinolone resistance occur primarily in a highly conserved region (the quinolone resistance-determining region [QRDR]) of DNA gyrase and topoisomerase IV (9, 19, 25, 37, 52, 55, 59, 60). Other secondary mechanisms, such as those that affect the regulatory gene *marA* (multiple antibiotic resistance) (9, 10) or *soxS* (superoxide) (2), generally cause decreased expression of the OmpF porin (11) and overexpression of the AcrAB efflux pump (40). These porin and pump changes lead to resistance not only to the quinolones but also to a number of structurally unrelated compounds (2, 10, 40).

In this study, we sought to determine whether the regulatory genes (*marA, soxS, acrR*), in addition to the structural genes (*gyrA*/*B, parC*), contained mutations which contribute to the high-level fluoroquinolone resistance of clinical *E. coli* isolates from China.

MATERIALS AND METHODS

Bacterial strains. Thirty clinical strains of *E. coli* with high levels of ciprofloxacin resistance (MIC > 32 μg/ml) were isolated from different patients in different wards from the 1,000-bed Peking Union Medical College Hospital in Beijing, China, from May to August 1999. These strains were selected for study because of their resistance to fluoroquinolones (Table 1). Additional strains used in this study were plasmid-free *E. coli* K-12 derivatives. Their properties are described in Table 2. Plasmid pHRP315, containing a spectinomycin cassette, was used for cloning (42), since all of the clinical strains were susceptible to spectinomycin. All isolates were grown in Luria-Bertani (LB) medium (Difco Laboratories, Detroit, Mich.) at 35°C, unless otherwise noted. Stock cultures were stored at −80°C in 30% glycerol or were lyophilized on dry disks until tested.

Antimicrobial susceptibility testing. Initial MIC profiles were screened by the disk diffusion method (M2-A6; National Committee for Clinical Laboratory Standards) and verified by E-test (AB Biodisk, Solna, Sweden) or gradient plate methodology (14). The MICs of ciprofloxacin, cefotaxime, imipenem, gentamicin, ticarcillin-clavulanic acid, and piperacillin-tazobactam were evaluated by E-test (a gift from AB Biodisk); in addition, ampicillin (400 μg/ml), tetracycline (250 μg/ml), chloramphenicol (300 μg/ml), and norfloxacin (250 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) susceptibilities were tested on LB agar using the gradient plate method. The results were recorded after 24 h of incubation at 35°C. *E. coli* strains ATCC 25922 and AG100 were included as controls. Each assay was performed three times on separate occasions.

Antimicrobial susceptibility of clinical strains bearing pHRP*acrR* was determined using gradient plates.

Organic solvent tolerance. Mid-logarithmic-phase cultures (A_{530}, 0.4 to 0.5) grown in LB broth were diluted in phosphate-buffered saline to approximately 10^6 to 10^7 cells/ml. Five microliters was spotted onto LB agar and allowed to dry. The surface of the agar was overlaid with either hexane (99%; Sigma-Aldrich Chemical Co., Milwaukee, Wis.), cyclohexane (Fisher Scientific), or a mixture of hexane and cyclohexane (3:1, 1:1, or 1:3 [vol/vol]) to a depth of ~2 to 3 mm. The plates were incubated at 30°C in a closed container to prevent evaporation of the solvent. After 24 to 36 h, the spots were scored for confluent growth, which demonstrated tolerance to the solvent(s) tested. Tests were run in duplicate, three times.

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TABLE 1. Sources and antibiotic phenotypes of 30 strains of ciprofloxacin-resistant clinical *E. coli* isolates (May to August 1999)

Strain	Specimen	MIC (μg/ml)[a]							
		Tet[b]	Chl[b]	Gen[c]	Amp[b]	Tim[c]	Pta[c]	Ftx[c]	Nor[b,d]
CH1	Urine	72	87	0.5	218	4	1.5	0.06	64
CH2	Urine	1.3	125	3	76	256	12	0.2	250
CH3	Drainage	>400	6	24	>400	256	3	256	167
CH4	Blood	153	10	24	191	24	4	0.4	92
CH5	Urine	83	250	1.5	258	16	4	0.3	194
CH6	Sputum	>400	16	24	262	32	12	4	250
CH7	Pus	>400	259	32	147	6	3	0.1	106
CH8	Pus	138	250	24	>400	128	1.5	0.3	69
CH9	Urine	302	7	0.8	>400	24	4	0.3	117
CH10	Blood	258	258	64	191	24	4	0.4	250
CH11	Drainage	>400	3	64	182	8	4	0.4	158
CH12	Pus	189	4	64	>400	32	4	0.4	97
CH13	Vaginal swab	50	316	192	>400	>256	2	0.06	25
CH14	Blood	156	46	>256	>400	32	2	0.09	67
CH15	Abscess	97	16	24	76	64	8	2	75
CH16	Blood	128	147	48	142	96	6	0.5	28
CH17	Blood	142	249	64	142	256	16	8	28
CH18	Urine	111	250	64	173	16	4	0.2	21
CH19	Vaginal swab	1.4	4	0.8	>400	256	4	>256	51
CH20	Urine	267	236	64	>400	256	3	>256	156
CH21	Sputum	160	187	1	129	32	3	0.2	58
CH22	Sputum	167	236	256	267	64	24	6	69
CH23	Vaginal swab	139	213	>256	>400	>256	64	>256	125
CH24	Throat	169	259	192	142	192	16	6	24
CH25	Sputum	236	6	4	209	64	3	0.4	28
CH26	Urine	230	14	32	316	16	4	0.4	117
CH27	Urine	44	8	32	244	96	4	0.5	236
CH28	Drainage	191	187	48	351	48	2	0.4	33
CH29	Blood	72	258	96	98	16	4	0.4	117
CH30	Urine	>400	19	32	222	48	3	0.3	158

[a] Tet, tetracycline; Chl, chloramphenicol; Gen, gentamicin; Amp, ampicillin; Tim, ticarcillin-clavulanate; Pta, piperacillin-tazobactam; Ftx, cefotaxime; Nor, norfloxacin.
[b] By gradient plate.
[c] By E-test.
[d] All strains showed ciprofloxacin MICs of >32 μg/ml. The susceptible *E. coli* K-12 strain AG100 showed a ciprofloxacin MIC of 0.015 μg/ml (by E-test) and a norfloxacin MIC of 0.08 μg/ml (by gradient plate).

PCR amplification and DNA sequencing of *gyrA, gyrB, parC*, and *acrR*. Mutations in the *gyrA, gyrB, parC*, and *acrR* genes of the *E. coli* isolates were identified by DNA sequencing of their PCR products. PCR amplification of the QRDRs of *gyrA* (nucleotides 100 to 368), *gyrB* (nucleotides 1223 to 1425), and *parC* (nucleotides 138 to 401) was performed with the following oligonucleotide primer pairs: the *gyrA* gene was amplified with 5′-^{12020}TGCCAGATGTCCGA GAT^{12004}-3′ and 5′-^{11753}GTATAACGCATTGCCGC^{11769}-3′ (AE000312; annealing temperature [T_m], 58°C), and *parC* was amplified with 5′-^{4664}TATGCG ATGTCTGAACTGGG^{4645}-3′ and 5′-^{4401}GCTCAATAGCAGCTCGGAAT^{4420}-3′ (AE000384; T_m, 54°C). Likewise, amplification of the *gyrB* gene was with primers 5′-^{1910}CAGACTGCCAGGAACGCGAT^{1891}-3′ and 5′-^{1707}AGCCAAG CGCGGTGATAAGC^{1726}-3′ (AE000447; T_m, 60°C). Wild-type *acrR* (DNA from AG100) was amplified in its entirety from bases 9175 to 9822 (AE000152; T_m, 58°C), including the promoter-operator region, with the oligonucleotide primer pairs 5′-GCTCTAGA ^{8900}ACTGTTACTACGCCAACG^{8918}-3′ and 5′-AAACTGCAG ^{9934}CTGAACCTGAAGAACGACCTG^{9913} - 3′. The underline denotes the *Xba*I and *Pst*I sites, respectively, introduced into each primer for cloning into pHRP315.

A single colony of each bacterial isolate was used as the template for PCR amplification. The PCRs were performed using high-fidelity platinum *Taq* DNA polymerase (Gibco BRL) in a GeneAmp PCR System 9700 (PE Applied Biosystems). The PCR products were purified using a Qiaquick PCR purification kit (Qiagen, Inc.). Direct cycle sequencing in both directions was performed with the same primers, using an automatic 377A DNA Sequencer (Applied Biosystems) at the Tufts University Core Facility.

Computer analyses of the sequences were performed using the ClustalW (version 1.8) multiple sequence alignment program (51).

Cloning of *acrR* gene. The 1.2-kb *acrR* PCR fragment was digested by *Xba*I and *Pst*I, gel purified (QiaexII Gel Extraction Kit; Qiagen, Inc.), and ligated into the *Xba*I- and *Pst*I-digested pHRP315. The resulting recombinant plasmid (pHRP/*acrR*) was first isolated in DH5α cells by selection with spectinomycin (30 μg/ml). It was then transformed by electroporation into clinical strains of *E. coli* (CH5, CH10, CH19, CH27, and CH29), with selection on LB agar plates containing 200 μg of spectinomycin/ml. CH15 was unable to be transformed using electroporation or $CaCl_2$ heat shock, perhaps because it contained many endogenous plasmids.

Protein electrophoresis and Western blot analysis. Freshly grown *E. coli* cells were harvested by centrifugation (794 × *g*, 10 min at 4°C), washed twice in ice-cold phosphate-buffered saline, resuspended in lysis buffer [20 mM Tris-HCl (pH 8), 100 mM NaCl, 30% glycerol, 2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 1 mM EDTA (pH 8), 1 mM dithiothreitol], and sonicated on ice. After centrifugation (4,900 × *g*, 10 min at 4°C), the concentration of total whole-cell protein in the supernatant was assessed (Bio-Rad Protein Assay) using bovine serum albumin as a standard.

Whole-cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 15% separating gel (10% gel was used for detecting AcrA) by standard methods (28). Twenty micrograms of total protein was loaded for detection of MarA, while 15 μg was loaded for the detection of AcrA. The proteins from the gel were transferred electrophoretically for 30 min at 25 V (Trans-Blot SD Semi-Dry Transfer Cell; Bio-Rad) to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, Mass.), according to the manufacturer's protocol. The membrane was blocked overnight at room temperature in Tris-buffered saline (TBS; 0.15% NaCl, 10 mM Tris-HCl, pH 7.4) containing 5% dried milk, washed three times in wash buffer (0.05% Tween 20 in TBS), and hybridized at room temperature with anti-MarA polyclonal antibody (1:5,000) (31) or anti-AcrA polyclonal antibody (1:15,000; gift from H. Nikaido of the University of California, Berkeley) diluted in buffer A (0.5% Triton X-100, 0.2% SDS, 0.5% dry milk [wt/vol] in TBS) for 1 h with shaking. After three washes in wash buffer, the membrane was incubated at room temperature for 1 h with horseradish peroxidase conjugated to anti-rabbit immunoglobulin G (1:2,000; Life Technologies) diluted in wash buffer. Finally, after three additional washes, the blots were developed using the Renaissance Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Inc., Boston, Mass.). AG100 and AG112 were used as negative and positive controls, respectively, for the detection of MarA. For the detection of AcrA, the following controls were used: AG100B (*acrR* mutant, AcrA overexpressed) was used to determine the location of overexpressed AcrA, and AG100A (*acrAB* deleted, no acrA expression) and AG100 (wild-type expression of AcrA) were employed to assess the relative amount of AcrA above wild-type levels. All Western blotting was run at least twice on separate occasions.

RNA extraction and Northern blot analysis. *soxS* expression in the clinical *E. coli* strains was detected by Northern blot analysis. Briefly, total RNA was extracted from bacterial cultures, which were grown at 35°C to an A_{530} of 0.4 to 0.5, by using a modified hot acidic phenol extraction method (Sigma-Genosys Biotechnologies, Inc., The Woodlands, Tex.). The concentration of total RNA was determined spectrophotometrically at 260 nm. Samples of total RNA (5 μg/lane) were fractionated by electrophoresis on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane (Hybond-N; Amersham Life Sciences, Inc.) overnight (48).

A 344-bp PCR fragment containing the complete *soxS* coding sequence was

TABLE 2. Laboratory strains and plasmids

Strain or plasmid	Description	Reference
Strains		
AG100	Wild-type *E. coli* K-12	16
AG112	*marR* mutant of AG100; 5-bp deletion (Δ1481–1485)	37
AG100A	AG100; Δ*acrAB*	40
AG100B	AG100; *acrR*::kan mutant	40
DJ901	GC4468, *soxRS*Δ *901*::Tn*10*Kan	18
JTG1078	GC4468, *soxR105 zjc-2204*::Tn*10*Kan	17
Plasmids		
pHRP315	Ap^r, pUCMB20 with Ω Sm^r/Sp^r cassette in *Bam*HI site	42
pHRP/*acrR*	*acrR* cloned into pHRP315	This study

amplified from AG100 chromosomal DNA with the SoxS primer pair (Sigma-Genosys) according to the manufacturer's instructions. The PCR-amplified fragment was gel purified, radiolabeled with [^{32}P]dCTP (New England Nuclear), and hybridized to the membrane-bound RNA overnight at 65°C, essentially as described by Barbosa and Levy (6). RNA from the strain with *soxRS* deleted (DJ901) and from the *soxS*-overexpressing strain (JTG1078) served as negative and positive controls, respectively (17, 18).

Effect of cloned *acrR* on AcrA expression. Bacterial strains containing pHRP/*acrR* bearing the wild-type *acrR* gene or the strain alone were grown to an A_{600} of 0.6 to 0.8, cells were harvested, and total proteins were fractionated by SDS-PAGE, Western blotted, and hybridized with anti-AcrA antibody (see above). Densitometric analysis of the blot was assessed using the National Institutes of Health *Image* program (http://rsb.info.nih.gov/hih-image/manual/tech.html#analyze). Negative (AG100A) and positive (AG100B) control strains for *acrA* were included.

RESULTS

Antibiotic and organic solvent susceptibility. Thirty fluoroquinolone-resistant *E. coli* isolates were obtained from a variety of clinical specimens from 13 different wards in one hospital, including samples of urine (nine samples), tissue drainage and abscess (seven samples), blood (six samples), and vaginal swabs (three samples). Each *E. coli* strain demonstrated high-level multidrug resistance (resistance to three or more structurally unrelated antibiotics) (Table 1). All 30 strains were highly resistant to ampicillin, and a large majority of the strains were resistant to tetracycline (28 of 30), chloramphenicol (23 of 30), gentamicin (23 of 30), ticarcillin-clavulanic acid (27 of 30), and piperacillin-tazobactam (24 of 30). Most strains were susceptible to cefotaxime, although four strains were highly resistant. All strains were resistant to ciprofloxacin (MIC, >32 μg/ml) and norfloxacin (MIC, ≥21 μg/ml) (Table 1).

All strains grew on LB agar overlaid with 99% hexane (log P_{ow}, 3.9). However, only 19 of the 30 strains were able to grow in a mixture of 3:1 hexane:cyclohexane (log P_{ow}, 3.4), and of these, only two strains (CH5 and CH29) could tolerate a 1:1 hexane:cyclohexane mixture (Table 3). These 19 strains were designated organic solvent tolerant (OST). None of the strains was able to grow in >50% cyclohexane.

Identification of mutations in DNA *gyrA, gyrB*, and *parC*. DNA sequencing of the 268-bp PCR product covering the entire QRDR of *gyrA* demonstrated the presence of mutations at codons 83 and 87 in all 30 of the isolates when compared to wild-type *E. coli* K-12. A third mutation in *gyrA* was also noted in six isolates (Table 3). In every case, the mutation at codon 83 was a C→T transversion in the codon TCG, resulting in the substitution of leucine for serine. For 27 strains, a mutation at codon 87 (G→A transversion of codon GAC) resulted in an asparagine substitution for an aspartate. In the remaining three strains, Asp-87 was replaced by Tyr (G→T transversion of codon GAC). Isolates CH3, CH9, CH11, CH14, and CH21 contained a third mutation, Ala-93 Thr substitution due to a G→A transversion at codon GCG, whereas one strain (CH16) substituted a Ser for Ala at position 93 (GCG→TCG). Of note, 23 of 30 isolates had the same nucleotide changes at positions 255 (CAG→CAA), 273 (GCG→GCA), 300 (TAT→TAC), and 333 (AGA→AGG), none of which resulted in amino acid substitutions.

Analogous to the Ser-83 Leu substitution in *gyrA*, 83% of the isolates (25 of 30) contained a mutation (G→T) at codon 80 in the QRDR of *parC*, resulting in the substitution of isoleucine for serine (Table 3). The remaining five isolates demonstrated a Glu-84 Lys replacement (analogous to Asp-87 in *gyrA*). Among the 25 strains with a Ser-80 Ile substitution, six isolates had additional mutations: three had an Ala-108 Val substitution, and three had a Glu-84 Gly substitution.

TABLE 3. Characterization of fluoroquinolone-resistant *E. coli* strains[a]

Strain	Topoisomerase mutations			AcrA overexpression	OST[b]
	GyrA	ParC	GyrB[c]		
CH1	S83L D87N	S80I	ND	−	−
CH2	S83L D87N	S80I E84G	WT	−	−
CH3	S83L D87N A93T	S80I	ND	+	+
CH4	S83L D87N	S80I A108V	ND	−	−
CH5	S83L D87N	S80I	ND	+	+
CH6	S83L D87N	S80I	WT	+	+
CH7	S83L D87N	S80I A108V	ND	+	+
CH8	S83L D87N	S80I	ND	−	−
CH9	S83L D87N A93T	E84K	ND	+	+
CH10	S83L D87N	S80I E84G	WT	+	+
CH11	S83L D87N A93T	S80I	ND	+	+
CH12	S83L D87N	E84K	ND	+	+
CH13	S83L D87N	S80I	ND	−	−
CH14	S83L D87Y A93T	S80I	ND	−	−
CH15	S83L D87N	S80I	ND	+	+
CH16	S83L D87Y A93S	S80I	ND	−	−
CH17	S83L D87N	S80I	ND	+	+
CH18	S83L D87N	S80I	WT	−	−
CH19	S83L D87N	E84K	ND	+	+
CH20	S83L D87Y	S80I	ND	+	+
CH21	S83L D87N A93T	S80I A108V	ND	+	+
CH22	S83L D87N	S80I	ND	−	−
CH23	S83L D87N	E84K	ND	+	+
CH24	S83L D87N	S80I	WT	+	+
CH25	S83L D87N	S80I	ND	−	+
CH26	S83L D87N	E84K	ND	+	+
CH27	S83L D87N	S80I	WT	+	+
CH28	S83L D87N	S80I E84G	ND	−	−
CH29	S83L D87N	S80I	ND	+	+
CH30	S83L D87N	S80I	ND	+	+

[a] None of the strains showed overexpression of MarA or SoxS.
[b] OST in hexane:cyclohexane (3:1).
[c] WT, wild-type; ND, not determined.

The QRDR of the *gyrB* gene (204-bp fragment) was amplified from four strains with norfloxacin MICs of >200 μg/ml (CH2, CH6, CH10, CH27), as well as from one strain for which the MIC of norfloxacin was 19.4 μg/ml (CH24). No mutations were found (Table 3).

Expression of *marA* and *soxS*. Overexpression of MarA protein was not observed in any of the 30 strains of *E. coli* by Western blot analysis, while MarA overexpression was clearly identified in the control Mar mutant strain, AG112 (data not shown). Likewise, Northern blot analysis was unable to detect the overexpression of *soxS* RNA in any of the clinical strains, while the 400-bp *soxS*-hybridizing band was easily detected in the control strain, JTG1078 (data not shown).

Genetic analysis of *acrR*. As measured by Western blot analysis, AcrA protein was overexpressed in 19 of the 30 strains of *E. coli* (Table 3 and Fig. 1), compared to control (AG100) levels and the Mar mutant AG112. All 19 of these strains were tolerant to organic solvents and, with the exception of five strains (CH15, CH17, CH19, CH21, and CH24), all demonstrated norfloxacin MICs of ≥100 μg/ml (Table 1 and 2). In contrast, none of the remaining 11 strains lacking AcrA overexpression could grow in the presence of organic solvents.

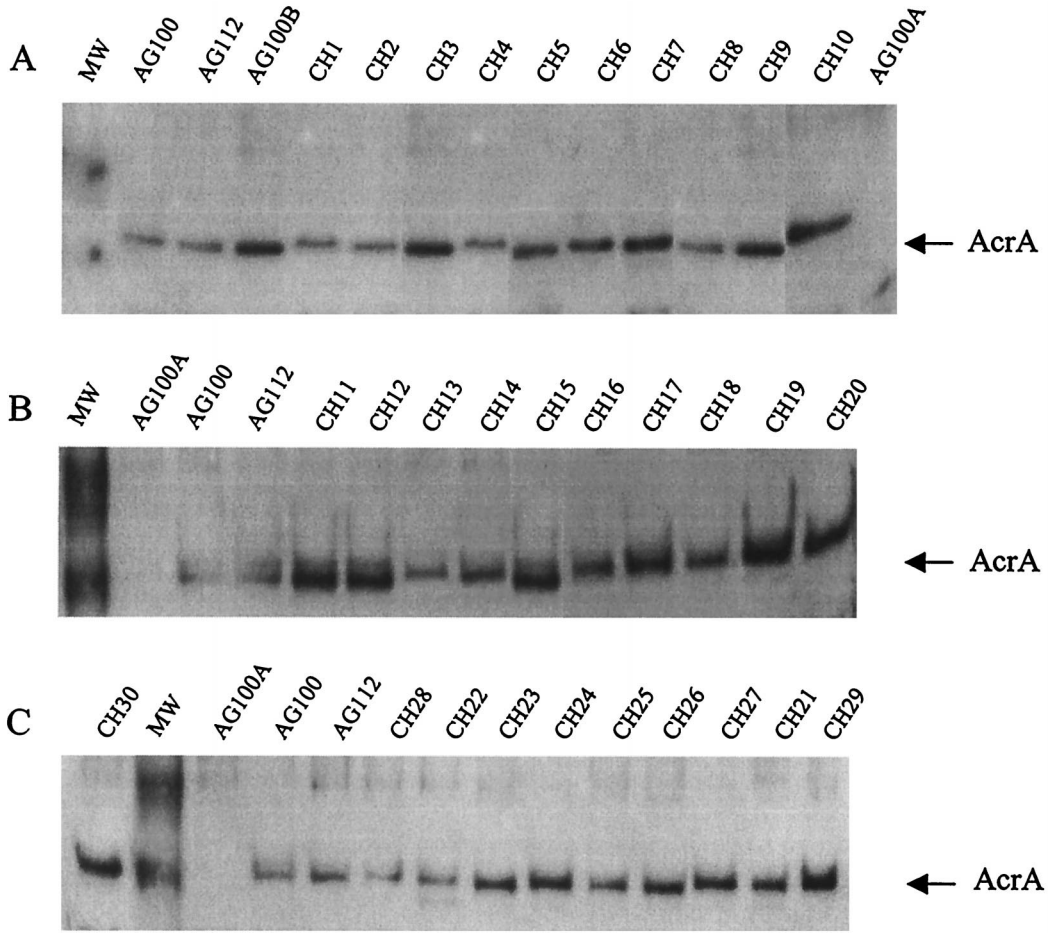

FIG. 1. Western blot analysis of AcrA prepared from clinical *E. coli* strains, separated by SDS-PAGE, blotted, and developed by chemiluminescence. AG100B (AcrR mutant, AcrA overexpressed) was used to determine the location of AcrA; AG100A (*acrAB* deleted, no AcrA expression) was a negative control; and AG100 (wild-type expression of AcrA) was used to assess the relative amount of AcrA above wild-type levels. AG112 (a MarR mutant) was included to examine AcrA levels in a constitutive MarA background. The arrow points to the 50-kDa AcrA protein. MW = molecular mass markers of 62 and 51 kDa. Western analysis was performed at least twice for each series.

Strains demonstrating overexpression of AcrA (but not MarA or *soxS*) were selected for evaluation of *acrR* (the repressor/regulator for *acrAB*, the genes encoding the major multidrug efflux pump). PCR amplification of the complete *acrR* gene was performed on eight clinical strains overproducing AcrA. DNA sequencing revealed that four isolates contained point mutations (CH5, CH10, CH15, and CH29) (Table 4). In addition, CH19 had a 9-bp deletion and CH27 had a 22-bp duplication. The mutations were random throughout the repressor and were not localized to one region (Fig. 2 and Table 4). CH24 had three silent mutations. Silent mutations were also noted in all but two strains tested. Strain CH21, in which AcrA was elevated, had no mutations in the repressor or the operator-promoter region for *acrR* (Fig. 2).

Complementation studies were undertaken in these eight strains to determine if wild-type AcrR protein would restore antibiotic susceptibility and, in addition, lower the expression of AcrA. Only five of the eight strains accepted the complementing plasmid. Sensitivity to the tested antibiotic was partially restored when the clinical strain was complemented with the wild-type *acrR* gene on pHRP/*acrR* (Table 5). Resistance to chloramphenicol decreased by 2- to 3.5-fold, and tetracycline resistance decreased 1.3- to 3-fold. Norfloxacin resistance decreased 1.5- to 6-fold. Kanamycin and gentamicin resistances were not affected by the AcrAB efflux pump, as expected (47).

TABLE 4. Mutations in *acrR*

Isolate	AcrA expression	DNA position	Codon substitution	No. of silent mutations[a]
CH5	+	9188 (AC<u>C</u>→A<u>A</u>C)	T5N	3
CH10	+	9209 (AC<u>G</u>→AT<u>G</u>)	T12M	4
		9812 (AC<u>T</u>→A<u>T</u>T)	T213I	
		9815 (AA<u>C</u>→AC<u>C</u>)	N214T	
CH15	+	9516 (C<u>C</u>A→C<u>T</u>A)	H115Y	2
CH19	+	Δ 9 nt (9383–9391)[b]	IGEL (70–73)→Y	
CH21	+		None	0
CH24	+		None	3
CH27	+	22-nt duplication (9715–9736)	FAPQSFDL (78–86) repeated, creating a frameshift	1
CH29	+	9256 (<u>G</u>GG→<u>A</u>GG)	G28R	0

[a] Changes in nucleotide sequence, with no change in amino acid.
[b] nt, nucleotide.

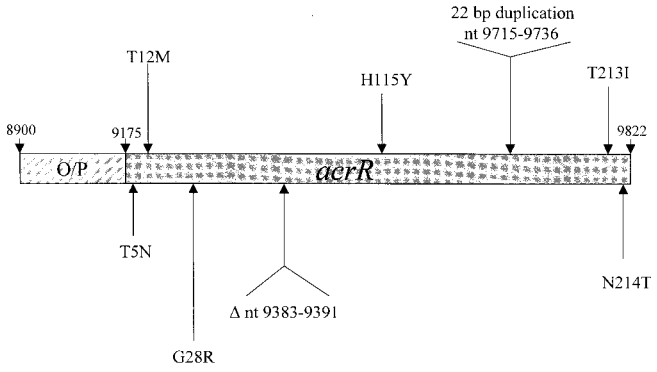

FIG. 2. Mutations within *acrR*, the repressor of the *acrAB* locus. The operator-promoter region is indicated by a hatched box, and nucleotide locations are indicated by the arrows.

AcrA protein expression was examined in the complemented strains by Western blot analysis and compared to its isogenic clinical strain by densitometry. In every case, AcrA was reduced in the complemented strain compared to its isogenic parent (Fig. 3), showing that the wild-type AcrR supplanted the host mutant AcrR and that the region regulating *acrAB* in the host was susceptible to wild-type AcrR.

DISCUSSION

High-level fluoroquinolone resistance in *E. coli* has become a major problem in China and other countries. In the present study, 30 clinical *E. coli* isolates from a hospital in China showed multiple chromosomal mutations, including mutations in the topoisomerase genes, *gyrA* and *parC*, a finding in accord with other studies of fluoroquinolone-resistant *E. coli* (8, 19–21, 46, 50). While silent mutations in *gyrA* suggest some strains may be related, the variation of silent mutations in *acrR* and the different phenotypes and mutant genotypes indicate that more than one clone is involved.

Point mutations in GyrA of Ser-83 to either Tyr or Leu (both of which convert the polar amino acid serine to a nonpolar amino acid [13, 22, 41, 58, 60]), or a double mutation of Ser-83 Leu and Asp-87 Gly (53), are the most frequent bases for fluoroquinolone resistance in clinical *E. coli* isolates. In addition, mutations in ParC at Ser-80, Gly-78, and Glu-84 (corresponding to Ser-83, Gly-81, and Asp-87 of GyrA, respectively) have also been noted (27). Higher levels of quinolone resistance (MICs, 8 to 64 μg/ml) in *E. coli* result from double mutations in *gyrA* (Ser-83 Leu and Asp-87 Gly) with a single *parC* mutation (Ser-80 or Glu-84) (5, 15, 18, 19, 23, 24, 52). The highest level of resistance generally results from four mutations: two in *gyrA* and two in *parC* (52). Such findings are supported by studies of sequential mutations of *gyrA* in laboratory strains (4, 24), which correlate higher levels of resistance with increasing numbers of mutations.

In the present study, all 30 clinical strains of *E. coli* had at least three mutations in the target genes; all shared the common mutations at Ser83 and Asp87 in GyrA. Most notably, 11 strains had more than three mutations (10 strains with four mutations, and five mutations in 1 strain) (Table 3). This number of multiple mutations from individual clinical strains has not been reported before and undoubtedly contributes to the high resistance to fluoroquinolones observed (Table 1). Importantly, mutations affecting Ala-93 in *gyrA* and Ala-108 in *parC* have not been described previously.

Although less common, mutations in the QRDR of *gyrB* have been found by others (34, 46, 57, 58), but no mutations were found in *gyrB* in the isolates studied here. A study by Everett et al. (15) examining high-level fluoroquinolone resistance in *E. coli* isolates from humans and animals was also unable to detect mutations in either *gyrB* or *parE*.

Besides target gene mutations (*gyrAB, parC/E*), studies have shown that high-level fluoroquinolone resistance can be influenced, at least in part, by mutations in one or more of the known global regulator loci (24), such as *marA* (9, 10, 16), *soxS* (2, 56), and *robA* (3, 33). Mutations in the repressors of these loci lead to overexpression of the transcriptional activator *marA* or *soxS* (36). When overexpressed, MarA or SoxS decreases the synthesis of OmpF porin (responsible for outer membrane permeability of low-molecular-weight hydrophilic molecules, including many antibiotics) (11) and increases the expression of the multidrug efflux pump, AcrAB/TolC (40), ultimately resulting in increased resistance to fluoroquinolones and other structurally unrelated antibiotics. In the present study, we were unable to detect overexpression of either MarA protein or *soxS* RNA, although 63% of the strains (19 of 30) were resistant to organic solvents and overexpressed AcrA (Table 2). Therefore, another mechanism for AcrA overexpression was involved. The AcrAB efflux pump has been associated with increased OST (55). Studies by Kern et al. (24) and Oethinger et al. (36) found that while half of high-level fluoroquinolone-resistant *E. coli* clinical strains were OST, only a proportion of the strains overexpressed *marA* or *soxS* genes. The *acrAB* locus was not examined.

We looked for possible mutations in *acrR*, the regulator of *acrAB*, that might be the basis for increased AcrA expression. Of eight strains tested, six demonstrated different amino acid substitutions, deletions, or duplications in the AcrR repressor. When the six were complemented with a wild-type repressor, AcrA levels decreased, in most cases, by half. Of interest were the two strains (CH21 and CH24) where no mutations could be found in the *acrR* gene or the *acrAB* promoter/operator region, yet they overexpressed AcrA, were OST, and were highly resis-

TABLE 5. Restoration of sensitivity to select antibiotics in clinical *E. coli* strains complemented with wild-type *acrR*

Strain	MIC (μg/ml)[a]		
	Tetracycline	Chloramphenicol	Norfloxacin
CH5	83	253	194
CH5(pHRP*acrR*)	27	73	138
CH10	258	258	250
CH10(pHRP*acrR*)	81	103	78
CH19	ND	ND	51
CH19(pHRP*acrR*)	ND	ND	18
CH27	44	8	236
CH27(pHRP*acrR*)	20	2	146
CH29	75	275	117
CH29(pHRP*acrR*)	56	80	21

[a] All susceptibilities were determined using gradient plates. ND, not done, since these strains were already relatively sensitive to tetracycline and chloramphenicol.

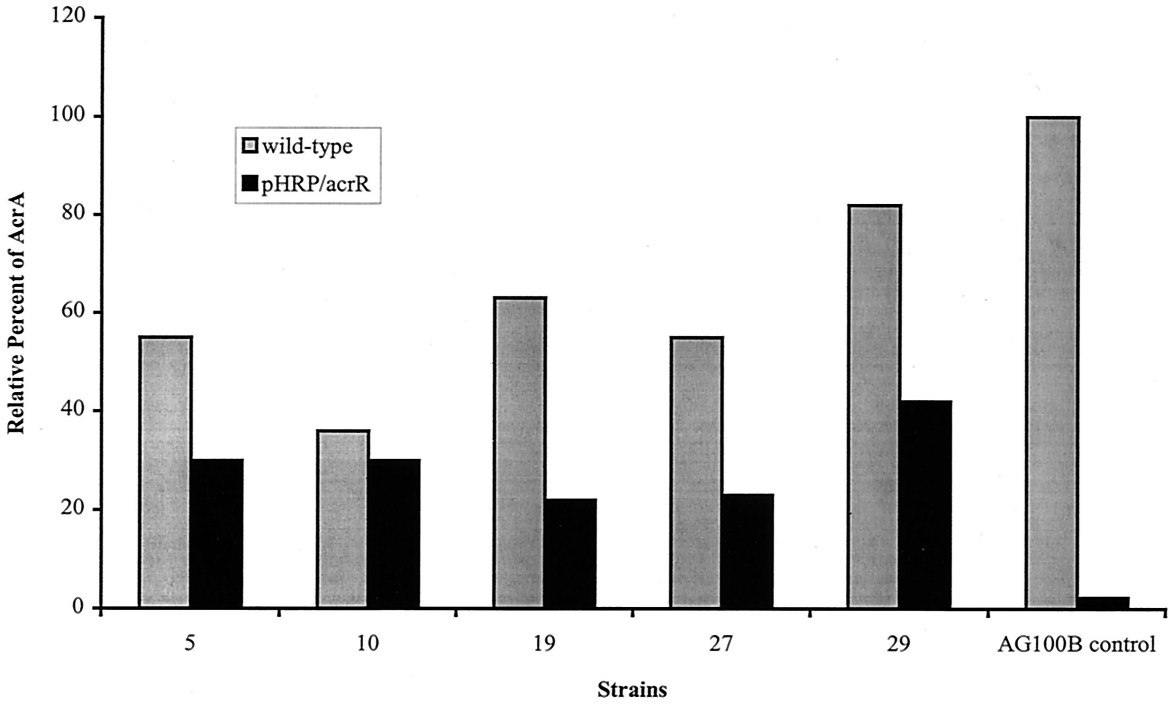

FIG. 3. Effect of wild-type AcrR on the expression of AcrA protein. Hatched bars indicate AcrA expression in the clinical *E. coli* strains relative to that in AG100B (mutant in AcrR), the positive control. Solid bars denote AcrA expression in each isogenic strain when complemented with the wild-type *acrR* on pHRP/*acrR*. AG100B (*acrR* mutant) served as the positive control.

tant to multiple antimicrobials, including the fluoroquinolones. That other unidentified genes may be involved in the up-regulation of AcrA has been reported in an earlier study in which general stress signals were able to regulate the *acrAB* operon lacking a functional repressor (29). In that study, deletion of *marRAB* or *soxRS* had little effect on the transcription of *acrAB*, demonstrating that up-regulation of *acrAB* expression was not mediated by the known global regulators, MarA and SoxS. By utilizing gel mobility shift assays, Ma and colleagues suggested that a factor other than AcrR was able to bind to the promoter region in response to global stress conditions (29). This unknown factor, or another, may be operational in CH21 and CH24. While our work was under review, another group (30) reported the overexpression of AcrA in clinical *E. coli* isolates, but the genetic basis, e.g., mutations in *acrR* or in a regulatory locus, was not described. This present study demonstrates that *acrR* gene mutations can now be included with other known chromosomal mutations to explain high-level fluoroquinolone resistance in clinical strains of *E. coli*.

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REFERENCES

1. **Aguiar, J. M., J. Chacon, R. Canton, and F. Baquero.** 1992. The emergence of highly fluoroquinolone-resistant *Escherichia coli* in community-acquired urinary tract infections. J. Antimicrob. Chemother. **29:**349–350.
2. **Amábile-Cuevas, C. F., and B. Demple.** 1991. Molecular characterization of the *soxRS* genes of *Escherichia coli*: two genes control a superoxide stress regulon. Nucleic Acids Res. **19:**4479–4484.
3. **Ariza, R. R., Z. Li, N. Ringstad, and B. Demple.** 1995. Activation of multiple antibiotic resistance and binding of stress-inducible promoters by *Escherichia coli* Rob protein. J. Bacteriol. **177:**1655–1661.
4. **Bachoual, R., J. Tankovic, and C. J. Soussy.** 1998. Analysis of mutations involved in fluoroquinolone resistance of in vivo and in vitro mutants of *Escherichia coli*. Microb. Drug Resist. **4:**271–276.
5. **Bagel, S., V. Hüllen, B. Wiedemann, and P. Heisig.** 1999. Impact of *gyrA* and *parC* mutations on quinolone resistance, doubling time, and supercoiling degree of *Escherichia coli*. Antimicrob. Agents Chemother. **40:**868–875.
6. **Barbosa, T. M., and S. B. Levy.** 2000. Differential expression of over 60 chromosomal genes in *Escherichia coli* by constitutive expression of MarA. J. Bacteriol. **182:**3467–3474.
7. **Blumberg, H. M., D. Rimland, D. J. Carroll, P. Terry, and I. K. Wachsmuth.** 1991. Rapid development of ciprofloxacin resistance in methicillin-susceptible and -resistant *Staphylococcus aureus*. J. Infect. Dis. **163:**1279–1285.
8. **Cambau, E., and L. Gutmann.** 1993. Mechanisms of resistance to quinolones. Drugs **45**(Suppl. 3)**:**5–23.
9. **Cohen, S. P., H. Hächler, and S. B. Levy.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175:**1484–1492.
10. **Cohen, S. P., L. M. McMurry, D. C. Hooper, J. S. Wolfson, and S. B. Levy.** 1989. Cross-resistance to fluoroquinolone in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemother. **33:**1318–1325.
11. **Cohen, S. P., L. M. McMurry, and S. B. Levy.** 1988. *marA* locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*. J. Bacteriol. **170:**5416–5422.
12. **Conrad, S., M. Oethinger, K. Kaifel, G. Klotz, R. Marre, and W. V. Kern.** 1996. *gyrA* mutations in high-level fluoroquinolone-resistant clinical isolates of *Escherichia coli*. J. Antimicrob. Chemother. **38:**443–455.
13. **Cullen, M. E., A. W. Wyke, R. Kuroda, and L. M. Fisher.** 1989. Cloning and characterization of a DNA gyrase A gene from *Escherichia coli* that confers clinical resistance to 4-quinolones. Antimicrob. Agents Chemother. **33:**886–894.
14. **Curiale, M. S., and S. B. Levy.** 1982. Two complementation groups mediate tetracycline resistance determined by Tn*10*. J. Bacteriol. **151:**209–215.
15. **Everett, M. J., Y. F. Jin, V. Ricci, and L. J. V. Piddock.** 1996. Contributions of individual mechanisms to fluoroquinolone resistance in 36 strains of *Esch-*

erichia coli isolated from humans and animals. Antimicrob. Agents Chemother. **40:**2380–2386.
16. **George, A. M., and S. B. Levy.** 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol. **155:**531–540.
17. **Greenberg, J. T., J. H. Chou, P. A. Monach, J. H. Chou, and B. Demple.** 1991. Activation of oxidative stress genes by mutations at soxQ/cfxB/marA locus in *Escherichia coli*. J. Bacteriol. **173:**4433–4439.
18. **Greenberg, J. T., P. A. Monach, J. H. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**6181–6185.
19. **Heisig, P.** 1996. Genetic evidence for a role of *parC* mutation in the development of high-level fluoroquinolone resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **40:**879–885.
20. **Heisig, P., H. Schedletzky, and H. Falkenstein-Paul.** 1993. Mutations in the *gyrA* gene of a highly fluoroquinolone-resistant clinical isolate of *Escherichia coli*. Antimicrob. Agents Chemother. **37:**696–701.
21. **Heisig, P., and R. Tschorny.** 1994. Characterization of fluoroquinolone-resistant mutants of *Escherichia coli* selected in vitro. Antimicrob. Agents Chemother. **38:**1284–1291.
22. **Hooper, D. C.** 1993. Quinolone mode of action—new aspects. Drugs **45** (Suppl. 3)**:**8–14.
23. **Hooper, D. C.** 1999. Mechanisms of fluoroquinolone resistance. Drug Resist. Updates **2:**38–55.
24. **Kern, W. V., M. Oethinger, A. S. Jellen-Ritter, and S. B. Levy.** 2000. Non-target gene mutations in the development of fluoroquinolone resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **44:**814–820.
25. **Khodursky, A. B., E. L. Zechiedrich, and N. R. Cozzarelli.** 1995. Topoisomerase IV is a target of quinolones in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **92:**11801–11805.
26. **Kresken, M., and B. Wiedemann.** 1988. Development of resistance to nalidixic acid and the fluoroquinolones after the introduction of norfloxacin and ofloxacin. Antimicrob. Agents Chemother. **32:**1285–1288.
27. **Kumagai, Y., J. Kato, K. Hoshino, T. Akasaka, K. Sato, and H. Ikeda.** 1996. Mutants of *Escherichia coli* DNA topoisomerase IV *parC* gene. Antimicrob. Agents Chemother. **40:**710–714.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
29. **Ma, D., M. Alberti, C. Lynch, H. Nikaido, and J. E. Hearst.** 1996. The local repressor AcrR plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol. Microbiol. **19:**101–112.
30. **Mazzariol, A., Y. Tokue, T. M. Kanegawa, G. Cornaglia, and H. Nakaido.** 2000. High-level fluoroquinolone-resistant clinical isolates of *Escherichia coli* overproduce multidrug efflux protein AcrA. Antimicrob. Agents Chemother. **44:**3441–3443.
31. **McDermott, P. F., D. G. White, I. Podglajen, M. N. Alekshun, and S. B. Levy.** 1998. Multidrug resistance following expression of the *Escherichia coli marA* gene in *Mycobacterium smegmatis*. J. Bacteriol. **180:**2995–2998.
32. **Muder, R. R., C. Brennen, A. M. Goetz, M. M. Wagener, and J. D. Rihs.** 1991. Association with prior fluoroquinolone therapy of widespread ciprofloxacin resistance among gram-negative isolates in a Veterans Affairs medical center. Antimicrob. Agents Chemother. **35:**256–258.
33. **Nakajima, H., K. Kobayashi, M. Kobayashi, H. Asako, and R. Aono.** 1995. Overexpression of the *robA* gene increases organic solvent tolerance and multiple antibiotic and heavy metal ion resistance in *Escherichia coli*. Appl. Environ. Microbiol. **61:**2302–2307.
34. **Nakamura, S., M. Nakmura, T. Kojima, and H. Yoshida.** 1989. *gyrA* and *gyrB* mutations in quinolone-resistant clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **33:**254–255.
35. **Oethinger, M., S. Conrad, K. Kaifel, A. Cometta, J. Belle, G. Klotz, M. P. Glauser, R. Marre, The International Antimicrobial Therapy Cooperative Group of the European Organization for Research and Treatment of Cancer, and W. V. Kern.** 1996. Molecular epidemiology of fluoroquinolone-resistant *Escherichia coli* bloodstream isolates from patients admitted to European cancer centers. Antimicrob. Agents Chemother. **40:**387–392.
36. **Oethinger, M., W. V. Kern, J. D. Goldman, and S. B. Levy.** 1998. Association of organic solvent tolerance and fluoroquinolone resistance in clinical isolates of *Escherichia coli*. J. Antimicrob. Chemother. **41:**111–114.
37. **Oethinger, M., W. V. Kern, A. S. Jellen-Ritter, L. M. McMurry, and S. B. Levy.** 2000. Ineffectiveness of topoisomerase mutations in mediating clinically significant fluoroquinolone resistance in *Escherichia coli* in the absence of the AcrAB efflux pump. Antimicrob. Agents Chemother. **44:**10–13.
38. **Oethinger, M., I. Podglajen, W. V. Kern, and S. B. Levy.** 1998. Overexpression of the *marA* or *soxS* regulatory gene in clinical topoisomerase mutants of *Escherichia coli*. Antimicrob. Agents Chemother. **42:**2089–2094.
39. **Ogle, J. W., L. B. Reller, and M. L. Vasil.** 1988. Development of resistance in *Pseudomonas aeruginosa* to imipenem, norfloxacin, and ciprofloxacin during therapy: proof provided by typing with a DNA probe. J. Infect. Dis. **157:**743–748.
40. **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.
41. **Oram, M., and L. M. Fisher.** 1991. 4-Quinolone resistance mutations in the DNA gyrase of *Escherichia coli* clinical isolates identified by using the polymerase chain reaction. Antimicrob. Agents Chemother. **35:**387–389.
42. **Parales, R. E., and C. S. Harwood.** 1993. Construction and use of a new broad-host-range *lacZ* transcriptional fusion vector, pHRP309, for Gram-negative bacteria. Gene **133:**23–30.
43. **Park, Y. H., J. H. Yoo, D. H. Huh, Y. K. Cho, J. H. Choi, and W. S. Shin.** 1998. Molecular analysis of fluoroquinolone-resistance in *Escherichia coli*. On the aspect of gyrase and multiple antibiotic resistance (mar) genes. Yonsie Med. J. **39:**534–540.
44. **Pea, C., J. M. Albareda, R. Pallares, M. Pujol, F. Tubau, and J. Ariza.** 1995. Relationship between quinolone use and emergence of ciprofloxacin-resistant *Escherichia coli* in bloodstream infections. Antimicrob. Agents Chemother. **39:**520–524.
45. **Peterson, L. R.** 1993. Quinolone resistance in clinical practice: occurrence and importance, p. 119–137. *In* D. C. Hooper and J. S. Wolfson (ed.), Quinolone antimicrobial agents, 2nd ed. American Society for Microbiology, Washington, D.C.
46. **Quabdesselam, S., D. C. Hooper, J. Tankovic, and C. J. Soussy.** 1995. Detection of *gyrA* and *gyrB* mutations in quinolone-resistant clinical isolates of *Escherichia coli* by single-strand conformational polymorphism analysis and determination of levels of resistance conferred by two different single *gyrA* mutations. Antimicrob. Agents Chemother. **39:**1667–1670.
47. **Rosenberg, E. Y., D. Ma, and H. Nikaido.** 2000. AcrD of *Escherichia coli* is an aminoglycoside efflux pump. J. Bacteriol. **182:**1754–1756.
48. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
49. **Tanaka, M., H. Nakayama, M. Haraoka, T. Saika, I. Kobayashi, and S. Naito.** 2000. Antimicrobial resistance of *Neisseria gonorrhoeae* and high prevalence of ciprofloxacin-resistant isolates in Japan, 1993 to 1998. J. Clin. Microbiol. **38:**521–525.
50. **Tavío, M. del M., J. Vila, J. Ruiz, A. M. Martín-Sánchez, and M. T. J. de Anta.** 1999. Mechanisms involved in the development of resistance to fluoroquinolone in *Escherichia coli*. J. Antimicrob. Chemother. **44:**735–742.
51. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
52. **Vila, J., J. Ruiz, P. Goñi, and M. T. J. de Anta.** 1996. Detection of mutations in *parC* in quinolone-resistant clinical isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **40:**491–493.
53. **Vila, J., J. Ruiz, F. Marco, A. Barcelo, P. Goín, E. Giralt, and T. J. de Anta.** 1994. Association between double mutations in *gyrA* gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. Antimicrob. Agents Chemother. **38:**2477–2479.
54. **Wang, H., X. Xie, Y. Xu, X. Zhang, B. Ren, and C. Chen.** 1997. Resistant rates of Gram-negative bacilli to 12 antibiotics by using Etest. Chin. J. Med. Lab. Sci. **20:**265–267.
55. **White, D. G., J. D. Goldman, B. Demple, and S. B. Levy.** 1997. Role of the *acrAB* locus in organic solvent tolerance mediated by expression of *marA, soxS*, or *robA* in *Escherichia coli*. J. Bacteriol. **179:**6122–6126.
56. **Wu, J., and B. Weiss.** 1991. Two divergently transcribed genes, *soxR* and *soxS*, control a superoxide response regulon of *Escherichia coli*. J. Bacteriol. **173:**2864–2871.
57. **Yamagishi, J., H. Yoshida, M. Yamayoshi, and S. Nakamura.** 1986. Nalidixic acid-resistant mutations of the *gyrB* gene of *Escherichia coli*. Mol. Gen. Genet. **204:**367–373.
58. **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.
59. **Yoshida, H., M. Bogaki, M. Nakamura, L. M. Yamanaka, and S. Nakamura.** 1991. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. Antimicrob. Agents Chemother. **35:**1647–1650.
60. **Yoshida, H., T. Kojima, J. Yamagishi, and S. Nakamura.** 1988. Quinolone resistant mutations of the gyrase *gyrA* gene of *Escherichia coli*. Mol. Gen. Genet. **211:**1–7.
61. **Zhang, X., H. Wang, Y. Hu, X. Xie, Y. Xu, M. Chen, G. Zhou, J. Wu, S. Xu, S. Zhang, Q. Wang, and X. Du.** 1997. Susceptibility of 5374 isolates to 18 antibiotics from 5 hospitals in Beijing. Natl. Med. J. China **77:**543–544.