High-Level Fluoroquinolone-Resistant Clinical Isolates of *Escherichia coli* Overproduce Multidrug Efflux Protein AcrA

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Immunoblotting with antibody against AcrA, an obligatory component of the AcrAB multidrug efflux system, showed that this protein was overexpressed by $\geq 170\%$ in 9 of 10 clinical isolates of *Escherichia coli* with high-level ciprofloxacin resistance (MICs, ≥ 32 $\mu\text{g/ml}$) but not in any of the 15 isolates for which the MIC was ≤ 1 $\mu\text{g/ml}$.

It is well known that resistance to fluoroquinolones usually requires alteration in the genes that code for the targets of these drugs, *gyrA* and *parC* (4–7, 16). On the other hand, it is not clear whether these mutations alone could produce very high levels of resistance, and one of the major factors that produce such resistance is sometimes thought to be an increased drug efflux. This hypothesis has been examined in several laboratories. Thus, Piddock and associates (2) examined 36 high-level ciprofloxacin-resistant isolates of *Escherichia coli* and found that 22 strains accumulated lower levels of ciprofloxacin than the wild type, in addition to the *gyrA* mutations found in all of them. Levy and associates (12) found that 21 of 57 high-level fluoroquinolone-resistant clinical isolates of *E. coli* showed tolerance to cyclohexane, suggesting an elevated broad-spectrum efflux activity. Furthermore, some of the regulatory factors that may cause the overproduction of AcrAB, the major, constitutively expressed multidrug efflux pump of *E. coli*, were examined: in a 1996 study dealing with 23 fluoroquinolone-resistant *E. coli* isolates (9), 3 were shown to produce MarA, a positive regulator of *acrAB* transcription (1), constitutively at high levels, and 8 were shown to produce MarA to higher levels than the wild type upon “induction” with tetracycline or salicylate; and in a 1999 study of 25 fluoroquinolone-resistant, cyclohexane-tolerant strains (13), 9 were shown to have mutations in *marR*, the repressor for MarA expression, or in *soxR*, the repressor for the expression of SoxS, a close homolog of MarA. All these studies, however, were somewhat indirect in view of our knowledge that the AcrAB pump alone contributes in a decisive manner to the MarA-induced multidrug resistance of *E. coli* (14). Thus, the identity of the efflux pump remained unknown in the direct efflux study (2) or in the solvent resistance study (12), and the examination of *mar* and *sox* systems (9, 13) left open the question of whether other regulatory pathways were altered. We have therefore examined directly the level of expression of AcrA, one of the essential components of the AcrAB-TolC multidrug efflux system (10), by immunoblotting.

The 25 clinical isolates of *E. coli*, randomly chosen from the isolates collected during the period of October 1995 through December 1998, were obtained from the Clinical Microbiology Laboratory of the University of Verona Hospital. Each strain was from a different patient. Their ciprofloxacin susceptibilities were tested by the broth microdilution method in Luria-Bertani broth at 37°C with a standard inoculum of 10^4 cells ml^{-1} . Ciprofloxacin was obtained from Sigma (St. Louis, Mo.). In terms of susceptibility to ciprofloxacin, these isolates fell into two groups: one composed of 15 strains containing susceptible strains and moderately resistant strains (MICs, up to 1.0 $\mu\text{g/ml}$) and the other composed of 10 strains with high levels of ciprofloxacin resistance (MICs, 32 $\mu\text{g/ml}$ or higher) (see Table 1).

We examined the AcrA expression levels of these strains by immunoblotting, as follows. The strains were grown in Luria-Bertani broth at 37°C until the culture attained a density of about 0.2 mg (dry weight) ml^{-1} from a previously constructed standard curve relating the optical density to cell density. These early-exponential-phase cells were used in order to avoid the problem of possible induction of AcrAB in the late exponential and stationary phases (8). The cells were harvested by centrifugation and were resuspended in the same volume of 1% sodium dodecyl sulfate (SDS), followed by heating in a boiling water bath for 10 min. The protein concentrations of the samples were determined by using the bicinchoninic acid assay (Pierce), and the solutions were adjusted to the same protein concentration of 2 $\mu\text{g/ml}$. Identical volumes of the samples were separated by SDS-slab-polyacrylamide gel electrophoresis with a 10% acrylamide gel. The proteins were transferred to a nitrocellulose membrane electrophoretically (4°C, 100 V, 1 h) by using a 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer (pH 11) containing 10% methanol. After blocking overnight with 5% dry milk, the proteins were stained by using anti-AcrA rabbit antibodies (17) as the first antibody (1 h) and horseradish-peroxidase-linked anti-rabbit immunoglobulin G (Amersham Pharmacia) as the second antibody, which was detected with the ECL Western blotting detection reagent (Amersham Pharmacia) and Kodak Biomax MR film developed with a Kodak RPX-OMAT processor. The intensities of the bands obtained were compared by using the analysis software of ImageMaster VDS (Pharmacia Biotech). Preliminary trials with the purified AcrA were run to define the range in which the response was proportional to the amount of

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TABLE 1. Ciprofloxacin susceptibilities, QRDR sequences in topoisomerases, and AcrA expression levels for clinical isolates of *E. coli*

Strain	Source	Ciprofloxacin MIC ^a		QRDR of <i>gyrA</i>	QRDR of <i>parC</i>	AcrA level
		Without inhibitor	With inhibitor			
AG100	Laboratory strain ^b	0.03	0.03	ND ^c	ND	1.0 ^d
AG102	Laboratory strain ^b	0.12	0.06	ND	ND	1.79
EC84	Urine	0.12	ND	ND	ND	0.30
EC85	Ascites	1	0.5	ND	ND	1.2
EC91	Wound fluid	0.03	0.03	ND	ND	1.2
EC92	Urine	0.015	0.015	ND	ND	0.33
EC97	Urine	0.03	ND	ND	ND	0.30
EC100	Urine	0.03	ND	ND	ND	0.45
EC101	Urine	0.03	ND	ND	ND	0.30
EC104	Urine	0.03	ND	ND	ND	1.0
EC105	Urine	0.03	ND	ND	ND	0.86
EC108	Wound fluid	0.06	ND	ND	ND	0.98
EC112	Blood	0.06	ND	ND	ND	1.2
EC120	Urine	0.03	ND	ND	ND	0.96
EC121	Pus	1	1	ND	ND	0.64
EC123	Biopsy specimen	0.06	ND	ND	ND	0.5
EC124	Urine	0.03	ND	ND	ND	0.83
EC44	Urine	32	16	S83L, D87N	E84K	0.95
EC45	Pus	64	32	S83L, D87N	E84K	2.7
EC82	Wound swab	64	16	S83L, D87N	S80R	2.2
EC93	Wound swab	512	512	S83L, D87N	S80I	5.0
EC107	Urine	>512	512	S83L, D87N	S80I	2.0
EC110	Blood	512	512	S83L, D87N	S80I	2.7
EC114	Pus	64	64	S83L, D87N	S80I, E84G	1.7
EC115	Bronchial aspirate	512	512	S83L, D87N	S80I	2.0
EC117	Blood	512	512	S83L, D87N	S80I	1.7
EC118	Blood	512	256	S83L, D87N	S80I	1.8

^a The ciprofloxacin MIC was determined in the absence and presence of 20 µg of the efflux inhibitor MC 207,110 per ml (15).

^b AG100 is a wild-type strain, and AG102 is its *marR1* derivative (3, 14).

^c ND, not determined.

^d The levels of expression of the AcrA protein were determined as described in the text and are expressed relative to that for strain AG100.

the protein applied, and only data within this range were used. In each experiment, AG100 (a laboratory strain showing a normal expression level for the AcrAB system [3, 14]) was included as a standard, and the AcrA levels in various strains were calculated as relative values in comparison with that for AG100. Each strain was usually tested at least twice; the levels found were quite similar; for example, the values for AG102 were 1.76 and 1.81, those for EC44 were 0.94 and 0.96, and those for EC85 were 1.05 and 1.29.

The results (Table 1) showed clearly that in the susceptible and low-level-resistant strains there was no significant overexpression of AcrA (and presumably also of AcrB, which belongs to the same operon), whereas in all except one of the high-level-resistant strains there was significant overexpression of AcrA, at levels $\geq 170\%$ of that found for control strain AG100. The sole exception was EC44, for which the MIC was the lowest among those for this group. Selected strains were examined for the levels of expression of AcrB by a similar immunoblot method with anti-AcrB rabbit antibody raised with the purified AcrB (18). With the AcrB expression level in AG100 taken as 1.0, the two ciprofloxacin-susceptible isolates EC104 and EC108 showed expression levels of 0.94 and 0.86, respectively, whereas the high-level-resistant isolates EC107 and EC110 showed expression levels of 2.3 and 2.7, respectively, in perfect agreement with the expression levels found in these strains for AcrA (Table 1). Considering that MarA or SoxS was overexpressed in only a fraction of a similar, highly resistant group of *E. coli* isolates (9, 13), our results may suggest that other regulatory factors, many of which remain to be identified (8), are involved in the elevated levels of expression of the AcrAB system.

The accumulation of ciprofloxacin was determined by a modification of the method of Piddock and coworkers (2). Thus, the exponential-phase cells were harvested from Luria-Bertani broth by centrifugation, washed and resuspended in 50 mM sodium phosphate buffer (pH 7.0) at an optical density at 600 nm of 1, and incubated with shaking at 37°C. Ciprofloxacin (10 mg/ml) was added, and 1-ml portions were filtered on 0.45-µm-pore-size Millipore filters and washed quickly with 5 ml of 0.1 M LiCl instead of the time-consuming centrifugation steps in the original method. The filter was extracted in 2.5 ml of 0.1 M glycine-HCl buffer (pH 3.0), and the ciprofloxacin was quantitated by fluorescence with a Shimadzu RF 5301 spectrofluorometer. The fluorescence intensity was calibrated by using a 0.01-µg/ml solution of ciprofloxacin in the same buffer. Steady-state accumulation values were calculated by averaging the data for five time points (1, 2, 3, 4, and 5 min); the standard deviation was usually below 10% of the average. In this assay, the two susceptible isolates, isolates EC91 and EC101, accumulated 91 and 109% of the level seen in control strain AG100, respectively. In contrast, all except one of the highly resistant isolates accumulated lower levels of ciprofloxacin than AG100 (average, 72% of the level in AG100), a result confirming that ciprofloxacin was pumped out more effectively in the strains that overexpressed AcrAB. Although EC110, the sole exception, accumulated more ciprofloxacin than AG100, in an earlier series of experiment it accumulated far less, and the reason for the erratic behavior of this strain remains unknown.

The nearly perfect correlation between the high-level resistance to fluoroquinolones and the overexpression of the AcrAB efflux system leads us to the question of whether the

very high levels of resistance always require the contribution of efflux. We know that the overexpression of AcrAB alone in AG102 (AG100 *marR1*) (3), without mutations in topoisomerases, produced only a modest increase in the MIC, to 0.12 µg/ml (Table 1). Fluoroquinolone-resistant isolates of clinical origin do contain an additional mutation(s) in the topoisomerase gene(s). However, Oethinger et al. (11) showed that in a strain containing both a mutation in *marR* and a mutation in the quinolone resistance-determining region (QRDR) of *gyrA*, the ofloxacin MIC for *E. coli* goes up only to 1 µg/ml. Thus, the very high MICs (higher than 32 µg/ml) observed for some strains studied here must involve additional mechanisms, most likely two or more mutations in the target genes (4–7, 16). Indeed, when sequencing of the QRDRs in *gyrA* and *parC* was carried out with our high-level-resistant isolates by the procedure described by Everett et al. (2), all of these strains were found to contain two mutations in *gyrA* and at least one mutation in *parC* (Table 1). The level of resistance achieved by mutations in the target genes alone cannot be assessed from most studies of laboratory-generated quinolone-resistant strains, which involved successive subculturing in drug-containing media (for example, see references 4 and 11), a procedure that is likely to introduce additionally up-regulation mutations in efflux pumps. However, there are a few studies in which the strains were constructed by the methods of bacterial genetics. Thus, Khodursky et al. (6) showed that the combination of *gyrA* (S83L) and *parC* (E84K) mutations (often found among our isolates; see Table 1) produced an apparent norfloxacin MIC of about 25 µg/ml, and Kumagai et al. (7) found that ciprofloxacin MICs for *E. coli* K-12 strains containing a quinolone resistance mutation in *gyrA*, in addition to mutations in the QRDR of *parC* (including ones identical to those observed in this study, such as S80I, S80R, and E84K), were as high as 12.5 µg/ml. These levels of resistance, however, are lower than those found in our isolates with high-level resistance, as exemplified by MICs in the range of 64 to >512 µg/ml (Table 1), suggesting that the very high level ciprofloxacin resistance observed here requires the up-regulation of AcrAB, in addition to multiple changes in the targets.

In order to confirm the participation of the overexpressed AcrAB pump to the high-level resistance, we transduced the Δ *acrAB::kan* mutation into EC114 with phage P1 *vir* from the strain AG100A, using the procedure described earlier (14), selecting for kanamycin resistance. The ciprofloxacin MIC for the resultant transductant, defective in the AcrAB pump, was 8 µg/ml, strongly decreased from the MIC of 64 µg/ml for strain EC114. This approach by use of transduction was not possible with the other high-level-resistant strains because they were already kanamycin resistant. We therefore determined ciprofloxacin MICs in the presence of an efflux inhibitor, MC 207,110 (purchased from Sigma as Phe-Arg-naphthylamide) (15), which is reported to inhibit AcrAB (J. Blais, D. Cho, K. Tangen, C. Ford, A. Lee, O. Lomovskaya, and S. Chamberland, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1266, 1999). There was a clear decrease in the MICs for several strains (Table 1). With other strains, however, the effect of the inhibitor was not apparent. In some

cases, this is possibly because the efflux was caused by systems other than AcrAB or because mutations in other target genes, such as *gyrB* or *parE* (5), contributed to resistance. In any case, our study lends support to the idea that increased production of the AcrAB pump is an important component in the development of high levels of resistance to fluoroquinolones.

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