

# Ineffectiveness of Topoisomerase Mutations in Mediating Clinically Significant Fluoroquinolone Resistance in *Escherichia coli* in the Absence of the AcrAB Efflux Pump

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**Fluoroquinolone-resistant mutants, selected from a wild-type *Escherichia coli* K-12 strain and its Mar mutant by exposure to increasing levels of ofloxacin on solid medium, were analyzed by Northern (RNA) blot analysis, sequencing, and radiolabelled ciprofloxacin accumulation studies. Mutations in the target gene *gyrA* (DNA gyrase), the regulatory gene *marR*, and additional, as yet unidentified genes (genes that probably affect efflux mediated by the multidrug efflux pump AcrAB) all contributed to fluoroquinolone resistance. Inactivation of the *acrAB* locus made all strains, including those with target gene mutations, hypersusceptible to fluoroquinolones and certain other unrelated drugs. These studies indicate that, in the absence of the AcrAB pump, gyrase mutations fail to produce clinically relevant levels of fluoroquinolone resistance.**

Fluoroquinolone (FQ) resistance in *Escherichia coli* can be caused by mutations in the target topoisomerases of the drugs, DNA gyrase (e.g., in *gyrA*) (11, 30) and topoisomerase IV (e.g., in *parC*) (10, 13, 14). Mutations that affect regulatory genes such as *marA* (3, 5) or *soxS* (2) also lead to FQ resistance. The latter genes regulate intracellular drug concentrations by producing decreased uptake and/or increased efflux of the drug (1). In *E. coli*, overexpression of MarA causes decreased expression of the OmpF porin (6) as well as increased expression of the multidrug efflux pump AcrAB (23), thereby conferring resistance to a large number of antimicrobial agents (for a review, see reference 1). Studies with mutants selected in vitro have been confined to descriptions of phenotypic differences (MICs, outer membrane profiles, accumulation of FQs) or have focused on mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA* and/or *parC* (10, 25, 27, 29). In this in vitro study we sought to understand the role of the AcrAB efflux pump in the FQ resistance mediated by topoisomerase mutations acquired during selection on ofloxacin. The AcrAB efflux pump was found to be critical to the FQ resistance level.

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## MATERIALS AND METHODS

**Antibiotics, chemicals, and media.** Ofloxacin (OFL) was kindly donated by Hoechst, Frankfurt, Germany. Radiolabelled [<sup>14</sup>C]ciprofloxacin ([<sup>14</sup>C]CIP) was a generous gift of Bayer AG, Leverkusen, Germany. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was purchased from Sigma Chemical Co., St. Louis, Mo., and organic solvents were purchased from Aldrich, Milwaukee, Wis. Strains were grown in Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) unless otherwise noted.

**Bacterial strains and plasmids.** All strains were derivatives of plasmid-free *E. coli* K-12 strain AG100 (8) and its Mar mutant AG112. The latter was selected on tetracycline in two steps (this study). Wild-type *E. coli* GC4468, its derived Sox mutant JTG1078 (*soxR105* [9]), and plasmid pSXS bearing the *soxS* gene on a 432-bp fragment (2) were a generous gift of B. Demple. Strain JZM120 (JC7623Δ*acrAB*::Tn903 Kan<sup>r</sup>) (17) was kindly supplied by H. Nikaido.

**Selection of FQ-resistant mutants.** Mutants 1-AG100, 2-AG100, 3\*-AG100, and 4\*-AG100 and mutants 1-AG112, 2-AG112, and 3-AG112 were sequential step mutants derived from strains AG100 and AG112, respectively, on solid media. For each step, about 10<sup>11</sup> cells of an overnight culture were plated on several LB agar plates supplemented with increasing concentrations of OFL (0.5 to 16 μg/ml). Single colonies were further purified on OFL-supplemented agar plates. Mutants 1-AG100 and 2-AG100 came out of one series, while mutants 3\*-AG100 and 4\*-AG100 came from a second series.

**Susceptibility testing.** The MICs of selected antimicrobial agents were determined by a standard broth microdilution procedure with cation-adjusted Mueller-Hinton broth (Becton Dickinson, Cockeysville, Md.) and an inoculum of 5 × 10<sup>5</sup> CFU/ml according to National Committee for Clinical Laboratory Standards (NCCLS) performance and interpretive guidelines (20). The antimicrobial agents in the commercially available microtiter plates (Merlin Diagnostics GmbH, Bornheim, Germany) included the FQs CIP, enoxacin, feroxacin, norfloxacin, OFL, pefloxacin, sparfloxacin (SPX), and trovafloxacin (TVF). They also included tetracycline (TET), chloramphenicol (CML), trimethoprim, cefoxitin (CFOX), cefaclor, cefixime, and loracarbef. The MICs of bile salts were determined by a broth microdilution procedure: sodium cholate and sodium deoxycholate were serially diluted in twofold steps in LB broth, and tubes were inoculated with 5 × 10<sup>5</sup> CFU/ml. The MICs of both bile salts (data not shown) and antibiotics were determined twice in independent experiments, with reproducible results.

**P1 transduction.** *acrAB*-deleted strains were constructed by P1 transduction (26) of Δ*acrAB*::Tn903 Kan<sup>r</sup> from strain JZM120 into AG100, AG112, and all derived FQ-resistant mutants (18). The AcrAB gene-deleted strains were designated with the suffix "AK", e.g., AG100AK, 1-AG100AK, and AG112AK. Two independent transductants were saved for each recipient. Deletion of the gene for AcrAB was confirmed by the absence of intact target DNA in a PCR assay and by greatly increased susceptibility to bile salts (data not shown), as reported previously (29). Besides positive and negative controls in each reaction, the *gapA* gene was used as the internal standard for determination of the validities of the PCR assays.

**DNA sequencing.** The QRDRs of *gyrA* (nucleotides 123 to 366) or *parC* (nucleotides 145 to 492) in the FQ-resistant mutants were amplified by PCR and were purified by use of Qiaquick spin columns (Qiagen, Hilden, Germany), as described previously (7; S. Conrad, L. Scheit, M. Oethinger, G. Klotz, R. Marre, and W. V. Kern, Abstr. 36th Intersci. Conf. Antimicrob. Agents Chemother. abstr. C9, 1996). *marOR* (which encodes the operator [*marO*] and repressor [*marR*] of the Mar operon) was amplified from base pairs 1311 to 1858 with primer pair ORAB2 and RK3 as described earlier (22). Direct cycle sequencing

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TABLE 1. FQ resistance mutations in *E. coli* AG100 and its Mar mutant AG112<sup>a</sup>

Strain or mutant	OFL MIC (μg/ml)	Fold increase in OFL MIC <sup>b</sup>	Substitution in <i>gyrA</i>	Mutation in <i>marOR</i>	<i>marA</i> expression	CIP uptake (% of deenergized accumulation)
AG100	0.03		None	None	Wild type	51
1-AG100	0.25	8	D87G	None	Wild type	43
2-AG100	1	32	D87G	Frameshift at aa 14 (position 1485 + 1 bp)	Overexpression	30
3*-AG100	2	64	D87G	Asp67Tyr (1643G→T)	Overexpression	9
4*-AG100	4	128	S83L, D87G	Asp67Tyr (1643G→T)	Overexpression	9
AG112	0.125	4	None	5-bp deletion (Δ1481–1485)	Overexpression	25
1-AG112	1	32	D87G	5-bp deletion (Δ1481–1485)	Overexpression	24
2-AG112 <sup>c</sup>	4	128	D87G	5-bp deletion (Δ1481–1485)	Overexpression	12
3-AG112 <sup>c</sup>	8	256	D87G	5-bp deletion (Δ1481–1485)	Overexpression	9

<sup>a</sup> Abbreviations and symbols: aa, amino acid; Δ, deletion of the bases at the indicated positions; \*, mutants were not derived from 2-AG100.

<sup>b</sup> Fold change in MIC compared to the MIC for AG100 (wild type).

<sup>c</sup> Strains have putative mutations at additional unknown loci that affect *acrAB*.

in both directions was performed with the same primers in an automatic 373A DNA Sequencer (Applied Biosystems).

**RNA extraction and Northern blot analysis.** Northern blot analysis was performed as described previously (22). In brief, RNA was harvested by a cesium chloride method from mid-logarithmic-phase cultures grown at 30°C. For assessment of the state of the *marRAB* operon or the *soxRS* operon, cultures were split and half the cultures were induced with 5 mM sodium salicylate (*marRAB* operon) or 1.3 mM paraquat (*soxRS* operon). The level of transcription from both operons was assessed by hybridization of radiolabelled DNA probes (*marA* or *soxS*) to the membrane-bound RNA (20 μg/lane), exposure on a Phosphor-Imager screen, and visualization with Image-Quant Software (Molecular Dynamics, Sunnyvale, Calif.), as described recently (22).

**Accumulation of [<sup>14</sup>C]CIP in whole cells.** Cultures were grown to the logarithmic phase in LB broth at 30°C, washed in 50 mM potassium phosphate–0.2% glucose (pH 7.4), and resuspended in the same buffer to an optical density at 600 nm (OD<sub>600</sub>) of 5 to 7. [<sup>14</sup>C]CIP (specific activity, 59 mCi/mmol) was added to 10 μM. Accumulation was measured at equilibrium after 5 and 15 min at 30°C by dilution of 50 μl of the cell-labelling suspension into 5 ml of 100 mM LiCl–50 mM KPO<sub>4</sub> (pH 7.4), collection of cells immediately on Metrical mixed-cellulose ester membrane filters (pore size, 0.45 μm; Gelman Sciences Inc., Ann Arbor, Mich.), and washing with 5 ml of the same buffer. The filters were dried, and the radioactivity was assayed with a liquid scintillation counter by using Betafluor (National Diagnostics, Somerville, N.J.). The counting efficiency was 90%. The amount of radiolabel that bound to filters in the absence of cells was subtracted. When used, CCCP, which destroys the proton motive force, was added at 20 min to a final concentration of 200 μM, and the levels of accumulation of CIP were assayed 5 and 15 min thereafter. The assay was designed in a way to investigate up to five strains in one experiment and to include, in addition, AG112 as a control strain. Results were calculated as accumulation of CIP in picomoles per OD<sub>600</sub> unit, where 1 OD<sub>600</sub> unit represented the number of cells in 1 ml when the OD<sub>600</sub> was equal to 1 (approximately 10<sup>9</sup> *E. coli* cells; about 0.3 mg of protein). The ratio (expressed as percent) of CIP accumulation of energized cells divided by that of deenergized (CCCP-treated) cells was used as an indirect measure of

active efflux (15). The values used to obtain this ratio were the separate averages of accumulation before (5 and 15 min) and after (25 min and 35 min) the addition of CCCP.

## RESULTS AND DISCUSSION

**Selection of FQ-resistant mutants in vivo.** The number of mutants observed for the total number of cells plated at the different levels of OFL ranged between  $8 \times 10^{-8}$  and  $1 \times 10^{-10}$ , which agrees well with previous data (10, 27, 29). None of the mutants was defective in growth. The first mutation step increased resistance to OFL by eightfold in both AG100 and its Mar mutant AG112 (Table 1). Subsequent increases were two- to fourfold in all steps (Table 1 and Table 2), with the highest MIC being that of OFL for 3-AG112 (MIC, 8 μg/ml). The MICs of all FQs increased in parallel, with the order of MICs being OFL > CIP > TVA = SPX (Table 2).

**Identification of chromosomal mutations in structural and regulatory genes and susceptibilities to unrelated antibiotics.** Sequencing of the QRDRs of *gyrA* revealed that an identical point mutation at codon 87 (substitution of glycine for aspartate) occurred during the first mutation step in both AG100- and AG112-derived mutants (Table 1). These mutations led to an increase in the MICs of FQs without an additional multiple resistance (Mar) phenotype. There are several reports that a *gyrA* mutation is the first “visible” mutation in the chain of events that leads to higher levels of FQ resistance during step-

TABLE 2. Effect of deletion of *acrAB* on susceptibility of FQ-resistant mutants of *E. coli* AG100 and AG112<sup>a</sup>

Strain	MIC <sup>a</sup> (μg/ml)													
	OFL		CIP		TVA		SPX		TET		CML		CFOX	
	+	Δ	+	Δ	+	Δ	+	Δ	+	Δ	+	Δ	+	Δ
AG100	0.03	≤0.015	≤0.015	≤0.015	0.06	≤0.03	≤0.015	≤0.015	1	0.5	4	1	4	0.5
1-AG100	0.25	0.06	0.25	0.06	0.25	0.06	0.125	≤0.015	2	1	4	1	4	0.5
2-AG100	1	0.06	0.5	0.06	0.25	0.06	0.25	≤0.015	2	0.5	16	1	16	0.5
3*-AG100	2	0.06	1	0.06	0.5	0.06	0.5	≤0.015	8	1	32	1	32	0.5
4*-AG100	4	0.125	2	0.125	2	0.125	2	≤0.015	8	1	64	1	32	1
AG112	0.125	≤0.015	0.06	≤0.015	0.125	≤0.03	0.06	≤0.015	4	1	16	1	32	1
1-AG112	1	0.06	0.5	0.03	0.5	0.06	0.25	≤0.015	4	0.5	32	1	16	1
2-AG112	4	0.06	2	0.03	1	0.06	1	≤0.015	16	1	64	1	32	0.5
3-AG112	8	0.06	4	0.03	2	0.06	2	≤0.015	8	0.5	64	1	64	0.5

<sup>a</sup> Determined by the broth microdilution method according to the standards of the National Committee for Clinical Laboratory Standards (20). Results are representative of experiments done in duplicate. +, *acrAB* present; Δ, *acrAB* was deleted.

wise in vitro mutagenesis (10, 25; W. V. Kern, M. Oethinger, A. S. Ritter, S. Conrad, R. Marre, and S. B. Levy, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-182, 1997). That the first *gyrA* mutations in both AG100 and AG112 were identical may be coincidental.

The second-step mutation in the AG100 background was a Mar mutation, as shown by overexpression of *marRAB* by Northern blot analysis of 2-AG100. Such overexpression was found in three independently selected second-step mutants of AG100 (data not shown). Constitutive overexpression of *marA* was associated with increased levels of resistance to TET, CML, and CFOX (Table 2). Sequencing of *marOR* of a third-step mutant, mutant 3\*-AG100, from a different series showed a different *mar* mutation but the same *gyrA* mutation.

As expected (9), *marRAB* was derepressed in Mar mutant AG112 and all subsequently derived mutants. Sequencing of *marOR* of the parental strain AG112 identified a 5-bp deletion after the codon for amino acid 12 of MarR, resulting in deletion of one amino acid and a change in the complete protein sequence thereafter (Table 1).

A single mutation in the *gyrA* gene conferred a somewhat higher level of FQ resistance than overexpression of *marA* by itself did: the OFL MICs were 0.25  $\mu\text{g/ml}$  (eightfold increase) and 0.125  $\mu\text{g/ml}$  (fourfold increase) for 1-AG100 and AG112, respectively (Table 1). The two mutations are multiplicative (OFL MICs, 1  $\mu\text{g/ml}$  [32-fold increase] for 2-AG100 and 1-AG112; Table 1). During further steps to higher levels of FQ resistance, a mutant in another series, mutant 4\*-AG100, acquired a second mutation in *gyrA*, at codon 83, substituting leucine for serine (Table 1). No additional mutations in *gyrA*, *parC*, or *marOR* could be identified in any of the more resistant mutants, nor did they constitutively overexpress *soxS* at any step (data not shown). Mutant 2-AG112, derived from *gyrA marR* double mutant 1-AG112, displayed increased levels of resistance to multiple drugs (Table 2), with no further mutation in *marOR*. The additional mutation possibly led to up-regulation of *acrAB* (see below). In contrast, the next step mutant, 3-AG112, had increased levels of resistance to only FQs and CFOX (Table 2). The genetic basis for this resistance is also unknown.

**Accumulation of [ $^{14}\text{C}$ ]CIP in whole cells.** The accumulation of CIP in whole energized cells reached a plateau by 5 min and averaged 103 pmol of CIP/OD<sub>600</sub> unit in AG100. When the proton motive force was dissipated by adding 200  $\mu\text{M}$  CCCP, the level of accumulation of [ $^{14}\text{C}$ ]ciprofloxacin doubled (201 pmol of CIP/OD<sub>600</sub> unit). These findings confirmed earlier studies with norfloxacin (4) that FQ-susceptible *E. coli* cells use energy to reduce the level of FQ accumulation; i.e., they show active efflux. This phenomenon was also observed for *Proteus vulgaris* with OFL (12). In comparison, accumulation in AG112 was 54% of that in the wild-type strain, averaging 56 pmol of CIP/OD<sub>600</sub> unit, and increased to 226 pmol CIP/OD<sub>600</sub> unit after the addition of CCCP. The rapid accumulation of CIP (this work) or norfloxacin (4) to the level in the AG100 parental strains upon deenergization of the cells rules out downregulation of outer membrane porins as the major mechanism of reduced levels of drug accumulation in Mar mutants. The amount of CIP accumulated by energized first-step (*gyrA*) mutants 1-AG100 and 1-AG112 and the increase in the level of drug uptake following deenergization were virtually identical to those for parental strains AG100 and AG112, respectively (data not shown). In second-step Mar mutant 2-AG100, the level of accumulation decreased to 65 pmol CIP/OD<sub>600</sub> unit (63% of that for the wild-type strain; Fig. 1). Independently isolated mutants 3\*-AG100 and 4\*-AG100 showed even lower levels of accumulation (16 and 19% of that for the wild-type

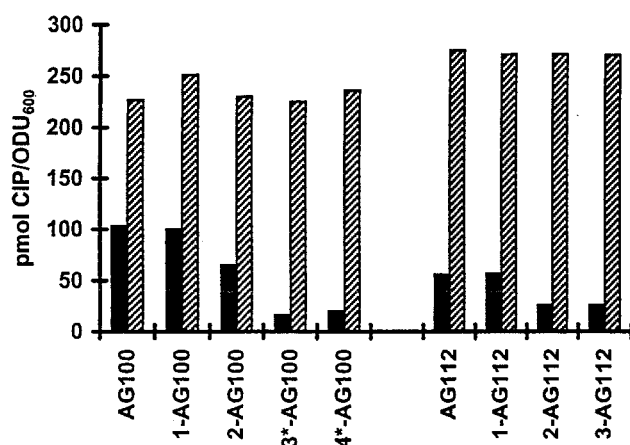


FIG. 1. Cellular accumulation of CIP. [ $^{14}\text{C}$ ]CIP accumulation by FQ-resistant mutants derived in vitro from *E. coli* K-12 strain AG100 and its Mar mutant AG112 was assayed at 30°C at equilibrium after addition of 10  $\mu\text{M}$  CIP, as described in Materials and Methods. Cells carried either the wild-type *acrAB* gene (solid bars) or had an *acrAB* deletion (hashed bars). ODU<sub>600</sub>, OD<sub>600</sub> units.

strain, respectively; Fig. 1). Similarly, mutants 2-AG112 and 3-AG112 accumulated considerably less CIP than Mar mutants AG112 and 1-AG112 (Fig. 1). With less drug accumulation, the cells can survive in the presence of higher external concentrations of FQ. The mutations also increased the cells' resistance to TET, CML, and CFOX.

**Effects of deletion of *acrAB*.** Upon deletion of the AcrAB multidrug efflux pump, the ability to actively efflux CIP was completely lost by all strains (data not shown). Energized AG100 cells bearing the *acrAB* deletion accumulated CIP to more than twice the level seen for energized *acrAB*-positive cells (Fig. 1). No difference in the levels of CIP uptake was noted between any *acrAB*-deleted strains, irrespective of their mutations in *marRAB* or *gyrA* (Fig. 1). Although all  $\Delta$ *acrAB* strains became profoundly hypersusceptible to all FQs, mutants with newly acquired mutations in *gyrA* (Table 1) still retained some *gyrA*-mediated FQ resistance (Table 2), although this was therefore well below the level of clinical significance. The OFL MIC for the *gyrA* double mutant 4\*-AG100  $\Delta$ *acrAB*, for instance, was only 0.125  $\mu\text{g/ml}$ . Interestingly, the differences in MICs of different FQs were no longer observed for  $\Delta$ *acrAB* strains, e.g., for 2-AG100  $\Delta$ *acrAB*, the OFL, CIP, and TVA MICs were 0.06  $\mu\text{g/ml}$ , while for *acrAB*-positive strain 2-AG100, the OFL MIC was 1  $\mu\text{g/ml}$ , the CIP MIC was 0.5  $\mu\text{g/ml}$ , and the TVA MIC was 0.25  $\mu\text{g/ml}$  (Table 2). The SPX MICs for all  $\Delta$ *acrAB* mutants were below the detection limit (MICs,  $\leq 0.015$   $\mu\text{g/ml}$ ). Deletion of *acrAB* also eliminated the multidrug resistance conferred by overexpression of *marA*, thus underlining previous findings that the AcrAB multidrug efflux pump plays a major role in the antibiotic resistance phenotype of Mar mutants (23). Effects of additional, as yet unknown mutations were also completely abolished by deletion of *acrAB* (Table 2). We conclude, therefore, that the effect of one or more additional mutations on drug resistance is also affected by *acrAB*.

**Concluding remarks.** In view of the large number of pumps in *E. coli* (21, 24), it is remarkable that none of the other pumps actively effluxes CIP in the absence of the AcrAB efflux pump. Thus, the AcrAB multidrug efflux pump appears to be the only, or at least the most important, pump in *E. coli* which uses CIP as a substrate.

Our findings correspond with recent data on the triclosan

susceptibility of *E. coli*, which was greatly affected by loss of the AcrAB pump (18). While overexpression of *acrAB*, *marA*, or *soxS* increased the cells' resistance to triclosan about twofold, a mutation in the target of triclosan, enoyl reductase (encoded by *fabI*), rendered the cell about 100-fold more resistant (19). However, deletion of *acrAB* reduced the level of resistance 10-fold in all strains, rendering the *fabI* mutation less effective, similar to the decrease in the effectiveness of topoisomerase mutations in the case of FQs.

The prominent finding of our work is that the AcrAB efflux pump has a powerful role in both the intrinsic and the acquired level of FQ resistance in *E. coli*. As previously reported by others for *Pseudomonas* (21), we show that efflux mechanisms significantly decrease the action of FQs also in *E. coli*, even though in the latter organism the hydrophilic drugs presumably traverse the outer membrane more rapidly through the more permeable porin channels (21). Mutations in unidentified chromosomal loci, not *marOR* or *soxRS*, modulate the level of resistance apparently by increasing efflux via AcrAB. Blockage of the AcrAB efflux pump would increase the potencies of drugs such as FQs even in the face of topoisomerase mutations. While this work was in progress, a report on the importance of the Mex multidrug efflux pumps for FQ resistance in *Pseudomonas aeruginosa* drew a parallel conclusion (16).

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