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Enhanced Expression of the Multidrug Efflux Pumps AcrAB and AcrEF Associated with Insertion Element Transposition in *Escherichia coli* Mutants Selected with a Fluoroquinolone

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The development of fluoroquinolone resistance in *Escherichia coli* may be associated with mutations in regulatory gene loci such as *marRAB* that lead to increased multidrug efflux, presumably through activation of expression of the AcrAB multidrug efflux pump. We found that multidrug-resistant (MDR) phenotypes with enhanced efflux can also be selected by fluoroquinolones from *marRAB*- or *acrAB*-inactivated *E. coli* K-12 strains having a single mutation in the quinolone-resistance-determining region of *gyrA*. Mutant 3-AG100MKX, obtained from a *mar* knockout strain after two selection steps, showed enhanced expression of *acrB* in a reverse transcriptase PCR associated with insertion of IS*186* into the AcrAB repressor gene *acrR*. In vitro selection experiments with *acrAB* knockout strains yielded MDR mutants after a single step. Enhanced efflux in these mutants was due to increased expression of *acrEF* and associated with insertion of IS2 into the upstream region of *acrEF*, presumably creating a hybrid promoter. These observations confirm the importance of efflux-associated nontarget gene mutations and indicate that transposition of genetic elements may have a role in the development of fluoroquinolone resistance in *E. coli*.

High-level fluoroquinolone resistance in *Escherichia coli* is associated with mutations in the genes coding for the target proteins DNA gyrase (*gyrA*) and topoisomerase IV (*parC*) (3, 33, 39). Mutations in regulatory loci such as *mar* (stands for multiple antibiotic resistance) and *sox* (stands for superoxide stress response) may play an important role during resistance development (19, 25, 36). Such mutations lead to overexpression of MarA, a transcriptional activator negatively regulated by MarR (9, 28), or of SoxS, the activator of the superoxide SoxRS regulon (1, 28), and thereby confer increased resistance to chemically unrelated antibiotics by activating or depressing a number of genetic loci in *E. coli* that contribute in a synergistic way to the multiple-antibiotic resistance or multidrug resistance (MDR) phenotype. After fluoroquinolone exposure in vitro, *E. coli* mutants selected from DNA gyrase single mutants typically exhibit a MDR phenotype with enhanced multidrug efflux but without additional target gene mutations (19). Previous studies have shown that MarA decreases expression of the OmpF porin and influences the expression of the *E. coli* multidrug efflux pump AcrAB (29, 32). AcrAB is a multicomponent efflux pump of the resistance-nodulation-division family that functions in association with the outer membrane protein TolC (12). Knockout experiments have shown that the AcrAB pump is critical for the resistance of *E. coli* to bile salts (40) and a number of antibiotics and that *mar* mutations are effective only in the presence of this pump (32). Upon deletion or inactivation of AcrAB, *E. coli* cells without topoisomerase mutations become hypersusceptible and resistance to fluoroquinolones can be reversed in topoisomerase mutants (31). We wondered whether efflux-associated mutations would be selectable in *E. coli* strains in which regulatory genes or the *acrAB* locus was inactivated. In initial experiments with a *mar* knockout parental strain, MDR phenotypes could be selected (19). The present report extends these observations, summarizes results obtained with *acrAB* knockout strains, and examines the mechanisms behind the enhanced active efflux measurable in the mutants.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain AG100 has been previously described (13). The *mar* deletion mutant AG100MK was constructed by P1 transduction from AG100/Kan as the donor strain into AG100. AG100/Kan was constructed by replacement of a chromosomal 1.24-kb *Bsp*HI fragment of the *mar* locus in AG100 by homologous recombination with the kanamycin resistance (Kan) cassette from pKMN33 (25). The mutant strain 1-AG100MKX was selected from strain AG100MK with 0.25 μg of ofloxacin per ml. This strain acquired a point mutation in the *gyrA* gene at codon 83 (serine→leucine), leading to an eightfold increase in the MICs of ofloxacin and ciprofloxacin (19).

The *acrAB* knockout mutant 1-AG100AK was constructed by P1 transduction (35) of a Δ*acrAB*::Tn*903*Kan^r sequence from strain JZM120 (27) into the *gyrA* single mutant 1-AG100. The AG100 *acrAB* knockout mutant 1-DC14PS was a *gyrA* single mutant obtained in a single step (selecting concentration, 0.03 μg of ofloxacin per ml) from DC14, which, in turn, had been constructed by inactivating *acrAB* with a Kan^r cassette by homologous recombination. Plasmid pCAB30 used for this replacement is a derivative of pCVD442 (10), which contains part of the *acrAB* gene cluster from AG100 and a Kan^r cassette inserted in *acrAB*, which caused the deletion of part of *acrA* and *acrB*.

Chemicals and media. Ofloxacin was obtained from Hoechst, Frankfurt, Germany. Sodium salicylate and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemicals, St. Louis, Mo. Mueller-Hinton broth and agar were from Oxoid, Basingstoke, England. Luria-Bertani (LB) broth and agar were prepared by standard protocols.

Selection of mutants. Strains were grown overnight in LB medium. Inocula of ~10^{10} CFU were plated on LB agar containing inhibitory concentrations (2 to 16 times the MIC) of ofloxacin. Plates were incubated for 24 to 48 h at 37°C. Three to six single colonies from each selecting concentration, if available, were purified on ofloxacin-supplemented plates and examined for antimicrobial susceptibility.

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Susceptibility testing. MICs of selected antimicrobial agents were determined by E-test (Viva Diagnostika, Hürth, Germany) or by a standard microdilution procedure with an inoculum of 5×10^5 CFU/ml according to NCCLS performance and inoculum guidelines (29) using commercially available microtiter plates from Merlin Diagnostics GmbH, Bornheim, Germany.

Fluoroquinolone uptake. Cells were grown to logarithmic phase in LB broth at 37°C, washed twice in 50 mM sodium phosphate buffer (pH 7.0), and resuspended in the same buffer containing 0.2% glucose. After an incubation of 10 min at 37°C, ofloxacin was added to a final concentration of 10 μg/ml. At timed intervals, 1-ml samples were removed and centrifuged immediately through silicone oil, and the pellet was resuspended in 0.1 M glycine hydrochloride (pH 3.0). After overnight incubation at room temperature, samples were centrifuged and the amount of released ofloxacin was determined spectrofluorometrically (excitation wavelength, 292 nm; emission wavelength, 496 nm) (8). The intracellular drug accumulation by the mutant strain was expressed as a percentage of the uptake measured in the corresponding parental strain. Experiments were done in triplicate and included repeated measurements after CCCP (200 μM) was added as an inhibitor of the proton-motive force.

DNA sequencing. The quinolone-resistance-determining regions (QRDRs) of *gyrA* (nucleotides 123 to 366), *parC* (nucleotides 145 to 492), and *marOR* were amplified by PCR and purified by use of Quiaquick spin columns (Qiagen, Hilden, Germany) as previously described (30). Direct cycle sequencing was performed in an automatic model 373 A DNA sequencer (Applied Biosystems).

RNA extraction and Northern blot analysis. Overnight cultures were diluted 1:100 in LB broth and grown to the mid-logarithmic phase at 37°C with shaking. Cultures were split, and sodium salicylate (5 mM final concentration) was added to one-half of the cultures for induction. RNA was isolated using an RNeasy Kit (Qiagen) according to the manufacterer's instructions. Northern blot analysis was performed by using 1.5% agarose–formaldehyde gels as described previously (2). As probes, a 387-bp *marA* fragment and a 432-bp *soxRS* fragment were used. Hybridization was performed with an ECL detection kit (Amersham Pharmacia Biotech, Freiburg, Germany).

Reverse transcription of total RNA and PCR of cDNA. Two hundred nanograms of total RNA (20 ng/μl) was mixed with 1 μl of $pd(N)_6$ random hexamers (100 ng/μl; Pharmacia) and incubated for 5 min at 65°C. The RNA was then immediately cooled on ice. Nine microliters of a reverse transcription reaction mixture containing 5× Superscript buffer, 2 μl of 10 mM dithiothreitol, 1 μl of deoxynucleoside triphosphates (10 mM), 10 U of RNasin (Promega), and 50 U of Supercript II reverse transcriptase (RT) (Gibco BRL) was added to the RNA-hexamer mix, and the solution was incubated at 42°C for 30 min.

Two microliters of the cDNA was used for the amplification of the specific cDNA species in a standard PCR. Expression of *gapA* (coding for D-glyceraldehyde-3-phosphate-dehydrogenase) (6) in the same cDNA preparation was used as a standard. The PCR was performed in a thermocycler (Biometra) using the following program: once for 5 min at 94°C; 28 cycles of 1 min at 94°C, 1 min at 51.5 to 55°C (depending on the primers used), and 1 min at 72°C; and finally once for 5 min at 72°C. The resulting PCR products were detected on a 1.5% agarose gel containing ethidium bromide. The bands were analyzed densitrometrically (Image Master 1D; Pharmacia), and results were normalized for the *gapA* band density.

RESULTS AND DISCUSSION

Selection of MDR phenotypes from *mar* and *acrAB* knockout strains. Mutants 2-AG100MKX (selecting concentration, 1 μg of ofloxacin per ml) and 3-AG100MKX (selecting concentration, 4 μg of ofloxacin per ml) were selected in a stepwise manner from the *mar* knockout strain 1-AG100MKX (single *gyrA* mutation). For both mutants increased MICs of the fluoroquinolones ofloxacin, ciprofloxacin (Table 1), and moxifloxacin (data not shown) were seen, but no additional mutations in the QRDRs of the target genes *gyrA* and *parC* were detected.

Mutant 3-AG100MKX acquired an MDR phenotype; for this strain MICs of tetracycline and chloramphenicol increased by fourfold and MICs of ampicillin, cefuroxime, and trimethoprim increased by twofold (Table 1). Activation of the *sox* regulatory gene locus was not an explanation for the MDR phenotype since *soxS* expression was unchanged compared to that of the parental strain in RT-PCR and Northern blot analyses (19).

Mutant 2-AG100AKX was obtained from 1-AG100AK, and mutant 2-DC14PS was obtained from 1-DC14PS by selection for ofloxacin resistance (selecting concentration, 0.5 μg of ofloxacin per ml) at frequencies of $\sim 2 \times 10^{-10}$ and $\sim 5 \times 10^{-10}$, respectively. All individual colonies of both 2-AG100AKX and 2-DC14PS had similar phenotypes, and for these strains increased MICs of fluoroquinolones and of chemically unrelated agents such as tetracycline, chloramphenicol, and others were seen (Table 1), results resembling the phenotype seen with mutants overexpressing the *mar* or *sox* locus. However, both 2-AG100AKX and 2-DC14PS had neither additional target gene mutations nor mutations in *mar* or *sox* that would lead to an overexpression of these loci (Table 1).

Mutant 3-AG100AKX was obtained from 2-AG100AKX in an additional selection step (ofloxacin concentration, 1 μg/ml; frequency, $\sim 3 \times 10^{-9}$). For mutant 3-AG100AKX, we found an increase in the MIC of chloramphenicol (from 8 to 32 μg/ml) and very modest increases in the MICs of the other agents, including the fluoroquinolones, but again, no additional mutations in the QRDRs of target genes or in the regulatory loci analyzed were detected (Table 1). The failure of selecting a mutation leading to constitutive expression of *marA* in *acrAB* knockout strains supports previous results which showed that the effects of MarA overexpression are mediated mainly by AcrAB (32).

Measurement of intracellular ofloxacin concentration with and without CCCP as an inhibitor of the proton-motive force confirmed that enhanced drug efflux was the mechanism of the MDR of mutants 3-AG100MKX and 2-AG100AKX. 3-AG100MKX showed a clear reduction of the intracellular ofloxacin concentration (59% of that of the parental strain), suggesting that AcrAB or another efflux pump was activated (Fig. 1). A decreased intracellular ofloxacin concentration was also clearly documented for 2-AG100AKX (42% of that of the parental strain) (Fig. 1). The enhanced efflux in this mutant in association with the acquired MDR phenotype was interesting. Since the mutant had a deletion in the gene coding for AcrAB, activation of an alternative efflux mechanism without involvement of the *mar* and *sox* regulatory loci was the likely resistance mechanism in this mutant.

Induction of the efflux pump genes *acrAB* or *acrEF*. Previous work has documented the importance of AcrAB for active efflux of fluoroquinolones in *E. coli* (32). We therefore initially examined the level of expression of *acrAB* in mutant strain 3-AG100MKX. RT-PCR analysis of *acrB* indicated that the AcrAB message relative to those seen in 1-AG100MKX and in 2-AG100MKX was slightly increased by about 1.5-fold (estimated from densitometrical analysis of RT-PCR bands [Fig. 1]). In view of the known role of AcrAB in solvent tolerance (41), this result was consistent with the observation that 3-AG100MKX showed increased tolerance to organic solvents compared with that of 2-AG100MKX (19).

We studied the expression of other different efflux pump systems (*emrAB, mdfA, acrD,* and *acrEF*) known to transport quinolones or showing homology to *acrAB* (11, 22–24). No induction of either of these genes was detected in RT-PCR experiments (data not shown). Also, the expression of *tolC*, which codes for the outer membrane component of the AcrAB

FIG. 1. Intracellular fluoroquinolone concentration and expression of the efflux pump genes *acrB* and *acrE* in *E. coli* mutants selected by ofloxacin. (Graphs) Steady-state intracellular concentrations of ofloxacin (external concentration, 10 μg/ml) in the absence of CCCP, measured spectrofluorometrically and expressed as percentages of that of the parental strain. (Gels) RT-PCR of *acrB* and *acrE* and of *gapA* (coding for D-glyceraldehyde-3-phosphate-dehydrogenase; used as the standard). (A) *mar* knockout mutants 1-AG100MKX (lane 1), 2-AG100MKX (lane 2), and 3-AG100MKX (lane 3). (B) *acrAB* knockout mutants 1-AG100AK (lane 1), 2-AG100AKX (lane 2), and 3-AG100AKX (lane 3).

pump complex (12), was unaffected, as was the expression of *tonB*, a gene coding for the TonB protein, which probably functions as an energy coupler (7) between inner and outer membranes and which was shown to influence the activity of the AcrAB homologous MexAB-OprM multidrug efflux pump in *Pseudomonas aeruginosa* (44). We concluded from these findings that *mar*- and *sox*-independent transcriptional activation of AcrAB most likely was responsible for the enhanced efflux and the MDR phenotype seen in mutant 3-AG100MKX.

The same experimental approach was used for study of the *acrAB* deletion mutants 2-AG100AKX and 3-AG100AKX. Expression of *emrAB, mdfA*, and *acrD* as well as of *tolC* and *tonB* was unchanged in these mutants (data not shown), but RT-PCR analysis showed that the expression of *acrE* was strongly induced (Fig. 1). The same result was obtained in RT-PCR experiments with 2-DC14PS, which strongly overexpressed *acrE. E. coli* AcrEF, which is homologous to AcrAB, is thought to be involved in indole efflux (18), and upregulation of AcrEF might compensate an AcrAB deletion (J. Xu, M. L. Nilles, and K. P. Bertrand, Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993, abstr. K-169, 1993). Only limited data have been reported on the substrate specificity of AcrEF (18, 23). Based on changes in the MICs for 2-AG100AKX and 2-DC14PS relative to those for their precursors, we can confirm that AcrEF has a broad substrate specificity similar to that of AcrAB and includes specificities not only for ofloxacin, ciprofloxacin, tetracyclines, and chloramphenicol but also for oxacillin, cefuroxime, trimethoprim, macrolides or azalides, clindamycin, and linezolid (Table 1), as well as quinupristin-dalfopristin (MIC change, ≥2-fold), mupirocine (MIC change, ≥8-fold), and the newer fluoroquinolones moxifloxacin (MIC change, 8-fold) and clinafloxacin (MIC change, 8-fold) (data not shown).

TABLE 1. Characterization of *E. coli* AG100 mutants selected in vitro by ofloxacin from *mar* knockout and *acrAB* knockout strains with a single *gyrA* mutation in the QRDR

Parental or mutant strain	Mutation in or status of[a]:					MIC[b] (μg/ml)									
	gyrA	*parC*	*mar*	*acrR* or *acrAB*	*acrS* or *acrEF*	OFX	CIP	TET	CHL	TMP	ERY	CLR	AZM	CLI	LNZ	AMP	CXM	OXA
AG100	wt	wt	wt	wt	wt	0.06	0.03	2	4	0.5	>256	64	24	128	128	2	2	>32
1-AG100MKX	S83L	wt	Inactivated	wt	wt	0.5	0.25	0.5	4	0.5	>256	64	24	64	64	2	2	>32
2-AG100MKX	S83L	wt	Inactivated	wt	wt	1	0.5	1	4	0.5	>256	96	24	96	64	2	2	NT
3-AG100MKX	S83L	wt	Inactivated	IS*186* inserted	wt	2	1	4	16	1	NT	>256	96	>256	>256	4	4	NT
1-AG100AK	D87G	wt	wt	Inactivated	wt	0.06	0.06	0.25	1	0.06	4	4	2	2	8	1	≤1	0.5
2-AG100AKX	D87G	wt	wt	Inactivated	IS2 inserted	0.5	0.25	2	8	1	>256	>256	>256	>256	>256	1	2	>32
3-AG100AKX	D87G	wt	wt	Inactivated	IS2 inserted	1	0.5	4	32	2	NT	NT	NT	NT	NT	1	4	NT
1-DC14PS	S83L	wt	wt	Inactivated	wt	0.06	0.06	0.12	0.5	0.06	4	4	2	2	8	1	≤1	0.5
2-DC14PS	S83L	wt	wt	Inactivated	IS2 inserted	2	1	1	4	2	>256	>256	>256	>256	>256	2	4	>32

[a] wt, wild type.
[b] OFX, ofloxacin; CIP, ciprofloxacin; TET, tetracycline; CHL, chloramphenicol; TMP, trimethoprim; ERY, erythromycin; CLR, clarithromycin; AZM, azithromycin; CLI, clindamycin; LNZ, linezolid; AMP, ampicillin; CXM, cefuroxime; OXA, oxacillin; NT, not tested.

Compared with MICs for 2-AG100AKX, the MICs of tetracyclines, chloramphenicol, trimethoprim, members of the macrolide-azalide-streptogramin group, and linezolid for mutant 3-AG100AKX showed further increases and the MICs of fluoroquinolones and cefuroxime showed very modest increases. No significant change in ofloxacin uptake (Fig. 1) was measurable in this mutant, indicating enhanced activity of a pump with limited fluoroquinolone affinity or the presence of an additional, so far unknown, resistance-enhancing mechanism.

Despite several attempts, we were unable to construct a Δ*acrAB*-Δ*acrEF* double knockout mutant from 2-AG100AKX to study in more detail the effect of pump inactivation on the resistance level. Similar observations were described by Kawamura-Sato et al. (18), suggesting that one of the two homologous pumps needs to be present in the cell. This hypothesis, however, has been challenged, given the recent description of the successful construction of a viable Δ*acrAB*-Δ*acrEF* double knockout mutant (D. Cho, D. Lofland, J. Blais, K. Tangen, D. Cotter, O. Lomovskaya, S. Chamberland, and M. N. Dudley, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1497, 2000).

Transposition of insertion sequence (IS) elements is responsible for induction of efflux pumps in the mutant strains. In the absence of *mar* and *sox* mutations, loss of transcriptional control of *acrAB* and *acrEF* was possibly due to alterations in the local repressor genes *acrR* and *acrS*, respectively, or to altered promoter regions in the corresponding operons. *acrR* mutations leading to enhanced MDR in a *mar* mutant strain have been described by Okusu et al. (32). Similar loss-of-function mutations in efflux pump repressor genes were previously described for *P. aeruginosa* and *Neisseria gonorrhoeae* (16, 38, 43, 45).

To further characterize the mechanism of efflux pump activation in 3-AG100MKX, the promoter-repressor gene region of *acrAB* was amplified. The PCR product obtained from the *mar* knockout mutant 3-AG100MKX (sense primer, 5′-CGA CGCAGTGAACCAGAA-3′; antisense primer, 5′-CCTGAG AGCATCAGAACG-3′) covering *acrR* and the *acrAB* promoter region was about 1.3 kb larger than the PCR product obtained from the parental strain. Nucleotide sequencing of the PCR product revealed that this enlargement was due to the integration of the IS element IS*186* (Fig. 2). This integration at nucleotide 93 of the *acrR* gene is likely to cause inactivation of AcrR, which in turn leads to the increased expression of *acrAB* seen in the RT-PCR experiments (Fig. 1).

In the *acrAB* deletion mutants, the promoter region and regulatory gene of *acrEF* were amplified. The PCR fragments amplified from 2-AG100AKX and 2-DC14PS (sense primer, 5′-CAACTGTGACGAACTGAA-3′; antisense primer, 5′-GG CATGTTTCGTCATTAC-3′) were about 1.3 kb larger than the fragments obtained from the parental strains 1-AG100AK and 1-DC14PS, respectively. Sequencing reactions confirmed that, in both mutants, 2-AG100AKX and 2-DC14PS IS element IS*2* had integrated in the promoter region of *acrE* (Fig. 2). Integration of IS*2* in the region between the potential repressor gene *acrS* and *acrE* at exactly the same site as that found in our mutant has been described by Klein et al. (20). Both for the strain examined previously and for our mutant strains, the findings indicate that the integration created a hybrid promoter responsible for the activation of *acrEF* expression.

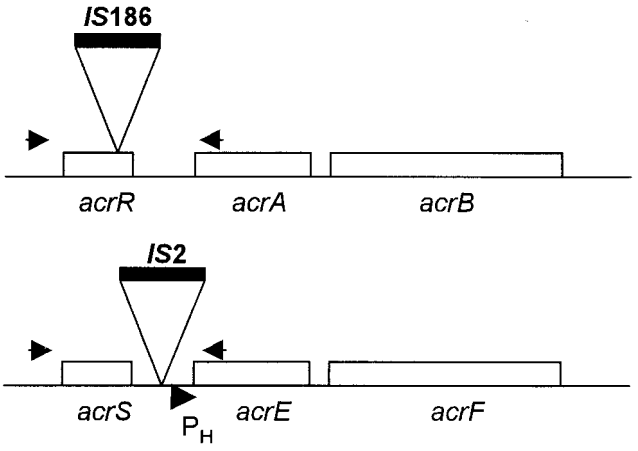

FIG. 2. Schematic diagram of the integration site of the IS elements IS*186* (upper panel) and IS*2* (lower panel) in the genes coding for the *E. coli* multidrug efflux pumps AcrAB and AcrEF. Arrows indicate the binding sites of the primers used for PCR and nucleotide sequencing. P_H designates a potential hybrid promoter created by the integration of IS*2*.

Concluding remarks. Previous in vitro selection experiments with *E. coli* indicated that mutations in regulatory genes follow an initial *gyrA* mutation and precede additional topoisomerase gene mutations (19). This finding is supported by the observations made in this study. Of note, mutations associated with enhanced efflux and selectable by fluoroquinolone exposure are not limited to the *mar* or *sox* regulon. Thus, there may be options for *E. coli* to acquire more than one mutation leading to enhanced fluoroquinolone and multidrug efflux. We postulate such double efflux-associated mutations for both 3-AG100 MKX and 3-AG100AKX as well as for some of the mutants reported earlier which had an initial *mar* mutation (leading to reduced drug uptake) and showed further reduction in intracellular drug uptake after a subsequent selection step (19). In view of the large number of proven or putative efflux pump genes on the *E. coli* chromosome, including *acrAB* homologous genes (37), and their complex regulation, involvement of more than one efflux pump system seems possible, but activation of a given efflux pump by more than one mechanism is another explanation. According to a recent study of selected fluoroquinolone-resistant clinical strains of *E. coli* with MDR phenotypes, some had mutations in both the *mar* and *sox* regulatory genes as well as in *acrR* (M. A. Webber and L. J. V. Piddock, Abstr. 40th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 759, 2000).

Gene activation and inactivation by transposition of IS elements is a well-known phenomenon. In some cases, the development of resistance has been causally linked to IS transposition (5, 17, 21, 34, 36, 42). Insertional inactivation of *acrR* in antibiotic-resistant mutants selected by fluoroquinolones has not previously been reported, but examples of IS element transposition and associated changes in permeability exist. They include *E. coli* K-12 *mar* mutants in which IS*2* integrated into *marOR*, resulting in MarA overexpression (14, 26). Also, porin-deficient, cefoxitin-resistant clinical isolates of *Klebsiella*

pneumoniae, for example, with IS elements in the *ompK36* gene have been reported (15). For the multidrug transporter MdfA, it was shown that the integration of an IS element creates a putative promoter that activates the expression of the corresponding gene (4), similar to the *acrEF* expression observed here after IS*2* transposition in mutants 2-AG100AKX and 2-DC14PS. More detailed analysis of several fluoroquinolone-resistant in vitro mutants selected in this laboratory indicates that IS element transposition is more prevalent than expected. However, functional consequences for the resistance levels of the mutants are not always obvious. Also, the specific role of fluoroquinolone exposure in such changes and the prevalence and significance of such changes under in vivo conditions remain to be determined.

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