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O'Regan, E., Quinn, T., Pagès, J-M., McCusker, M., Piddock, L., & Fanning, S. (2009). Multiple Regulatory Pathways Associated with High-Level Ciprofloxacin and Multidrug Resistance in Salmonella enterica Serovar Enteritidis: Involvement of ramA and Other Global Regulators. DOI: 10.1128/AAC.01005-08

Published in:
Antimicrobial Agents and Chemotherapy

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
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***Antimicrob. Agents Chemother.* 2009, 53(3):1080. DOI: 10.1128/AAC.01005-08.**
Published Ahead of Print 22 December 2008.

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2009, p. 1080–1087 Vol. 53, No. 3
0066-4804/09/$08.00+0 doi:10.1128/AAC.01005-08

Multiple Regulatory Pathways Associated with High-Level Ciprofloxacin and Multidrug Resistance in *Salmonella enterica* Serovar Enteritidis: Involvement of *ramA* and Other Global Regulators▽

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Received 27 July 2008/Returned for modification 9 October 2008/Accepted 12 December 2008

Mechanisms of antibiotic resistance were examined in nalidixic acid-resistant *Salmonella enterica* serovar Enteritidis field isolates displaying decreased susceptibility to ciprofloxacin and in in vitro-derived ciprofloxacin-resistant mutants (104-cip and 5408-cip). All field isolates harbored a single *gyrA* mutation (D87Y). Deletion of *acrB* and complementation with wild-type *gyrA* increased quinolone susceptibility. Selection for ciprofloxacin resistance was associated with the development of an additional *gyrA* (S83F) mutation in 104-cip, novel *gyrB* (E466D) and *parE* (V461G) mutations in 5408-cip, overexpression of *acrB* and decreased susceptibility to nonquinolone antibiotics in both mutants, and decreased OmpF production and altered lipopolysaccharide in 104-cip. Complementation of mutated *gyrA* and *gyrB* with wild-type alleles restored susceptibility to quinolones in 104-cip and significantly decreased the ciprofloxacin MIC in 5408-cip. Complementation of *parE* had no effect on quinolone MICs. Deletion of *acrB* restored susceptibility to ciprofloxacin and other antibiotics tested. Both *soxS* and *marA* were overexpressed in 104-cip, and *ramA* was overexpressed in 5408-cip. Inactivation of each of these global regulators lowered ciprofloxacin MICs, decreased expression of *acrB*, and restored susceptibility to other antibiotics. Mutations were found in *soxR* (R20H) and in *soxS* (E52K) in 104-cip and in *ramR* (G25A) in 5408-cip. In conclusion, both efflux activity and a single *gyrA* mutation contribute to nalidixic acid resistance and reduced ciprofloxacin sensitivity. Ciprofloxacin resistance and decreased susceptibility to multiple antibiotics can result from different genetic events leading to development of target gene mutations, increased efflux activity resulting from differential expression of global regulators associated with mutations in their regulatory genes, and possible altered membrane permeability.

Salmonella enterica serovar Enteritidis is the most common etiological agent of food-borne salmonellosis worldwide. Ciprofloxacin is the antibiotic of choice for the treatment of severe *Salmonella* infections when therapeutic intervention is warranted. To date, fluoroquinolone resistance (MIC of ciprofloxacin, ≥4 μg/ml) remains relatively uncommon in *Salmonella*. However, the incidence of nalidixic acid resistance in *Salmonella* isolates from humans and food animals has increased, with these isolates showing decreased susceptibility to fluoroquinolones (6, 27). Of paramount concern to public health are reports of therapeutic failure of ciprofloxacin in cases of invasive salmonellosis associated with isolates displaying reduced fluoroquinolone susceptibility (26, 30, 48).

Currently well-recognized mechanisms of quinolone resistance in *Salmonella* include target gene mutations, increased efflux pump activity, and plasmid-mediated protection of target topoisomerases (13, 18). The contribution of changes in the cell envelope, including porin loss or alterations of the lipopolysaccharide (LPS), to quinolone resistance is currently unclear (14, 29, 36). Nalidixic acid resistance and decreased susceptibility to ciprofloxacin have been largely associated with single *gyrA* mutations at codon S83 or D87 (7, 18, 36). Double mutations at both residues 83 and 87 have been found in fluoroquinolone-resistant strains, often in association with mutations in other topoisomerase genes (3, 4, 7). Overexpression of the multidrug efflux pump AcrAB-TolC has been shown to directly contribute to fluoroquinolone and multidrug resistance (MDR) in *Salmonella* (3–5).

Much of our knowledge on the regulation of expression of AcrAB comes from work carried out in *Escherichia coli* (34). At a local level *acrAB* expression is modulated by the local repressor AcrR, and at a global level it is modulated by MarA, SoxS, and Rob, which belong to the AraC/XylS family of transcriptional regulators (2). In addition to activating *tolC* and *acrAB* genes, these transcriptional activators activate transcription of *micF*, an antisense RNA that inhibits synthesis of the OmpF outer membrane porin (34). Mutations within the local repressor AcrR have also been shown to contribute to *acrB* overexpression (33). The *mar* locus consists of two transcription units, *marC* and *marRAB*, which are divergently transcribed from a central putative operator-promoter region, *marO* (10, 45). MarA regulates its own transcription as well as regulating the expression of the *mar* regulon, whereas MarR

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▽ Published ahead of print on 22 December 2008.

TABLE 1. Phenotypic and genotypic characteristics of the serovar Enteritidis strains used in this study

Strain	MIC (μg/ml) of drug[a,b]:		Amino acid substitution(s)[c,d]			Fold change in expression of gene[e]:				
	NAL	CIP	*gyrA*	*gyrB*	*parE*	*acrB*	*soxS*	*marA*	*ramA*	*rob*
CUH48	6 (0.5)	0.016 (0.012)	*	*	*					
CUH52	3 (0.75)	0.016 (0.008)	*	*	*					
NCTC 13349	8 (0.38)	0.023 (0.008)	*	*	*					
54	>256 (16)	0.19 (0.064)	D87Y	*	*					
58	>256 (24)	0.125 (0.064)	D87Y	*	*					
51	>256 (24)	0.19 (0.094)	D87Y	*	*					
51 *acrB*::*aph*	32	0.032	D87Y	*	*					
CUH60	>256 (32)	0.19 (0.094)	D87Y	*	*					
CUH60 *acrB*::*aph*	24	0.032	D87Y	*	*					
4931	>256 (16)	0.19 (0.064)	D87Y	*	*					
4931 *acrB*::*aph*	32	0.047	D87Y	*	*					
104	>256 (24)	0.19 (0.094)	D87Y	*	*					
104 *gyrA*$^+$	4	0.047	*	*	*					
104 *acrB*::*aph*	32	0.032	D87Y	*	*					
104-cip	>256 (>256)	>32 (2)	D87Y, S83F	*	*	6.1 ± 1.5	26.1 ± 4.0	8.9 ± 0.6	1.2 ± 0.2	−4.6 ± 0.2
104-cip *gyrA*$^+$	8	0.094	*	*	*					
104-cip *acrB*::*aph*	>256	0.38	D87Y, S83F	*	*					
104-cip *soxS*::*aph*	>256	1.5	D87Y, S83F	*	*	−4.2 ± 1.0		−0.6 ± 1.6		
104-cip *marA*::*aph*	>256	4	D87Y, S83F	*	*	1.4 ± 0.3	1.4 ± 0.2			
5408	>256 (24)	0.19 (0.094)	D87Y	*	*					
5408 *gyrA*$^+$	4	0.032	*	*	*					
5408 *acrB*::*aph*	32	0.047	D87Y	*	*					
5408-cip	>256 (16)	>32 (2)	D87Y	E466D	V461G	5.4 ± 1.6	−3.4 ± 0.5	1.3 ± 0.2	33.7 ± 4.0	−2.3 ± 1.0
5408-cip *gyrA*$^+$	>256	1	*	E466D	V461G					
5408-cip *gyrB*$^+$	>256	3	D87Y	*	V461G					
5408-cip *parE*$^+$	>256	>32	D87Y	E466D	*					
5408-cip *acrB*::*aph*	>256	0.5	D87Y	E466D	V461G					
5408-cip *ramA*::*aph*	>256	4	D87Y	E466D	V461G	1.6 ± 0.1				

[a] NAL, nalidixic acid; CIP, ciprofloxacin.
[b] Values in parentheses are the MICs determined in the presence of PAβN at 80 μg/ml.
[c] *, wild-type allele (no mutation).
[d] D, aspartic acid; Y, tyrosine; S, serine; F, phenylalanine; E, glutamic acid; V, valine; G, glycine.
[e] Gene expression data represent the means ± standard deviations of three independent total RNA extractions. Changes in gene expression are relative to the parental strain.

acts by repressing *marRAB* transcription. The functions of MarB and MarC are unknown. *marRAB* transcription can also be activated by the MarA homologs SoxS and Rob (2, 10, 45). SoxS is the effector of the *soxRS* global superoxide response regulon. SoxR is a constitutively expressed homodimeric transcriptional regulator that contains redox-active iron-sulfur clusters [2Fe-2S]. Oxidation of these clusters activates SoxR to trigger transcription of the *soxS* gene (22, 50). Increased expression of these global regulators may be associated with mutations in the regulatory genes of the operons (20, 21, 32) or the selective binding of inducers (38, 39). As in *E. coli*, increased expression of *marA* and *soxS* has been associated with fluoroquinolone resistance and MDR in *Salmonella* (7, 12). However, the contribution of these global regulators to overexpression of *acrAB* in fluoroquinolone resistance and MDR *Salmonella* phenotypes is still currently unclear. Furthermore, little is known about *rob* and its contribution to antibiotic resistance in *Salmonella*. More recently, RamA, which displays close homology to MarA and is absent from *E. coli*, has been implicated in MDR in *Salmonella* and other bacteria (1, 16, 19, 42). Overexpression of *ramA* has been associated with increased expression of *acrB* in *Salmonella* and other *Enterobacteriaceae* (1, 19, 42).

In this study we examined the mechanisms of quinolone resistance in nalidixic acid-resistant serovar Enteritidis field isolates showing decreased susceptibility to ciprofloxacin. We generated in vitro ciprofloxacin-resistant mutants from two of these isolates in order to assess the contribution of target gene mutations, altered membrane permeability, and active efflux to the development of fluoroquinolone resistance and MDR. The role of the global regulators *marA*, *soxS*, *rob*, and *ramA* in upregulation of *acrB* and consequently in efflux-mediated MDR was investigated. Local and global regulatory genes of AcrAB-TolC were examined for the presence of mutations.

MATERIALS AND METHODS

Bacterial strains. Nine serovar Enteritidis strains of animal (54, 58, 51, and 104) and human (CUH48, CUH52, CUH60, 4931, and 5408) origin were used in this study (Table 1). The serovar Enteritidis reference strain PT4 NCTC 13349 was also included. Ciprofloxacin-resistant mutants (104-cip and 5408-cip) were selected from serovar Enteritidis strains 104 and 5408. *Salmonella enterica* serovar Typhimurium knockout strains L110 *acrB*::*aph*, L130 *marA*::*aph*, L133 *ramA*::*aph*, and L135 *soxS*::*aph* derived from SL1344 (12, 41) and donor *E. coli* strains carrying the plasmids pBP513 *gyrA*$^+$, pBP548 *gyrB*$^+$, and pBP568 *parE*$^+$ were also used and kindly provided to L. Piddock by P. Heisig (15).

Selection of ciprofloxacin-resistant *Salmonella* mutants in vitro. Ciprofloxacin-resistant mutants (104-cip and 5408-cip) were obtained by seven serial passages on tryptone soy agar (Oxoid, Hampshire, United Kingdom) containing doubling concentrations of ciprofloxacin (0.25 to 16 μg/ml; Sigma-Aldrich, Ireland). Colonies from the highest-concentration selecting plates were subcultured five times on antibiotic-free medium before antibiotic sensitivities were determined. Mutants were stored on beads in cryopreservation fluid at −80°C (Technical Service Consultants Ltd., Lancashire, England).

Antimicrobial susceptibility testing. MICs of nalidixic acid, ciprofloxacin, ampicillin, chloramphenicol, tetracycline, and sulfamethoxazole-trimethoprim were determined by Etest on Mueller-Hinton agar following the manufacturer's instructions (AB-Biodisk, Solna, Sweden) and according to the Clinical and Laboratory Standards Institute (CLSI) (9). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control organisms. To assess the contribution of efflux pump activity to intrinsic and acquired antibiotic resistance, Etests were performed in the presence and absence of the efflux pump

TABLE 2. Primers used in this study

Primer purpose and gene	Primer sequence (5′–3′)	Annealing temp (°C)	Amplicon size (bp)	Reference or accession no.
QRDR				
gyrA	TGTCCGAGATGGCCTGAAGC CGTTAATCACTTCCGTCAG	55	470	Modified from reference 6
gyrB	GAAATGACCCGTCGTAAAGG TACAGTCTGCTCATCAGAAAG	54	710	AE008878
parC	ATGAGCGATATGGCAGAGCG TGACCGAGTTCGCTTAACAG	52	413	6
parE	GACCGAGCTGTTCCTTGTGG GCGTAACTGCATCGGGTTCA	52	493	6
RT-PCR				
16S rRNA	GCGGCAGGCCTAACACAT GCAAGAGGCCCGAACGTC	59/60	182	X80681
acrB	TTTTGCAGGGCGCGGTCAGAATAC TGCGGTGCCCAGCTCAACGAT	59	184	This study
soxS	AAATCGGGCTACTCCAAGTG CTACAGGCGGTGACGGTAAT	59	217	9
marA	ATCCGCAGCCGTAAAATGAC TGGTTCAGCGGCAGCATATA	59	180	9
rob	CATTACGGCTGGGCGAGTTTACC CTGGCGGAATAGTTGGCGAATGAC	60	180	This study
ramA	CGTCATGCGGGGTATTCCAAGTG CGCGCCGCCAGTTTTAGC	60	107	This study
Regulation				
acrR	CAGTGGTTCCGTTTTTAGTG ACAGAATAGCGACACAGAAA	58	992	40
soxRS	CGAACAGGGCGTCGTCGCTT CTGGTTGCTAAAACGCGGCG	60	1,199	40
marORAB	ACGGTGGTTAGCGGATTGGC AGCGGCGGACTTGTCATAGC	58	1,329	40
ramA	TTGCCGCTTCCAGTAATGCTTGTT CTTTATCTGGCGGCGCTGGTTTTC	60	724	NC_003197
ramR	CGTGTCGATAACCTGAGCGG AAGGCAGTTCCAGCGCAAAG	60	934	1
Gene knockout verification				
acrB	GGATCACACCTTATTGCCAG CGGCCTTATCAACAGTGAGC	52	3,541[a]/2,107[b]	15
marA	GCGGACTTGTCATAGCCAGA GCTGGATATCACCGCAACAC	52	1,037[a]/1,818[b]	49
soxS	TACCGGCTATTCGAACTTGC CTCGCTTAACGTATGTCCTT	50	989[a]/1,912[b]	49
ramA	CCGCTTCCAGTAATGCTTGT GAATCATTGATGACCGCTGC	50	918[a]/1,755[b]	49

[a] Without cassette.
[b] With cassette.

inhibitor Phe-Arg-β-naphthylamide (PAβN [80 μg/ml]; Sigma-Aldrich). MICs of PAβN were >640 μg/ml in all isolates.

PCR amplification and sequencing of QRDRs of quinolone target genes and local and global regulators of AcrAB-TolC. Sequences of the primers used in the PCR amplifications are given in Table 2. Genomic DNA was extracted from overnight cultures in tryptone soy broth (Oxoid) at 37°C using a Wizard genomic DNA purification kit (Promega, Madison, WI). PCR mixtures contained 100 ng of template DNA, 100 pmol of each quinolone resistance-determining region (QRDR) primer or 10 pmol of each regulator primer (MWG-Biotech AG, Ebersberg, Germany), 200 μM deoxynucleoside triphosphates (Promega), 1 U *Taq* DNA polymerase (New England Biolabs, Ipswich, MA), and 1× PCR buffer containing 2.5 mM $MgCl_2$. After an initial denaturation step of 3 min at 94°C, amplification was performed over 30 cycles, with each cycle consisting of 1 min at 94°C, 1 min at appropriate annealing temperature (Table 2), and 1 min at 72°C, with a final extension step of 10 min at 72°C. PCR products were purified with the Qiaquick spin PCR purification kit (Qiagen, West Sussex, United Kingdom) and sequenced commercially (Qiagen, Hilden, Germany).

Sequence analysis was carried out online using the programs BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and Transeq (http://www.ebi.ac.uk/emboss/transeq/).

Accumulation of ciprofloxacin. Ciprofloxacin accumulation was measured by a fluorometric method described by Mortimer and Piddock (31). Accumulation experiments were performed with and without the addition of efflux pump inhibitor PAβN (final concentration, 80 μg/ml) 6 min after the addition of ciprofloxacin (final concentration, 10 μg/ml). Fluorescence was measured with a spectrofluorimeter (spectraMax Gemini; Molecular Devices, Sunnyvale, CA) at excitation and emission wavelengths of 279 and 447 nm, respectively. The amount of ciprofloxacin accumulated was calculated by comparison with a standard curve for ciprofloxacin (0.02 to 2.5 μg/ml) in 0.1 M glycine hydrochloride (pH 3.0). Results are expressed as nanograms of ciprofloxacin incorporated per milligram (dry weight) of bacteria. All experiments were performed at least three times to ensure reproducibility.

Expression analyses of efflux transporter gene *acrB* and global regulators. Reverse transcription-PCR (RT-PCR) was used to assess gene expression of *acrB* and global regulators *soxS*, *marA*, *ramA*, and *rob*. Overnight cultures were diluted 1 in 100 in prewarmed LB broth and grown to mid-logarithmic phase

(optical density at 600 nm [OD_{600}], 0.6) with shaking at 37°C. A 1-ml aliquot of each culture was pelleted by centrifugation at 15,339 × *g* for 10 min, and RNA was extracted immediately using a RiboPure-Yeast (Ambion, Texas) kit. Contaminating genomic DNA was eliminated by two DNase I treatments according to the manufacturer's instructions (Ambion), and its absence was confirmed by including a reverse transcriptase-minus control on each RNA sample. Total RNA concentration was estimated by OD_{260} using a Nanodrop ND-1000 spectrophotometer (Thermoscientific, Delaware). Real-time quantification of RNA templates by real-time One-Step RT-PCR was performed in a Rotor Gene 3000 thermocycler (Corbett Research, Sydney, Australia) using a QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). The RT-PCR was carried out in a 25-μl reaction mixture containing 12.5 μl 2× QuantiTect SYBR Green RT-PCR Master Mix, 10 pmol of each primer (Table 2), 5 ng of purified RNA template, and 0.25 μl of QuantiTect RT mix. Initial reverse transcription at 50°C for 30 min was followed by a denaturation step of 15 min at 95°C. Amplification was then performed over 35 cycles, with each cycle consisting of 15 s at 94°C, 30 s at appropriate annealing temperature (Table 2), and 30 s at 72°C, with a final melting step. Within bacterial cells, the level of 16S rRNA was assumed to be transcribed at a constant rate throughout the growth conditions in this study. Relative gene expression was calculated using the threshold cycle method (24).

Phenotype microarray. Ciprofloxacin-sensitive parents and ciprofloxacin-resistant mutants were examined for cellular phenotypes using Omnilog phenotype microarrays (PM11-20) (Biolog, Inc., Hayward, CA). Briefly, bacteria were grown on blood agar overnight at 37°C. Colonies were picked with a sterile cotton swab and suspended in 10 ml IF-0a (Biolog), and the cell density was adjusted to an OD_{600} of 0.035 on a spectrophotometer (Biomate 5; Thermospectronic, Cambridge, United Kingdom). A 750-μl aliquot of this cell suspension was added to 150 ml IF-10 (Biolog). Microtiter plates were inoculated with 100 μl of cell suspension per well, incubated at 37°C for 48 h in the Omnilog, and monitored continuously for color changes in the wells. Kinetic data were analyzed with Omnilog PM software.

Complementation. The relevance of target gene mutations in 104-cip and 5408-cip was evaluated by complementation assays. Plasmids containing the wild-type alleles were introduced into the mutant cells by electrotransformation, and transformants were selected on LB agar supplemented with 50 μg/ml kanamycin and incubated at 37°C overnight. Quinolone MICs of transformants were compared to those of parent and mutant strains using Etest strips.

P22 transduction. Gene deletions (*acrB*, *marA*, *soxS*, and *ramA*) from SL1344 mutants were transduced into bacterial isolates by using P22 phage according to standard procedures. The resulting deletional mutants were selected on LB agar containing 50 μg/ml kanamycin, and insertion of the kanamycin resistance gene into mutants was confirmed by PCR using primers listed in Table 2.

Analysis of LPS: cell lysis and proteinase K digestion. LPS was isolated by proteinase K treatment of bacterial cells as described by Hitchcock and Brown (17). LPS preparations were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with a 4% (wt/vol) stacking gel and a 15% (wt/vol) separating gel. LPS from *Salmonella enterica* serotype Minnesota (Sigma-Aldrich) was used as a smooth LPS control. A full-range rainbow molecular weight marker was used as the size standard (Amersham, England). Following electrophoresis, the LPS was visualized by silver staining as previously described by Tsai and Frasch (47).

SDS-polyacrylamide gel electrophoresis analyses and immunocharacterization. Exponential-phase bacteria in LB broth were pelleted and solubilized in boiling buffer at 96°C as previously described (25). Equal amounts of total cell protein (OD_{600} = 0.01) were loaded onto an SDS-polyacrylamide gel (10% polyacrylamide, 0.1% SDS). Gels were stained with Coomassie brilliant blue R-250 (0.25% [wt/vol]). For Western blots, proteins were electrotransferred onto nitrocellulose membranes in transfer buffer (25). An initial saturating step was performed overnight at 4°C with Tris-buffered sodium (TBS; 50 mM Tris-HCl [pH 8.0], 150 mM NaCl) containing skim milk powder (10%). The nitrocellulose sheets were then incubated in TBS containing skim milk powder (10%) and Triton X-100 (0.2%) for 2 h at room temperature in the presence of polyclonal antibodies (1:2,000 dilution) directed against denatured OmpF porin or with F4 polyclonal antibody directed against the L3 internal loop of *E. coli* porins (11). These antibodies directed against denatured OmpF and the L3 internal porin loop recognized the denatured enterobacterial porins including *Salmonella* F and D porins (44). The detection of antigen-antibody complexes was performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit immunoglobulin G antibodies as previously reported (25).

TABLE 3. Antimicrobial susceptibilities of wild-type serovar Enteritidis strains, their isogenic ciprofloxacin-resistant mutants, and deletional mutants

Strain	MIC (μg/ml) of drug[a]:			
	TC	TS	AM	CL
104	1 (0.19)	0.094 (0.023)	1 (0.75)	2 (0.75)
104-cip	12 (3)	0.19 (0.023)	16 (1)	96 (1)
104-cip *acrB*::*aph*	0.25	0.008	0.125	0.75
104-cip *marA*::*aph*	1.5	0.064	1.5	3
104-cip *soxS*::*aph*	0.75	0.016	0.5	1.5
5408	1 (0.19)	0.064 (0.023)	1 (1)	3 (0.5)
5408-cip	1.5 (0.094)	0.25 (0.032)	4 (2)	16 (0.38)
5408-cip *acrB*::*aph*	0.094	0.023	0.094	0.5
5408-cip *ramA*::*aph*	1	0.064	1	3

[a] AM, ampicillin; CL, chloramphenicol; TC, tetracycline; TS, trimethoprim-sulfamethoxazole. Values in parentheses are disk diffusion results in the presence of efflux pump inhibitor PAβN at 80 μg/ml. Values represent means of three separate determinations.

RESULTS

Antimicrobial susceptibility. Two field isolates (CUH48 and CUH52) and the reference strain (NCTC 13349) were fully susceptible to nalidixic acid (MICs, ≤8 μg/ml) and ciprofloxacin (MICs, ≤0.023 μg/ml). Seven strains showing high-level nalidixic acid resistance (MIC, ≥256 μg/ml) displayed decreased susceptibility to ciprofloxacin (MICs between 0.125 and 0.19 μg/ml). The ciprofloxacin-selected mutants, 104-cip and 5408-cip, displayed high-level quinolone resistance (nalidixic acid MICs, ≥256 μg/ml; ciprofloxacin MICs, ≥32 μg/ml). Additionally, 104-cip showed decreased susceptibility to tetracycline, ampicillin, and chloramphenicol and 5408-cip showed decreased susceptibility to sulfamethoxazole-trimethoprim, ampicillin, and chloramphenicol (Table 3). Phenotype microarray analysis showed that 104-cip tested resistant to 36 antimicrobials including beta-lactams, narrow- and expanded-spectrum cephalosporins, chloramphenicol, tetracycline, fungicides, and biocides. Similarly, 5408-cip tested resistant to 23 antimicrobials, including beta-lactams, narrow- and expanded-spectrum cephalosporins, chloramphenicol, folate synthesis inhibitors, macrolides, aminoglycosides, and chelators (data not shown).

Contribution of target gene mutations to quinolone resistance. Fully quinolone-susceptible strains had no mutations in the target genes (Table 1). A single *gyrA* mutation (D87Y) was present in all strains showing high-level nalidixic acid resistance. Complementation of *gyrA* in both 104 and 5408 reduced nalidixic acid MICs to 4 μg/ml in both isolates and decreased ciprofloxacin MICs to 0.047 μg/ml and 0.032 μg/ml, respectively. In 104-cip an additional mutation in *gyrA* (S83F) was associated with the development of high-level ciprofloxacin resistance. In 5408-cip novel mutations were detected in *gyrB* (E466D) and *parE* (V461G) (Table 1). Complementation of *gyrA* in 104-cip (S83F, D87Y) restored susceptibility to nalidixic acid (MIC, 8 μg/ml) and ciprofloxacin (MIC, 0.094 μg/ml). Complementation of *gyrA* and *gyrB* in 5408-cip reduced ciprofloxacin MICs to 1 and 3 μg/ml, respectively. Complementation of *parE* in 5408-cip had no effect on quinolone MICs (Table 1).

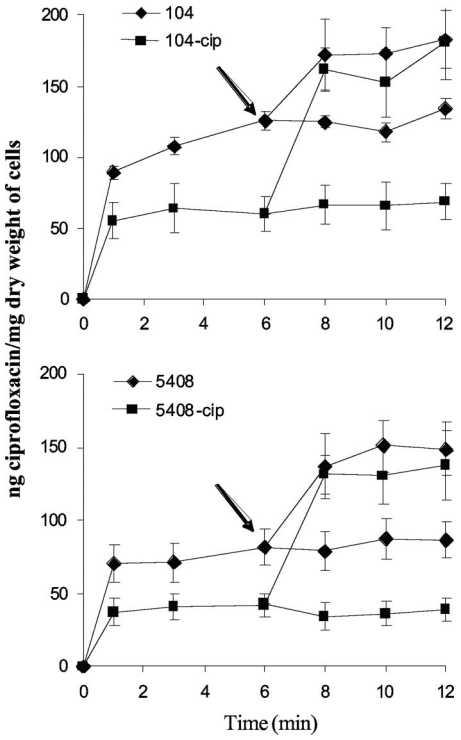

FIG. 1. Accumulation of ciprofloxacin in in vitro-selected ciprofloxacin-resistant serovar Enteritidis isolates (104-cip and 5408-cip) and their isogenic parent strains (104 and 5408) in the presence and absence of PAβN (80 μg/ml). Ciprofloxacin (10 μg/ml) was added to each bacterial suspension at time zero. PAβN was added at 6 min as indicated by the arrow. Each value represents the mean ± standard error of the mean of three or four separate experiments.

Contribution of efflux pump activity to antibiotic resistance. The contribution of efflux pump activity to antibiotic resistance was assessed by use of the efflux pump inhibitor PAβN. PAβN decreased the MIC of nalidixic acid and ciprofloxacin in all wild-type strains (Table 1). It decreased the MIC of ciprofloxacin in 104-cip to 2 μg/ml but had no effect on the MIC of nalidixic acid. The MICs of both nalidixic acid and ciprofloxacin in 5408-cip were reduced to 16 μg/ml and 2 μg/ml, respectively, in the presence of PAβN (Table 1). The susceptibility of ciprofloxacin-resistant mutants to other antibiotics was also restored in the presence of PAβN (Table 3). Both mutants accumulated less ciprofloxacin than did their isogenic parents (Fig. 1). Addition of PAβN increased ciprofloxacin accumulation in both mutants and parents, the two eventually reaching identical steady-state levels of cell-associated ciprofloxacin.

Contribution of AcrAB-TolC to antibiotic resistance. 104-cip and 5408-cip showed increased expression of *acrB* (6.1- ± 1.5- and 5.4- ± 1.6-fold increases, respectively). Deletion of *acrB* decreased the MIC of ciprofloxacin from ≥32 to 0.38 μg/ml in 104-cip and from ≥32 to 0.5 μg/ml in 5408-cip and had no effect on the MICs of nalidixic acid (Table 1). Susceptibility to other classes of antibiotics was also restored in both deletional mutants (Table 3). Deletion of *acrB* also decreased the MICs of ciprofloxacin and nalidixic acid in isolates (51, CUH60, 4931, 104, and 5408) showing nalidixic acid resistance and decreased susceptibility to ciprofloxacin (Table 1).

Contribution of global regulators to antibiotic resistance. In 104-cip there was a 26.1- ± 4.0-fold increase in *soxS* expression and an 8.9- ± 0.6-fold increase in *marA* expression. Deletion of *soxS* in 104-cip decreased the expression of *acrB* by −4.2- ± 1.0-fold and the expression of *marA* by −0.6- ± 1.6-fold and decreased the ciprofloxacin MIC to 1.5 μg/ml (Table 1). Deletion of *marA* in 104-cip decreased the expression of *acrB* by 1.4- ± 0.3-fold and the expression of *soxS* by 1.4- ± 0.2-fold and decreased the ciprofloxacin MIC to 4 μg/ml. Both *marA* and *soxS* deletional mutants remained nalidixic acid resistant (≥256 μg/ml) but lost their MDR phenotype (Table 3). In 5408-cip there was significantly increased expression of *ramA* (33.7- ± 4.0-fold). Deletion of *ramA* in 5408-cip reduced *acrB* expression by 1.6- ± 0.1-fold and decreased the MIC of ciprofloxacin to 4 μg/ml. It had no effect on the MIC of nalidixic acid (≥256 μg/ml) (Table 1). The *ramA* deletional mutant became susceptible to other classes of antibiotics (Table 3). Expression of *rob* was found to be decreased in both mutants (Table 1).

Genetic analysis of local and global regulators. Mutations were found in *soxR* (R20H) and in *soxS* (E52K) in 104-cip. The *soxR* mutation mapped to the helix-turn-helix region of the SoxR protein. A mutation was also found in *ramR* (G25A) in 5408-cip, which is found upstream of *ramA*. No mutations were found in the local regulator *acrR* or the global regulators *marORAB* and *ramA*.

Porin expression and LPS profiles. Immunodetection of OmpF with polyclonal antibodies directed against the denatured OmpF porin and with an F4 polyclonal antibody directed against the L3 internal loop of *E. coli* porins revealed a decrease in the production of OmpF in 104-cip (Fig. 2). All isolates displayed a smooth LPS phenotype. The LPS profile of 104-cip showed significant loss of short and intermediate O-chain LPSs compared to its isogenic parent (data not shown). No changes were observed in the LPS or OmpF profile of 5408-cip.

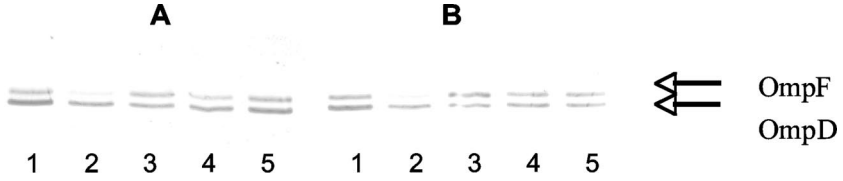

FIG. 2. The detection of porins was carried out using the polyclonal antibodies directed against denatured OmpF porin (A) or the F4 polyclonal antibody directed against the L3 internal loop of *E. coli* porins (B). Lanes 1, 104; lanes 2, 104-cip; lanes 3, 5408; lanes 4, 5408-cip, lanes 5, NCTC 13349. Arrows indicate the migration of F and D porins, respectively.

DISCUSSION

In this study, we confirmed that both AcrAB-TolC efflux pump activity and a D87 mutation in *gyrA* contribute to quinolone resistance in serovar Enteritidis field isolates displaying high-level nalidixic acid resistance and decreased susceptibility to fluoroquinolones (7). Ciprofloxacin-resistant and MDR mutants were readily selected in vitro from two of these field isolates, highlighting the ease with which resistance to fluoroquinolones and other clinically important antibiotics could potentially emerge during prolonged fluoroquinolone therapy in infected patients. Selection for high-level ciprofloxacin resistance was associated with the development of an additional *gyrA* mutation in 104-cip and hitherto-undocumented mutations in *gyrB* and *parE* in 5408-cip, overexpression of *acrB* and the development of an MDR phenotype in both isolates, and a decrease in OmpF expression and altered LPS in 104-cip.

Double mutations in *gyrA* have been widely reported in ciprofloxacin-resistant *Salmonella* isolates, whereas mutations in *gyrB* and *parE* in *Salmonella* are rarely detected (18). By complementation, we defined a significant role for the double *gyrA* mutations (D87Y and S83F) in quinolone resistance in 104-cip and for the single *gyrA* (D87Y) and the novel *gyrB* (E466D) mutation in ciprofloxacin resistance in 5408-cip. Although complementation with wild-type *parE* was without effect, the possibility that *parE* mutations may indirectly contribute to high-level fluoroquinolone resistance by increasing the level of resistance in isolates already harboring target gene mutations cannot be excluded (23).

Consistent with increased expression of an RND multidrug efflux pump, both mutants displayed an MDR phenotype. The increase in *acrB* expression was reflected in the decrease observed in ciprofloxacin accumulation, which increased following addition of PAβN. Inactivation of *acrB* restored susceptibility to ciprofloxacin and other nonquinolone antibiotics tested in accordance with previous reports that overexpression of AcrAB-TolC contributes to fluoroquinolone resistance and MDR in *Salmonella* (3–5). Similar effects were observed with PAβN, highlighting its utility as a pharmacological tool to screen for efflux-mediated antibiotic resistance in *Salmonella*. Interestingly, we observed a reproducible discrepancy between the effects of PAβN and deletion of *acrB* on nalidixic acid resistance in 5408-cip. The possibility of the contribution of an unidentified efflux pump, sensitive to PAβN, to nalidixic acid resistance in this isolate cannot be excluded.

The increased expression of *acrB* was associated with differential expression of global regulators, with 104-cip showing increased expression of both *soxS* and *marA* and 5408-cip showing increased expression of *ramA*. By deleting each of these regulators, we provided direct evidence for their involvement in fluoroquinolone resistance and MDR in serovar Enteritidis through *acrB* activation. To the best of our knowledge, this is the first report to document a direct contribution of *marA* and *soxS* to AcrAB-mediated MDR in *Salmonella*. Compared to the *marA* deletional mutant, the *soxS* deletional mutant showed greater downregulation of *acrB* and displayed lower MICs of ciprofloxacin and other antibiotics tested. These data suggest that *soxS* plays a greater role than *marA* in MDR in 104-cip. Expression of *marA* decreased following deletion of *soxS* and vice versa, highlighting the cross-regulation that exists between these transcriptional factors (12, 43). Recently, *ramA* has been reported to contribute to fluoroquinolone resistance and MDR in serovar Typhimurium through activation of *acrB* (1). Similarly our data clearly define a regulatory role for *ramA* in AcrAB-mediated MDR in serovar Enteritidis and furthermore show that *ramA* activates the MDR cascade independently of *marA*. As overexpression of *rob* has been shown to confer MDR in *E. coli* through activation of *acrB* (46), it is reasonable to assume a lack of involvement of this regulator in the development of MDR in this study. The decreased expression of *rob* in 104-cip is likely due to downregulation by *soxS* and *marA* (28, 43). The nature and extent of the cross talk between *ramA* and other global regulators are currently unknown. However, based on our data it would be interesting to speculate that it may downregulate both *soxS* and *rob*.

Increased expression of *soxS* and *marA* in *E. coli* has been attributed to mutations in the *soxR* gene that render *soxR* active independent of oxidative stress or mutations in *marR* that alleviate its repression of *marA* (21, 32, 49). To date, there is only one report documenting the contribution of a mutation in *soxR* to increased *soxS* expression and MDR in *Salmonella* (20). We identified mutations in both *soxR* and *soxS* in 104-cip. The same *soxR* mutation has been linked to increased *soxS* expression and multiple antibiotic resistance in *E. coli* (21). Mutations in *soxS* have been reported in *E. coli* isolates overexpressing *soxS*, but their significance was not determined (49). Similar to other studies of *Salmonella* (7, 12), we found no mutations in the *marR* or *marO* region that could explain the increased expression of *marA*. Therefore, it most likely resulted from feed-forward activation by SoxS. Sequence analysis also revealed a hitherto-unreported mutation within the recently defined local repressor *ramR* (G25A) of *ramA* in 5408-cip. Mutations in *ramR* have been reported to play a role in upregulation of *ramA* and AcrAB and consequently the efflux-mediated MDR phenotype in serovar Typhimurium (1). The significance of these mutations in *soxR*, *soxS*, and *ramR* is currently being investigated.

OmpF expression was decreased in 104-cip, consistent with the role of *marRAB* and *soxRS* in the control of its expression (37, 40). This mutant also displayed alterations in the LPS ladder. In contrast to the findings in *Enterobacter aerogenes* and *E. coli* (8), *ramA*-mediated MDR in 5408-cip was not associated with downregulation of porins. Few studies have investigated OmpF expression in fluoroquinolone-resistant *Salmonella*, and its contribution to resistance is unclear (14, 29, 35). Only one study to date has documented alterations in the LPS profile in fluoroquinolone-resistant *Salmonella*, and the authors suggested that the increase in the proportion of long O-chain LPSs observed could result in a lower level of antibiotic accessibility to the porins (14). Further studies are warranted to evaluate the exact contribution of altered porin expression and LPS to antibiotic resistance in 104-cip. Nonetheless, based on our data showing that both 104-cip and its isogenic parent accumulated the same amount of ciprofloxacin at steady state following the addition of PAβN, it would appear that the decreased accumulation of ciprofloxacin observed in 104-cip was mainly due to enhanced efflux activity rather than decreased influx resulting from altered membrane permeability. Furthermore, the hypersusceptibility to ampicillin, tetracycline, and chloramphenicol (which also enter through the porin

pathway) observed following deletion of *acrB* also suggests that active efflux is the main mechanism associated with MDR in this isolate.

Finally, this study revealed that different mechanisms were involved in the development of MDR following ciprofloxacin exposure, as the two mutants displayed different phenotypes of resistance to nonquinolone antibiotics. It is possible that these differences may be due to pleiotropic effects associated with the different expression profiles of global regulators observed in these isolates.

In summary, this study highlights that a high-level ciprofloxacin resistance and MDR phenotype in serovar Enteritidis can result from different genetic events associated with multiple resistance mechanisms. It provides direct evidence that quinolone resistance and MDR in serovar Enteritidis result from interplay between target gene mutations and increased AcrAB-TolC efflux activity and defines a role for a novel *gyrB* mutation in ciprofloxacin resistance. Increased AcrAB-TolC efflux activity in fluoroquinolone-resistant and MDR isolates can be due to differential expression of the global regulators *soxS*, *marA*, and *ramA* associated with mutations in their regulatory genes.

ACKNOWLEDGMENTS

We thank Jonathan Caddick and Vito Ricci (University of Birmingham) for technical training in complementation and phage transduction experiments.

This work was supported by COST Action BM0701 "ATENS".

REFERENCES

1. **Abouzeed, Y. M., S. Baucheron, and A. Cloeckaert.** 2008. *ramR* mutations involved in efflux-mediated multidrug resistance in *Salmonella enterica* serovar Typhimurium. Antimicrob. Agents Chemother. **52:**2428–2434.
2. **Alekshun, M. N., and S. B. Levy.** 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. Antimicrob. Agents Chemother. **41:**2067–2075.
3. **Baucheron, S., E. Chaslus-Dancla, and A. Cloeckaert.** 2004. Role of TolC and *parC* mutation in high-level fluoroquinolone resistance in *Salmonella enterica* serotype Typhimurium DT204. J. Antimicrob. Chemother. **53:**657–659.
4. **Baucheron, S., H. Imberechts, E. Chaslus-Dancla, and A. Cloeckaert.** 2002. The AcrB multidrug transporter plays a major role in high-level fluoroquinolone resistance in *Salmonella enterica* serovar Typhimurium phage type DT204. Microb. Drug Resist. **8:**281–289.
5. **Baucheron, S., S. Tyler, D. Boyd, M. R. Mulvey, E. Chaslus-Dancla, and A. Cloeckaert.** 2004. AcrAB-TolC directs efflux-mediated multidrug resistance in *Salmonella enterica* serovar Typhimurium DT104. Antimicrob. Agents Chemother. **48:**3729–3735.
6. **Carrique-Mas, J. J., C. Papadopoulou, S. J. Evans, A. Wales, C. J. Teale, and R. H. Davies.** 2008. Trends in phage types and antimicrobial resistance of *Salmonella enterica* serovar Enteritidis isolated from animals in Great Britain from 1990 to 2005. Vet. Rec. **162:**541–546.
7. **Chen, S., S. Cui, P. F. McDermott, S. Zhao, D. G. White, I. Paulsen, and J. Meng.** 2007. Contribution of target gene mutations and efflux to decreased susceptibility of *Salmonella enterica* serovar Typhimurium to fluoroquinolones and other antimicrobials. Antimicrob. Agents Chemother. **51:**535–542.
8. **Chollet, R., J. Chevalier, C. Bollet, J. M. Pages, and A. Davin-Regli.** 2004. RamA is an alternate activator of the multidrug resistance cascade in *Enterobacter aerogenes*. Antimicrob. Agents Chemother. **48:**2518–2523.
9. **CLSI.** 2004. Performance standards for antimicrobial susceptibility testing. CLSI document M100-S14. CLSI, Wayne, PA.
10. **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.
11. **De, E., A. Basle, M. Jaquinod, N. Saint, M. Mallea, G. Molle, and J. M. Pages.** 2001. A new mechanism of antibiotic resistance in *Enterobacteriaceae* induced by a structural modification of the major porin. Mol. Microbiol. **41:**189–198.
12. **Eaves, D. J., V. Ricci, and L. J. Piddock.** 2004. Expression of *acrB*, *acrF*, *acrD*, *marA*, and *soxS* in *Salmonella enterica* serovar Typhimurium: role in multiple antibiotic resistance. Antimicrob. Agents Chemother. **48:**1145–1150.
13. **Giraud, E., S. Baucheron, and A. Cloeckaert.** 2006. Resistance to fluoroquinolones in *Salmonella*: emerging mechanisms and resistance prevention strategies. Microbes Infect. **8:**1937–1944.
14. **Giraud, E., A. Cloeckaert, D. Kerboeuf, and E. Chaslus-Dancla.** 2000. Evidence for active efflux as the primary mechanism of resistance to ciprofloxacin in *Salmonella enterica* serovar Typhimurium. Antimicrob. Agents Chemother. **44:**1223–1228.
15. **Heisig, P.** 1993. High-level fluoroquinolone resistance in a *Salmonella* typhimurium isolate due to alterations in both *gyrA* and *gyrB* genes. J. Antimicrob. Chemother. **32:**367–377.
16. **Hernandez-Urzua, E., D. S. Zamorano-Sanchez, J. Ponce-Coria, E. Morett, S. Grogan, R. K. Poole, and J. Membrillo-Hernandez.** 2007. Multiple regulators of the flavohaemoglobin (*hmp*) gene of *Salmonella enterica* serovar Typhimurium include RamA, a transcriptional regulator conferring the multidrug resistance phenotype. Arch. Microbiol. **187:**67–77.
17. **Hitchcock, P. J., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. J. Bacteriol. **154:**269–277.
18. **Hopkins, K. L., R. H. Davies, and E. J. Threlfall.** 2005. Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. Int. J. Antimicrob. Agents **25:**358–373.
19. **Keeney, D., A. Ruzin, and P. A. Bradford.** 2007. RamA, a transcriptional regulator, and AcrAB, an RND-type efflux pump, are associated with decreased susceptibility to tigecycline in *Enterobacter cloacae*. Microb. Drug Resist. **13:**1–6.
20. **Koutsolioutsou, A., E. A. Martins, D. G. White, S. B. Levy, and B. Demple.** 2001. A *soxRS*-constitutive mutation contributing to antibiotic resistance in a clinical isolate of *Salmonella enterica* (serovar Typhimurium). Antimicrob. Agents Chemother. **45:**38–43.
21. **Koutsolioutsou, A., S. Pena-Llopis, and B. Demple.** 2005. Constitutive *soxR* mutations contribute to multiple-antibiotic resistance in clinical *Escherichia coli* isolates. Antimicrob. Agents Chemother. **49:**2746–2752.
22. **Li, Z., and B. Demple.** 1994. SoxS, an activator of superoxide stress genes in *Escherichia coli*. Purification and interaction with DNA. J. Biol. Chem. **269:**18371–18377.
23. **Ling, J. M., E. W. Chan, A. W. Lam, and A. F. Cheng.** 2003. Mutations in topoisomerase genes of fluoroquinolone-resistant salmonellae in Hong Kong. Antimicrob. Agents Chemother. **47:**3567–3573.
24. **Livak, K. J., and T. D. Schmittgen.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods **25:**402–408.
25. **Mallea, M., J. Chevalier, C. Bornet, A. Eyraud, A. Davin-Regli, C. Bollet, and J. M. Pages.** 1998. Porin alteration and active efflux: two *in vivo* drug resistance strategies used by *Enterobacter aerogenes*. Microbiology **144:**3003–3009.
26. **McCarron, B., and W. C. Love.** 1997. Acalculous nontyphoidal salmonellal cholecystitis requiring surgical intervention despite ciprofloxacin therapy: report of three cases. Clin. Infect. Dis. **24:**707–709.
27. **Meakins, S., I. S. Fisher, C. Berghold, P. Gerner-Smidt, H. Tschape, M. Cormican, I. Luzzi, F. Schneider, W. Wannett, J. Coia, A. Echeita, and E. J. Threlfall.** 2008. Antimicrobial drug resistance in human nontyphoidal *Salmonella* isolates in Europe 2000–2004: a report from the Enter-net International Surveillance Network. Microb. Drug Resist. **14:**31–35.
28. **Michan, C., M. Manchado, and C. Pueyo.** 2002. SoxRS down-regulation of *rob* transcription. J. Bacteriol. **184:**4733–4738.
29. **Miro, E., C. Verges, I. Garcia, B. Mirelis, F. Navarro, P. Coll, G. Prats, and L. Martinez-Martinez.** 2004. Resistance to quinolones and beta-lactams in *Salmonella enterica* due to mutations in topoisomerase-encoding genes, altered cell permeability and expression of an active efflux system. Enferm. Infecc. Microbiol. Clin. **22:**204–211. (In Spanish.)
30. **Molbak, K., D. L. Baggesen, F. M. Aarestrup, J. M. Ebbesen, J. Engberg, K. Frydendahl, P. Gerner-Smidt, A. M. Petersen, and H. C. Wegener.** 1999. An outbreak of multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype Typhimurium DT104. N. Engl. J. Med. **341:**1420–1425.
31. **Mortimer, P. G., and L. J. Piddock.** 1991. A comparison of methods used for measuring the accumulation of quinolones by *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. J. Antimicrob. Chemother. **28:**639–653.
32. **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.
33. **Olliver, A., M. Valle, E. Chaslus-Dancla, and A. Cloeckaert.** 2004. Role of an *acrR* mutation in multidrug resistance of *in vitro*-selected fluoroquinolone-resistant mutants of *Salmonella enterica* serovar Typhimurium. FEMS Microbiol. Lett. **238:**267–272.
34. **Piddock, L. J.** 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin. Microbiol. Rev. **19:**382–402.
35. **Piddock, L. J., D. J. Griggs, M. C. Hall, and Y. F. Jin.** 1993. Ciprofloxacin resistance in clinical isolates of *Salmonella typhimurium* obtained from two patients. Antimicrob. Agents Chemother. **37:**662–666.
36. **Piddock, L. J., V. Ricci, I. McLaren, and D. J. Griggs.** 1998. Role of mutation in the *gyrA* and *parC* genes of nalidixic-acid-resistant salmonella serotypes

isolated from animals in the United Kingdom. J. Antimicrob. Chemother. **41:**635–641.
37. **Pomposiello, P. J., and B. Demple.** 2000. Identification of SoxS-regulated genes in *Salmonella enterica* serovar Typhimurium. J. Bacteriol. **182:**23–29.
38. **Prouty, A. M., I. E. Brodsky, S. Falkow, and J. S. Gunn.** 2004. Bile-salt-mediated induction of antimicrobial and bile resistance in *Salmonella* typhimurium. Microbiology **150:**775–783.
39. **Randall, L. P., and M. J. Woodward.** 2001. Multiple antibiotic resistance (*mar*) locus in *Salmonella enterica* serovar Typhimurium DT104. Appl. Environ. Microbiol. **67:**1190–1197.
40. **Randall, L. P., and M. J. Woodward.** 2002. The multiple antibiotic resistance (mar) locus and its significance. Res. Vet. Sci. **72:**87–93.
41. **Ricci, V., P. Tzakas, A. Buckley, and L. J. Piddock.** 2006. Ciprofloxacin-resistant *Salmonella enterica* serovar Typhimurium strains are difficult to select in the absence of AcrB and TolC. Antimicrob. Agents Chemother. **50:**38–42.
42. **Ruzin, A., M. A. Visalli, D. Keeney, and P. A. Bradford.** 2005. Influence of transcriptional activator RamA on expression of multidrug efflux pump AcrAB and tigecycline susceptibility in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **49:**1017–1022.
43. **Schneiders, T., and S. B. Levy.** 2006. MarA-mediated transcriptional repression of the *rob* promoter. J. Biol. Chem. **281:**10049–10055.
44. **Simonet, V., M. Mallea, D. Fourel, J. M. Bolla, and J. M. Pages.** 1996. Crucial domains are conserved in *Enterobacteriaceae* porins. FEMS Microbiol. Lett. **136:**91–97.
45. **Sulavik, M. C., M. Dazer, and P. F. Miller.** 1997. The *Salmonella typhimurium mar* locus: molecular and genetic analyses and assessment of its role in virulence. J. Bacteriol. **179:**1857–1866.
46. **Tanaka, T., T. Horii, K. Shibayama, K. Sato, S. Ohsuka, Y. Arakawa, K. Yamaki, K. Takagi, and M. Ohta.** 1997. RobA-induced multiple antibiotic resistance largely depends on the activation of the AcrAB efflux. Microbiol. Immunol. **41:**697–702.
47. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
48. **Vasallo, F. J., P. Martin-Rabadan, L. Alcala, J. M. Garcia-Lechuz, M. Rodriguez-Creixems, and E. Bouza.** 1998. Failure of ciprofloxacin therapy for invasive nontyphoidal salmonellosis. Clin. Infect. Dis. **26:**535–536.
49. **Webber, M. A., and L. J. Piddock.** 2001. Absence of mutations in *marRAB* or *soxRS* in *acrB*-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of *Escherichia coli*. Antimicrob. Agents Chemother. **45:**1550–1552.
50. **Wu, J., W. R. Dunham, and B. Weiss.** 1995. Overproduction and physical characterization of SoxR, a [2Fe-2S] protein that governs an oxidative response regulon in *Escherichia coli*. J. Biol. Chem. **270:**10323–10327.