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In Vivo Increase in Resistance to Ciprofloxacin in *Escherichia coli* Associated with Deletion of the C-Terminal Part of MarR

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We recovered two isolates (EP1 and EP2) of *Escherichia coli* from the same patient that had identical pulsed-field gel electrophoresis patterns but required different MICs of ciprofloxacin (CIP): 16 and 256 mg/liter for EP1 and EP2, respectively. Both isolates had mutations in the quinolone resistance-determining regions of GyrA (Ser83Leu and Asp87Tyr) and ParC (Ser80Ile), but not in those regions of GyrB or ParE. Isolate EP2 was also more resistant to chloramphenicol, tetracyclines, cefuroxime, and organic solvents. A deletion of adenine (A) 1821 was found in *marR* of isolate EP2, which resulted in an 18-amino-acid C-terminal deletion in the MarR protein. The causative relationship between ΔA1821 and the Mar phenotype was demonstrated both by the replacement of the wild-type *marR* by *marR* ΔA1821 in isolate EP1 and by complementation with the wild-type *marR* in *trans* in isolate EP2. In isolate EP2 complemented with wild-type *marR*, susceptibility to chloramphenicol was restored completely, whereas susceptibility to CIP was restored only incompletely. Northern blotting demonstrated increased expression of *marA* and *acrAB* but not of *soxS* in isolate EP2 compared to EP1. In conclusion, the deletion of A1821 in *marR* in the clinical isolate EP2 caused an increase in the MICs of CIP and unrelated antibiotics. Presumably, the C-terminal part of MarR is necessary for proper repressor function.

Resistance mechanisms of *Escherichia coli* against fluoroquinolones (FQ) have been well studied, and three mechanisms have been identified. Point mutations in the quinolone resistance-determining regions (QRDRs) of topoisomerases II (*gyrAB*) and IV (*parCE*) lead to a stepwise acquisition of resistance (7, 9, 12). Active efflux of FQ by multidrug resistance pumps like AcrAB, and reduced uptake due to OmpF, both regulated by the transcription factor MarA, are also implicated in resistance (5, 18). These mechanisms, often in combination, have been found in strains from both in vitro and clinical investigations. However, few data are available about the development of resistance in patients, that is, the order of acquisition of the respective mechanisms and their contributions to the resistance phenotype. We investigated two clinical isolates of *E. coli*, with different levels of resistance to ciprofloxacin (CIP) but identical pulsed-field gel electrophoresis (PFGE) patterns, from one patient. By gene exchange and complementation we demonstrated the role of a C-terminal deletion in MarR resulting in increased efflux of FQ in the more resistant strain.

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MATERIALS AND METHODS

Bacterial strains. *E. coli* isolates EP1 and EP2 were isolated from the vagina and the urine of a patient with generalized follicular lymphoma who had received 750 mg of CIP twice daily for selective decontamination of the gut. *E. coli* ATCC 25922 was obtained from the American Type Culture Collection (ATCC). *E. coli* EP2 *acrA*::Tn*10*-Km was obtained from the clinical isolate EP2 by transposon mutagenesis and screening for susceptibility against FQ. *E. coli* S17 λpir harboring the plasmid pLOF/Km was kindly donated by V. de Lorenzo (6). *E. coli* S17 containing plasmid pBP591 with wild-type *marR* has been described previously (V. Hüllen, P. Heisig, and B. Wiedemann, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. C-64, p. 57, 1997).

PFGE. A rapid PFGE procedure was performed as described previously (8). Macrorestriction was performed with *Xba*I at 37°C for 3 h. Electrophoresis was performed with the contour-clamped homogeneous electric field (CHEF) mapper system (Bio-Rad, Hercules, Calif.). The run time was 14 h, with an initial switch time of 2.16 s and a final switch time of 35 s.

Susceptibility testing. MICs of CIP were determined according to NCCLS recommendations for microdilution assays (16). MICs of chloramphenicol and tetracycline were determined by Etest (AB BIODISK, Solna, Sweden). For testing of organic solvent tolerance (OST), isolates were inoculated on Mueller-Hinton agar at a concentration of 10^5 CFU per spot and the plate was overlaid with hexane (H), cyclohexane (CH), and H-CH mixtures at ratios of 3:1, 1:1, and 1:3 to generate different levels of organic solvent toxicity. *n*-Hexane has a p_{ow} of 3.9, and cyclohexane has a p_{ow} of 3.4 (3). The plates were checked for growth after 2 days.

DNA amplification and nucleotide sequence determination. Primers (Table 1) and PCR conditions for amplification of *gyrA*, *gyrB*, and *parE* were used as previously described (7, 12). Primers and annealing temperatures for the amplification of *parC*, the *mar* operon, *marA* and *marB*, and *acrA* are listed in Table 1. Complementary strands were sequenced in duplicate on a 310 DNA sequencer (Perkin-Elmer, Foster City, Calif.) using either PCR primer (6 μmol).

Plasmids and DNA manipulations. Strains and plasmids used in this study are listed in Table 2. Introduction of the *marR* frameshift mutation from isolate EP2 into the wild-type *marR* of isolate EP1 required subcloning of the *Eco*RI/*Pst*I-digested PCR fragment (with primers marR-f_{1452} and marR-r_{2229}) into the corresponding pUC18 site to yield plasmid pmarR-ΔC18. Probes for Northern blot analysis were generated by subcloning of *marA* and *soxS* into plasmid pcDNA3 (Invitrogen, Groningen, The Netherlands) and in vitro transcription from the SP6 and T7 promoters, respectively. The complete *marA* gene (1893 to 2282) was amplified in a DNA thermocycler using primers marA-f_{1893} and marA-r_{2281}. For the amplification and cloning of *soxS*, primers soxS-f_{331} and soxS-r_{1319} were used. Genomic DNA from *E. coli* ATCC 25922 served as a template. The PCR fragments were cloned directionally into plasmid pcDNA3 to generate plasmids pc-marA and pc-soxS, respectively. Recombinant DNA techniques, transformation, and restriction enzyme digestions followed standard protocols (19).

Gene replacement. Introduction of the *marR* mutation into the wild-type *marR* gene of isolate EP1 was accomplished by homologous recombination between plasmid pmarR-ΔC18 and the bacterial chromosome, essentially as described previously (11). Plasmid pmarR-ΔC18 was introduced into isolate EP1 by electroporation (Genepulser; Bio-Rad). Resulting transformants were grown on Luria-Bertani (LB) agar plates supplemented with 50 mg of ampicillin/liter overnight at 37°C. Recombinant bacteria were then propagated in LB broth

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TABLE 1. Primers used in this study

Target	Primer	Oligonucleotide (5′→3′)[a]	Position	T_{an}[b] (°C)	Remarks
Subunit A topoisomerase IV	parC-f_{58}	TGA ATT TAC GGA AAA CGC CTA C	58–79	60	Sequencing
	parC-r_{591}	GCC ACT TCA CGC AGG TTA TG	591–572	60	Sequencing
marA operon, *marR*	maropR-f_{1394}	TTG CCT GGG CAA TAT TAT C	1394–1411	45	Sequencing
	maropR-r_{1950}	GTC CAA AAT GCT ATG AAT G	1950–1928	45	Sequencing
	marop-f_{1140}	GCCAGGCCAAGAAATAAC	1140–1157	50	Sequencing
	marop-r_{1556}	ATCCAGCGGAGACAGATAC	1556–1538	50	Sequencing
marA, *marB*	marAB-f_{1840}	GGC AAC ACT TGA GTA TTT GC	1840–1859	50	Sequencing
	marAB-r_{2704}	AGT AGG ACT GGC AAG TGC	2704–2687	50	Sequencing
marA	marA-f_{1893}	ATT *AAG CTT* ATG ACG ATG TCC AGA CG	1893–1909	50	*Hin*dIII site
	marA-r_{2281}	ATT *TCT AGA* ACT AGC TGT TGT AAT G	2282–2267	50	*Xba*I site
marR	marR-f_{1452}	AAT *GAA TTC* GTA CCA GCG ATC TGT TCA ATG	1452–1472	50	*Eco*RI site
	marR-r_{2229}	AAT *CTG CAG* TGG TCA TCC GGT ATT TAT GC	2229–2210	50	*Pst*I site
soxS	soxS-f_{331}	ATA *GAA TTC* ACC AAT AAA ATT ACA GGC GG	3331–3350	50	*Eco*RI site
	soxS-r_{1319}	ATA *CTC GAG* ATG TCC CAT CAG AAA ATT ATT CAG	1319–1297	50	*Xho*I site
acrA	acrA-f_{31}	GCG GTC GTT CTG ATG CTC TC	31–48	50	Northern probe
	acrA-r_{330}	ACC TTT CGC ACT GTC GTA TG	330–310	50	Northern probe

[a] Restriction sites are italicized.
[b] T_{an}, annealing temperature.

containing 32 mg of CIP/liter for selection of *E. coli* carrying the desired recombination. Segregation of the plasmid after passaging of single colonies for 1 week was shown by growth inhibition of recombinant clones on LB agar plates containing ampicillin and by inability to amplify the specific *marR* DNA fragment by PCR with plasmid DNA preparations as templates using plasmid-specific primers.

Insertion mutagenesis. Transposon mutagenesis was performed using a suicide vector system as described previously (6, 10). The exconjugates were selected for reduced CIP MICs by transfer of single colonies to agar plates containing 64 or 2 μg of CIP/ml. Colonies growing selectively on plates with 2 μg of CIP/ml were further analyzed.

RNA extraction and Northern blot analysis. Overnight cultures were diluted 100-fold in LB broth and grown with shaking to mid-logarithmic phase at 37°C. Paraquat was added for the induction of the *sox* operon (45 min; final concentration, 1.3 mM; Sigma, Deisenhofen, Germany). For production of the *marA* probe, plasmid pc-marA was linearized by *Hin*dIII digestion and in vitro RNA runoff transcription was performed with the RiboProbe Kit (Promega). Similarly, the *soxS* probe was obtained by *Xho*I digestion of plasmid pc-soxS and in vitro transcription. Digoxigenin (DIG)-labeled RNA probes were purified with the RNeasy Purification Kit (QIAGEN). The *acrA* probe was obtained by PCR. Northern blotting was performed using standard techniques (19).

Complementation assays. For the complementation of the mutant *marR* of isolate EP2, wild-type *marR* under the control of the *bla* promoter was introduced into isolate EP2 by mobilization with the filter mating technique. The donor strain, *E. coli* S17 carrying plasmid pBP591, and isolate EP2 were mixed in a 1:1 ratio and incubated at 37°C for 12 h on a minimal agar plate. Cells were resuspended in LB broth and plated on LB agar plates containing 50 μg of kanamycin/ml for selection.

Data analysis. SPSS 8.0 for Windows was used for calculation of Mann-Whitney U test results.

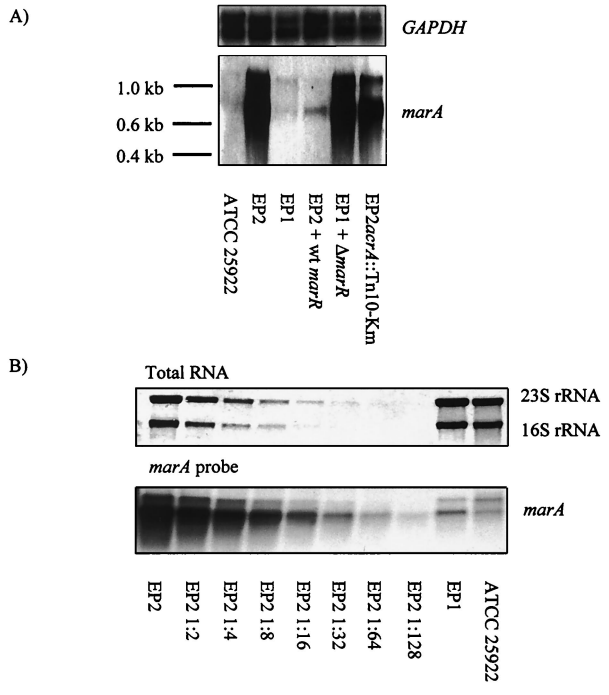

FIG. 1. Northern blot analyses of *marA*. (A) Total RNA of *E. coli* was prepared, transferred to Hybond-N^+ membranes, and probed with DIG-labeled *marR* RNA. The prominent transcripts and Boehringer RNA molecular weight standard III are indicated. (B) A twofold serial dilution of total RNA of *E. coli* isolate EP2 and undiluted RNAs from *E. coli* isolate EP1 and strain ATCC 25922 were transferred to Hybond-N^+ membranes, stained with methylene blue, and probed with DIG-labeled *marR* RNA.

RESULTS AND DISCUSSION

PFGE typing generated identical patterns of *Xba*I-digested total genomic DNA for isolates EP1 and EP2 but different patterns for three epidemiologically unrelated strains used as a

TABLE 2. Bacterial strains and plasmids used in this study

E. coli strain or plasmid	Description	Reference or source
Strains		
ATCC 25922	*E. coli*	ATCC
EP1	MIC of CIP, 16 mg/liter	Clinical isolate
EP2	MIC of CIP, 256 mg/liter	Clinical isolate
EP2 *acrA*::Tn*10*-Km	Derivative of isolate EP2 in which *acrA* was inactivated by transposon mutagenesis	This study
S17 λpir + pLOF/Km	Suicide vector for insertion mutagenesis	10
S17 + p591	Wild-type *marR* in *trans*	Hüllen et al., 37th ICAAC
Plasmids		
pmarR-ΔC18	Derived from pUC18 containing the PCR product of primers marR-f_{1452} and marR-r_{2229}	This study
pc-marA	Derived from pcDNA3 containing the PCR product of primers marA-f_{1893} and marA-r_{2281}	This study
pc-soxS	Derived from pcDNA3 containing the PCR product of primers soxS-f_{331} and soxS-r_{1319}	This study

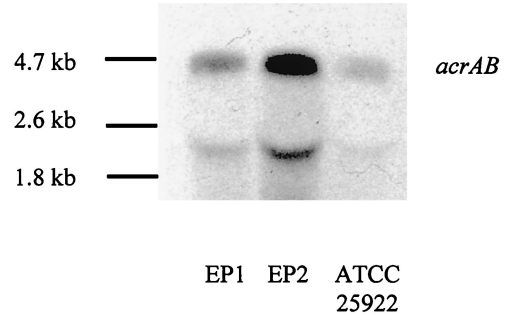

FIG. 2. Northern blot analysis of *acrAB*. Total RNA was prepared, transferred to Hybond-N$^+$ membranes and probed with DIG-labeled *acrA* PCR product. The prominent transcript and Boehringer RNA molecular weight standard II are indicated.

control. Further evidence for the high genetic relatedness of the two isolates was given by the finding of a 61-bp deletion and a 785-bp insertion at the same site in both strains between bp 1252 and 1313 of the published *mar* wild-type sequence (4) and by observation of identical patterns in randomly primed PCR using five different primers (data not shown).

The MICs of CIP for EP1 and EP2 were different, 16 and 256 mg/liter, respectively; however, identical changes in critical residues of the QRDRs of *gyrA* (Ser83Leu, Asp87Tyr) and *parC* (Ser80Ile) were found. This combination of amino acid alterations in critical residues can explain a CIP MIC of 16 mg/liter (12); however, no other substitutions in the QRDR of *gyrB* or *parE* were detected in any strain.

Compared to EP1, EP2 also required higher MICs of chloramphenicol (32 versus 4 mg/liter), tetracycline (32 versus 8 mg/liter), and cefuroxime (>16 versus 4 mg/liter), and EP2 but not EP1 grew on Mueller-Hinton agar overlaid with hexane or a 3:1 hexane-cyclohexane mixture. These properties of EP2 were consistent with a Mar phenotype, and consequently increased expression of the *marA* transcript was demonstrated by Northern blotting (Fig. 1A). Increased expression was demonstrated independently by quantitative PCR using TaqMan technology after a reverse PCR step (data not shown). Compared to wild-type transcription of *marA* in strain ATCC 25922, transcription of *marA* in isolate EP2 was estimated to be 64-fold, as determined by twofold serial dilutions of the target sequence of isolate EP2 (Fig. 1B). Expression of *soxS*, another transcription factor of the *acr* locus (15), with and without stimulation with paraquat, was similar in both isolates (Northern blot, data not shown). The increased transcription of *marA* in isolate EP2 led to increased expression of *acrA*, as could be expected (1). In the *acrA* Northern blot, two transcripts of approximately 4.5 and 2.2 kb were observed (Fig. 2). The size of the larger transcript corresponds to the expected length of the *acrAB* transcript of 4.344 kb, and this transcript is also dominant. Sequencing of the *mar* operons of isolates EP1 and EP2 indicated a deletion of adenine 1821 of *marR* in EP2. This deletion resulted in a frameshift and a concomitant loss of 18 amino acids in the C-terminal region of the MarR protein.

To investigate the possible role of the truncation of MarR, gene exchange and complementation experiments were designed. When we introduced the defective *marR* into the chromosomal DNA of isolate EP1 by homologous recombination, the MIC of CIP rose to 64 to 128 mg/liter, suggesting diminished repressor activity of the truncated MarR. Likewise, in isolate EP2, in *trans* complementation with the wild-type *marR* resulted in a lower CIP MIC of 64 mg/liter. The putative role of the frameshift in *marR* affecting the regulation of transcription of the AcrAB efflux pump was corroborated by the knockout mutant EP2 *acrA*::Tn*10*-Km, for which the MIC of CIP fell back to 32 mg/liter. The characteristics of EP1, EP2, and their derivatives are summarized in Table 3.

Neither the introduction of the wild-type *marR* into isolate EP2 nor the introduction of the defective *marR* into isolate EP1 resulted in a complete reversal of CIP MICs, indicating an additional, yet unknown resistance mechanism(s). The presence of additional resistance mechanisms is also suggested by the author of a recent study of *E. coli* with GyrA and MarA mutations generated in vitro, in which no clinically relevant resistance to CIP was detectable in *acrAB* knockout mutants (17). Efflux pump inhibitors, which restored susceptibility to FQ in the presence of target mutations (13), may not be effective in the *E. coli* clinical isolate EP2 analyzed in this study.

The multiple antibiotic resistance locus (*mar*) of *E. coli* controls intrinsic susceptibility to multiple antibiotics, organic solvents, oxidative stress agents, and the disinfectant triclosan (for reviews see references 1, 2, and 14). Presumably, the N-terminal and central regions of MarR, where a helix-turn-helix motif has been identified, are responsible for the specific interactions with the two binding sites in *marO* (1). The finding of this study indicates that the C terminus of MarR is also necessary for proper repressor function.

In conclusion, using genetic exchange and complementation techniques in an otherwise genetically indistinguishable pair of clinical isolates of *E. coli*, we have identified a unique C-terminal deletion in MarR resulting in a Mar phenotype affecting the MICs of FQ, tetracycline, chloramphenicol, and cefuroxime, as well as OST. Changes in the target enzyme and active efflux both add to the resistance phenotype. This is yet another example of the versatility of bacterial acquisition of antimicrobial resistance.

TABLE 3. Characteristics of *E. coli* ATCC 25922, EP1, EP2, EP2 *acrA*::Tn*10*-Km, EP1 Δ*marR*, and EP2 complemented with wild-type *marR*

E. coli strain[a]	MIC (mg/liter)[b]					OST				*mar* sequence	Transcription of:	
	CIP	Tetracycline	Chloramphenicol	Cefazolin	Cefuroxime	H	H-CH, 3:1	H-CH, 1:1	CH		*marA*	*acrA*
ATCC 25922	0.006	3	3	ND	ND	−	−	−	−	wt	+	+
EP1	16	>256 (8)[c]	4	<2	4	−	−	−	−	wt	++	+
EP2	256	>256 (32)[c]	32	4	>16	+	+	−	−	ΔA1821	++++	++
EP2 *acrA*::Tn*10*-Km	32	ND	0.75	<2	<2	−	−	−	−	ΔA1821	++++	ND
EP1 Δ*marR*	64–128	ND	32	ND	ND	ND	ND	ND	ND	ΔA1821	++++	ND
EP2 + wt *marR*	64	ND	4	ND	ND	ND	ND	ND	ND	wt	++	ND

[a] wt, wild type.
[b] ND, not determined.
[c] Two subpopulations.

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REFERENCES

1. **Alekshun, M. N., and S. B. Levy.** 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. Antimicrob. Agents Chemother. **41:**2067–2075.
2. **Alekshun, M. N., and S. B. Levy.** 1999. The *mar* regulon: multiple resistance to antibiotics and other toxic chemicals. Trends Microbiol. **7:**410–413.
3. **Aono, R.** 1998. Improvement of organic solvent tolerance level of *Escherichia coli* by overexpression of stress-responsive genes. Extremophiles **2:**239–248.
4. **Cohen, S. P., H. Hachler, and S. B. Levy.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175:**1484–1492.
5. **Cohen, S. P., L. M. McMurry, D. C. Hooper, J. S. Wolfson, and S. B. Levy.** 1989. Cross-resistance to fluoroquinolones 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.
6. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. Methods Enzymol. **235:**386–405.
7. **Everett, M. J., Y. F. Jin, V. Ricci, and L. J. Piddock.** 1996. Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. Antimicrob. Agents Chemother. **40:**2380–2386.
8. **Gautom, R. K.** 1997. Rapid pulsed-field gel electrophoresis protocol for typing of *Escherichia coli* O157:H7 and other gram-negative organisms in 1 day. J. Clin. Microbiol. **35:**2977–2980.
9. **Heisig, P.** 1996. Genetic evidence for a role of *parC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. Antimicrob. Agents Chemother. **40:**879–885.
10. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
11. **Kiel, J. A., J. P. Vossen, and G. Venema.** 1987. A general method for the construction of *Escherichia coli* mutants by homologous recombination and plasmid segregation. Mol. Gen. Genet. **207:**294–301.
12. **Lehn, N., J. Stoewer-Hoffmann, T. Kott, C. Strassner, H. Wagner, and W. Schneider-Brachert.** 1996. Characterization of clinical isolates of *Escherichia coli* showing high levels of fluoroquinolone resistance. J. Clin. Microbiol. **34:**597–602.
13. **Lomovskaya, O., A. Lee, K. Hoshino, H. Ishida, A. Mistry, M. S. Warren, E. Boyer, S. Chamberland, and V. J. Lee.** 1999. Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**1340–1346.
14. **McMurry, L. M., M. Oethinger, and S. B. Levy.** 1998. Overexpression of *marA*, *soxS*, or *acrAB* produces resistance to triclosan in laboratory and clinical strains of *Escherichia coli*. FEMS Microbiol. Lett. **166:**305–309.
15. **Miller, P. F., and M. C. Sulavik.** 1996. Overlaps and parallels in the regulation of intrinsic multiple-antibiotic resistance in *Escherichia coli*. Mol. Microbiol. **21:**441–448.
16. **National Committee for Clinical Laboratory Standards.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed. Approved standard. NCCLS document M7-A4. National Committee for Clinical Laboratory Standards, Wayne, Pa.
17. **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.
18. **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.
19. **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.