Constitutive SoxS Expression in a Fluoroquinolone-Resistant Strain with a Truncated SoxR Protein and Identification of a New Member of the *marA-soxS-rob* Regulon, *mdtG*[∇]

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Elevated levels of fluoroquinolone resistance are frequently found among *Escherichia coli* clinical isolates. This study investigated the antibiotic resistance mechanisms of strain NorE5, derived *in vitro* by exposing an *E. coli* clinical isolate, PS5, to two selection steps with increasing concentrations of norfloxacin. In addition to the amino acid substitution in GyrA (S83L) present in PS5, NorE5 has an amino acid change in ParC (S80R). Furthermore, we now find by Western blotting that NorE5 has a multidrug resistance phenotype resulting from the overexpression of the antibiotic resistance efflux pump AcrAB-TolC. Microarray and gene fusion analyses revealed significantly increased expression in NorE5 of *soxS*, a transcriptional activator of *acrAB* and *tolC*. The high *soxS* activity is attributable to a frameshift mutation that truncates SoxR, rendering it a constitutive transcriptional activator of *soxS*. Furthermore, microarray and reverse transcription-PCR analyses showed that *mdtG* (*yceE*), encoding a putative efflux pump, is overexpressed in the resistant strain. SoxS, MarA, and Rob activated an *mdtG::lacZ* fusion, and SoxS was shown to bind to the *mdtG* promoter, showing that *mdtG* is a member of the *marA-soxS-rob* regulon. The *mdtG* marbox sequence is in the backward or class I orientation within the promoter, and its disruption resulted in a loss of inducibility by MarA, SoxS, and Rob. Thus, chromosomal mutations in *parC* and *soxR* are responsible for the increased antibiotic resistance of NorE5.

Escherichia coli is the most frequent pathogen isolated from patients with urinary tract infections (UTIs). The prevalence of this microorganism in uncomplicated UTIs is between 71 and 90% throughout the world (3, 18), but the percentage is lower for complicated infections (1, 3). The common therapy for UTIs is ampicillin or trimethoprim-sulfamethoxazole. However, the increasing frequency of resistance to these agents (reaching maximum levels of >40% and >20%, respectively, in countries such as Spain, Portugal, Ireland, and Korea [1, 17, 18]) often necessitates the use of a second antibiotic, such as ciprofloxacin. However, resistance to ciprofloxacin is also increasing in some geographic areas, to >20% (17, 18). Reasonable alternatives for treating uncomplicated infections are nitrofurantoin and fosfomycin, due to the low rates of resistance detected so far (3, 17).

The mechanisms of quinolone resistance in *E. coli* strains have been studied in detail. They result from both chromosomally encoded mutations and plasmid-mediated quinolone resistance (10). The first mechanism includes mutations of the target genes *gyrA* and *gyrB*, which encode DNA gyrase, and *parC* and *parE*, encoding topoisomerase IV (29, 35, 41, 43, 44), as well as mutations responsible for a decrease in quinolone permeability, either by increasing the efflux or by decreasing the expression of outer membrane proteins used as entrance

channels (5, 7, 9, 34). The second mechanism, the prevalence of which is steadily increasing, relies on the presence of plasmid mediated determinants, such as *qnr*, *qepA*, and *aac(6′)-Ib-cr* (14, 48, 49).

The acquisition of quinolone resistance occurs sequentially. For *E. coli* strains, the first step usually involves mutations within the quinolone resistance-determining regions (QRDRs) of the target genes and is associated with 32- and 10-fold increases in the MICs of nalidixic acid and ciprofloxacin, respectively (32). The second-step mutations are often within the regulatory loci that control efflux pump expression and usually show a 2- to 8-fold increase in quinolone resistance levels (16). At this step, a multidrug resistance (MDR) phenotype is detected, since efflux pumps have a wide range of exportable substrates, so that cross-resistance with other antibiotics results (7, 10). AcrAB is the main efflux pump described for *Enterobacteriaceae* and acts in conjunction with TolC (11, 32, 34). Five regulators that play roles in AcrAB expression have been described so far (10, 13). AcrR, the local repressor encoded upstream of *acrA* (20), and AcrS, located upstream of the *acrEF* operon (13), repress *acrAB*. Mutations acquired within the *acrR* locus have been reported to trigger a truncated/inactivated repressor (45, 46). The three other regulators, SoxS, MarA, and Rob, are members of the AraC/XylS family of transcriptional activators (25). The *soxRS* region contains two loci divergently expressed; when oxidized, e.g., by treatment with superoxide-generating agents such as paraquat (PQ), SoxR transcriptionally activates *soxS* expression (2, 8), which in turn activates a wide number of genes, the *marA-soxS-rob* regulon (25). Constitutive expression of SoxS results from mu-

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TABLE 1. Bacteria, plasmids, and phages

Bacterium, plasmid, or phage	Parent strain	Relevant genotype and/or characteristics	Source or reference
Bacteria			
PS5		*E. coli* clinical isolate susceptible to fluoroquinolones	42
NorE5		Fluoroquinolone-resistant mutant selected from PS5 with norfloxacin	42
GC4468		*E. coli* K-12 (Δlac $soxRS^+$)	12
DJ901	GC4468	$\Delta soxRS$	12
JTG936	GC4468	*soxR*(Con)	12
GC48-F	GC4468	pRS551 *fpr::lacZ*	This study
P5-F	PS5	pRS551 *fpr::lacZ*	This study
N5-F	NorE5	pRS551 *fpr::lacZ*	This study
M4450	GC4468	*mdtG67::lacZ* (−159 to +6)	This study
M4452	GC4468	*mdtG77::lacZ* (−98 to +6)	This study
M9948	834(D3lysS)	pRGM9948	21
Plasmids			
pRS551		pBR322 derivative; Amp^r	40
pRGM9948		pET15b carrying the *soxS* gene; Amp^r	21
Phage, λRS45		λimm21; Kan^r	40

tations acquired within the C terminus of SoxR, which activate the protein (31). The *marRAB* operon also encodes a regulator, MarA, that is autorepressed by MarR, the first gene of the operon (6). Mutations within *marR* result in a loss of repressor activity and allow increased *marA* expression (6, 33). Exposure to salicylate also reduces MarR repression and increases *marA* expression (37). Rob is posttranscriptionally activated upon treatment of cells with a bile salt, decanoate or 2,2′-dipyridyl (DIP) (4, 36, 38). However, clinical significance has been associated only with the mutation of AcrR (45, 46) and the overexpression of MarA and SoxS.

SoxS, MarA, and Rob have highly overlapping regulons (25). They bind as monomers to a 20-bp asymmetric sequence with the degenerate consensus sequence AYNGCACNNWNNRY YAAAYN (where N stands for any base, R stands for A or G, W stands for A or T, and Y stands for C or T). This binding site is referred to as the "marbox." A marbox is present upstream of the promoters of all the regulon genes (such as *acrAB*, *tolC*, and *marRAB* itself, which is autoactivatable) (21). SoxS and MarA bind to the marboxes of different genes and activate them to different extents, such as the *fpr* promoter, which is more susceptible to activation by SoxS than by MarA (22).

Here we characterize the mechanisms of fluoroquinolone resistance acquired *in vitro* by a fluoroquinolone-susceptible *E. coli* clinical isolate upon exposure to increasing concentrations of norfloxacin. A mutation in *parC* and a novel mutation in *soxR* appear to be responsible.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All cultures were grown in LB broth at 37°C with shaking or on LB plates supplemented with ampicillin (100 μg/ml) or kanamycin (35 μg/ml) when necessary. The indicator 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to LB plates at a final concentration of 40 μg/ml.

Cells were treated with 50 μM PQ, 5 mM sodium salicylate (SAL), or 5 mM DIP and were incubated at 37°C with shaking for 1 h where indicated.

Susceptibility testing. MICs of chloramphenicol, tetracycline, amoxicillin, erythromycin, trimethoprim, and amikacin for strains PS5 and NorE5 were determined by Etest (AB Biodisk, Solna, Sweden) on Mueller-Hinton (MH) plates according to the manufacturer's recommendations.

Microarray analysis. Total RNA from PS5 and NorE5 was extracted from a mid-exponential-phase culture (optical density at 600 nm [OD_{600}], 0.6) using RNeasy spin columns (Qiagen, Chatsworth, CA). A total of 20 μg of total RNA was labeled with Cy-3-dUTP (RNA from strain PS5) or Cy-5-dUTP (RNA from strain NorE5) in a standard reverse transcriptase (RT) reaction using Superscript II(+) (Gibco BRL, Carlsbad, CA) with 1 μg of random hexamer (Amersham Pharmacia, Piscataway, NJ) primers. After purification through Microcon-30 membranes (Millipore, Billerica, MA), Cy-3- and Cy-5-labeled cDNA samples were mixed with SSC (final concentration, 2.5×; 1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate [pH 7]), 0.25% sodium dodecyl sulfate (SDS), and 40 μg of *E. coli* rRNA (Boehringer Mannheim, Ingelheim, Germany) in a final volume of 16 μl and were hybridized with the DNA microarray for 5 h at 65°C. The DNA microarray contained 4,058 open reading frames (ORFs) representing 95% of *E. coli* ORFs, and hybridization was performed as described in the MGuide (http://cmgm.stanford.edu/pbrown/mguide/index.html). The glass slide was washed and scanned using an Axon Scanner GENPIX 1.0 (Axon Instruments, Foster City, CA) at a resolution of 10 μm per pixel. The resulting 16-bit tagged-image format file (TIFF) images were analyzed using SCANALYZE software. The reproducibility of the technique was assessed in two separate experiments. A normalized relative Cy5/Cy3 ratio of >2 was considered a significant increase in expression, and a normalized relative Cy3/Cy5 ratio of >2 was considered a significant decrease in expression, when observed for both of the two different experiments performed.

RT-PCR. Fresh overnight cultures of PS5, NorE5, GC4468, DJ901, and JTG936 were diluted 1/100 into 15 ml LB medium and were aerated at 37°C until strains reached OD_{600} values of 0.5 to 0.6. Three milliliters was then taken and treated with 6 ml of RNAprotect Bacteria reagent (Qiagen, Hilden, Germany). Mixtures were processed according to the manufacturer's instructions. Pellets were resuspended in 200 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA [pH 8.0]) supplemented with 3 mg/ml lysozyme, vortexed, and incubated at 32°C for 10 min with shaking. The RNA was extracted by using an RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Samples were subsequently treated with DNA-free DNase (Ambion, Austin, TX) according to the manufacturer's recommendations until RNA samples were totally DNA free when checked by PCR using *gapA* (a housekeeping gene) primers. RT-PCR was performed using the AccessQuick RT-PCR system (Promega, Madison, WI) according to the manufacturer's recommendations with primers gapA.1 (GTATCAACGGTTTTGGCCG) and gapA.2 (AGCTTTAGCAGCAC CGGTA) for *gapA* and primers yceE.RT1 (GCCAGTTCGCGGACATAC) and yceE.RT2 (CTGCGGGCGCTTCTTGGGTTACTT) for *mdtG*. The retrotranscription process was performed using 500 ng of RNA at 45°C for 45 min, followed by a standard PCR program. Samples were loaded in a GeneGel Excel (GE Healthcare, Uppsala, Sweden) at 600 V, 25 mA, and 15 W for 1.5 h. Gels were stained with a DNA silver-staining kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer's recommendations. Results were corroborated from two independent mRNA extractions and amplifications.

TABLE 2. Mutations detected within the quinolone resistance-determining regions and MIC determinations for the strains

Strain	Amino acid substitution[a]		MIC (µg/ml)[b]								
	GyrA	ParC	NOR	CIP	NAL	CHL	TET	AMX	ERY	TMP	AMK
PS5	S83L	—	0.5	0.5	1,024	8	2	48	64	0.5	12
NorE5	S83L	S80R	32	8	2,048	64	8	>256	>256	8	12

[a] S, serine; L, leucine; R, arginine; —, no mutation found.
[b] NOR, norfloxacin; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; AMX, amoxicillin; ERY, erythromycin; TMP, trimethoprim; AMK, amikacin. Data for NOR, CIP, NAL, and TET are from reference 38.

Sequencing of the *soxRS* region. The whole *soxRS* regions of strains GC4468, PS5, and NorE5 were amplified by PCR using the same primers as previously described (soxRS.F [GGCGAAGCTTCCGCAGGTGTTTATGC] and soxRS.R [CGTCGGGGGAAGCTTTCCTGTGTACC]) (19). The amplified fragments were directly used for sequencing and were compared for the detection of mutations.

Western blotting. Bacterial strains were grown overnight in 50 ml LB medium and were harvested by centrifugation. The pellet was rinsed twice with 10 mM Tris supplemented with 1% NaCl and was resuspended in 3 ml of the same buffer. Bacterial samples were sonicated on ice on a Vibra-Cell VCX 130 (Sonics) for a total of 3 min (30 s for each cycle of sonication, followed by 30 s of rest) with an amplitude of 50%. Cell debris was removed by centrifugation for 20 min at 4°C and 3,500 rpm, and the supernatant was collected and centrifuged again for 90 min at 4°C and 16,000 rpm. The final pellet was resuspended in 1× phosphate-buffered saline (PBS) (Roche, Mannheim, Germany). Protein quantification was performed using the RC DC protein assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's indications.

Ten micrograms of each protein sample was loaded onto an 8% SDS-polyacrylamide gel electrophoresis (PAGE) gel (Mini Protean II). Transfer from the gel to a nitrocellulose membrane was performed for 2 h at 60 V on ice. The membranes were blocked using 1× PBS containing Tween 20 diluted 1/2,000 (PBS-T) and 5% skim milk for 1 h at room temperature, followed by an overnight incubation at 4°C with the primary antibodies against the AcrB and TolC proteins (Antibody Bcn, Barcelona, Spain) diluted 1/500 in PBS-T. The membranes were washed three times with PBS-T and once with PBS before the secondary antibody, anti-rabbit IgG (GE Healthcare, Buckinghamshire, United Kingdom), diluted 1/2,000 in PBS-T, was added for 1 h of incubation at room temperature. Then the membranes were washed as described above and processed using EZ-ECL (Biological Industries, Kibbutz Beit Haemek, Israel) for chemiluminescence detection of bands in a Fuji LAS-3000 instrument.

DNA manipulations. Strains GC48-F, P5-F, and N5-F were derived from parental strains GC4468, PS5, and NorE5, respectively, by transformation with plasmid pRS551 (40) harboring the *fpr::lacZ* transcriptional fusion. The resulting strains were assayed for β-galactosidase activity.

A *lacZ* transcriptional fusion was constructed with the *mdtG* promoter (40). Amplification of the promoter was carried out by PCR using chromosomal DNA from strain GC4468 as the template. The *mdtG67* fragment was 165 bp long (from −159 to +6, relative to the ATG codon) and was amplified with primers 1756 (AAAAAAGAATTCGGATGCTTCAGAATGGCATCCGGCATTACCACA [the EcoRI site is underlined]) and 1757 (AAAAAAGGATCCTGACATAGCAATCCGCTGTTGGTGCGCCA [the BamHI site is underlined]). A shorter fusion, containing the 104-bp *mdtG77* fragment (from −98 to +6), was also constructed, using primers 1767 (AACCAAGAATTCTCTCTGGATTGCGCCCCCTGGAAGT [the EcoRI site is underlined]) and 1757. The amplified DNA fragments were digested with EcoRI and BamHI and were ligated to the similarly cut vector pRS551. Recombinant plasmids were isolated in DH5α cells by selection for ampicillin resistance and were verified by sequence analysis. Recombination between the pRS551 derivatives and λRS45 resulted in lysates bearing the transcriptional fusions. Single λ lysogens of GC4468 were obtained (40) by selection for kanamycin resistance. The *mdtG::lacZ* fusion lysogens were designated M4450 and M4452, respectively.

β-Galactosidase assays. Strains M4450 and M4452, as well as strains GC48-F, P5-F, and N5-F, were assayed for β-galactosidase activity, expressed in Miller units, as previously described (26). Bacterial growth to log phase and treatments for 1 h with PQ, SAL, or DIP, where indicated, and at the concentrations mentioned above, were performed as reported previously (38, 39). All assays were carried out twice in duplicate and agreed within 15%.

DNA-binding assays. SoxS was overexpressed using the *soxS* gene cloned into a pET15b vector (pRGM9948) by induction of strain M9948 with isopropyl-β-

D-thiogalactopyranoside (IPTG) (21). SoxS was then purified to homogeneity, and the histidine tag was removed as previously described (15). Gel mobility experiments were performed three times in 6% acrylamide gels in TAE buffer as previously reported (24). The 20-bp oligonucleotide 5′-AGAGCTTTTATCGCTAAATC-3′ was labeled at its 5′ end using polynucleotide kinase (New England Biolabs, Beverly, MA) according to the manufacturer's recommendations and was annealed with a complementary 20-bp oligonucleotide. Trace quantities (approximately 10 fmol/sample) of the radiolabeled oligonucleotide in 9 µl of buffer containing 1× TAE buffer in 25% glycerol, 50 fmol of nonradioactive dA · dT (20 bp in length), and 0.1 µg serum albumin were mixed with 1-µl samples of purified SoxS that had been diluted in buffer containing 50 mM HEPES (pH 8.0), 0.5 M NaCl, and 25% glycerol to give the final concentrations indicated in Fig. 4. The samples were subjected to electrophoresis at 150 V for 35 min. The amounts of unbound and SoxS-complexed DNA were quantified by analysis on a Molecular Dynamics PhosphorImager.

RESULTS

Fluoroquinolone resistance acquisition and MDR phenotype. Strain NorE5 was selected *in vitro* from strain PS5, an *E. coli* clinical isolate, after exposure to increasing concentrations of norfloxacin as previously reported (42). The target gene mutations were described in the previous study (PS5 had an amino acid substitution in GyrA [Ser83Leu], whereas NorE5 acquired an additional change in ParC [Ser80Arg]). In the present study, a broader characterization of the antibiotic susceptibility profile was performed (Table 2). The results showed significant increases in the MICs of nalidixic acid, norfloxacin, and ciprofloxacin, as previously found, and of other classes of antibiotics—chloramphenicol, tetracycline, amoxicillin, erythromycin, and trimethoprim—whereas the levels of susceptibility to amikacin remained unchanged.

Changes in gene expression as determined by microarray and Western blot analyses. To determine the basis of this MDR, gene expression in strains PS5 and NorE5 was studied using cDNA microarrays. In comparison to PS5, NorE5 overexpressed *acrA* and *acrB* approximately 2.5-fold. Since these genes encode two components of the very important AcrAB-TolC drug efflux pump, this finding suggested a likely explanation for the MDR phenotype of NorE5. The ratios of increased expression for each gene, with their known roles in antimicrobial resistance, are listed in Table 3. Protein analysis using antibodies against AcrB corroborated its increased expression in NorE5. Furthermore, overexpression of TolC was also observed in Western blot gels (Fig. 1). AcrAB-TolC does not pump aminoglycosides out of the cell, consistent with the unchanged MIC of amikacin.

The microarrays also revealed significantly increased expression of *soxS* and *marA* in NorE5, about 8.4- and 2.8-fold, respectively (Table 3). In addition, *ompF* expression was decreased 7.8-fold, in agreement with the previous study, which

TABLE 3. Altered gene expression in NorE5 as determined by microarrays

Gene	Product	Fold change[a]		
		Expt 1	Expt 2	Expt 3
Upregulated				
soxS	Transcriptional activator of regulator of superoxide response regulon	8.3	6.2	10.6
marA	Transcriptional activator of multiple antibiotic resistance	3.6	1.7	3.2
acrA	MDR efflux membrane fusion protein	2.9	1.8	3.0
acrB	MDR efflux pump	2.3	2.8	2.3
mdtG	Predicted drug efflux pump	2.5	1.6	2.0
Downregulated (ompF)	Outer membrane porin 1a	9.1	5.9	8.3

[a] The ratio of expression in strain NorE5 to expression in strain PS5, as determined by microarray analysis, is shown for three independent experiments.

showed decreased amounts of OmpF in the NorE5 outer membranes (42). The microarrays also showed that *mdtG* (locus b1053 of *E. coli* K-12 NC_000913, also known as *mdtG*) was upregulated approximately 2-fold in NorE5 (Table 3). This gene has been reported to encode a putative transport protein and to be involved in fosfomycin resistance (30).

A truncated SoxR protein detected in NorE5 is associated with elevated levels of SoxS transcriptional activity. The significantly increased expression levels of *soxS* in NorE5 detected by the microarrays could be responsible for the MDR phenotype. We sequenced the full *soxRS* regions of GC4468, PS5, and NorE5 in order to detect any mutation that may have been acquired during the process of selection with norfloxacin. The results showed two mutations in NorE5 that differ from that in GC4468 and would affect the structure of the SoxR protein. One encodes a Gly74-to-Arg change, which is also present in PS5. The second mutation, an insertion of two adenines at nucleotide position 402, was detected only in NorE5. This second change in *soxR* encodes a frameshift starting at position Lys134 and generates a stop codon (TAG) 7 codons later. This would remove the last 21 amino acids of the C terminus of SoxR. No other amino acid change within SoxS, and no other mutation within the promoter region, was detected in the two strains in comparison with GC4468.

In order to evaluate *in vivo* the transcriptional activity of the SoxR protein in GC4468, PS5, and NorE5, each strain was transformed with plasmid pRS551 carrying the SoxS-activatable *fpr*::*lacZ* transcriptional fusion. These strains (GC48-F, P5-F, and N5-F) were either left untreated or treated with PQ for 1 h and were then assayed for β-galactosidase activity (Table 4). In the absence of PQ, the *fpr*::*lacZ* transcriptional fusion was 8.2- and 5.1-fold more active in N5-F than in

GC48-F and P5-F, respectively, indicating constitutive expression. When GC48-F and P5-F cells were treated with PQ, the activity significantly increased over that obtained without treatment, showing that *soxS* was inducible in these strains, as expected. In contrast, the activity detected in N5-F was almost the same in the absence as in the presence of PQ, indicating that the ability of SoxR to induce SoxS is constitutively elevated in this strain (Table 4). Thus, the overexpression of *acrAB* and *tolC* can be attributed to the *soxR* mutation.

Increased expression of *mdtG* in the *soxS*-overexpressing strains, NorE5 and JTG936. The microarray results showed significantly increased expression of the *mdtG* gene in NorE5. Further analysis by RT-PCR using the RNA extracts from PS5 and NorE5 corroborated this finding; *mdtG* was clearly overexpressed in NorE5 (Fig. 2A). Similarly, a further RT-PCR analysis of RNA extracts from strain GC4468, strain DJ901 (GC4468 Δ*soxRS*), and the constitutively *soxR* expressing strain JTG936 [GC4468 *soxR*(Con)] showed that *mdtG* is also upregulated in the *soxR*(Con) strain JTG936 (Fig. 2B). This suggests that *mdtG* is a member of the *soxS* regulon.

***mdtG*, a new gene activated by SoxS, MarA, and Rob.** To further characterize the regulation of *mdtG* expression, the *mdtG* promoter was cloned to obtain a *lacZ* transcriptional fusion. Strain M4450, a single-copy-number lysogen containing the whole *mdtG* promoter (starting 159 bp upstream of the ATG codon), was constructed. This strain was assayed for β-galactosidase activity in the absence and presence of PQ, SAL, and DIP. The results showed clear induction of the promoter in the presence of all three compounds, with maximum activity upon PQ induction (Table 5). Furthermore, a putative marbox sequence was found within the promoter re-
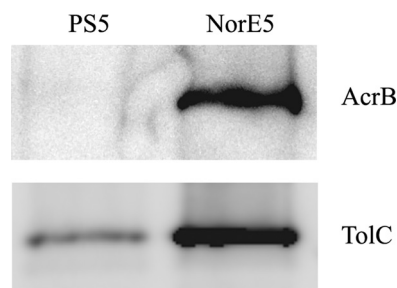

FIG. 1. Western blot analysis of PS5 and NorE5 using antibodies against AcrB and TolC.

TABLE 4. Constitutive expression of *fpr* in N5-F

Strain	Genetic background	β-Galactosidase activity (Miller units)[a]		Fold induction[b]
		−PQ	+ PQ	
GC4468		4.8	5.8	1.2
GC48-F	*fpr*::*lacZ*	2,149.1	19,744.6	9.2
PS5		5.3	6.0	1.1
P5-F	*fpr*::*lacZ*	3,460.8	26,360.7	7.6
NorE5		4.9	6.6	1.3
N5-F	*fpr*::*lacZ*	17,548.8	29,066.1	1.7

[a] − PQ, without PQ; + PQ, with the addition of 50 μM PQ.
[b] Calculated as the β-galactosidase activity of the *fpr* fusion in the presence of PQ divided by the value obtained in the absence of PQ.
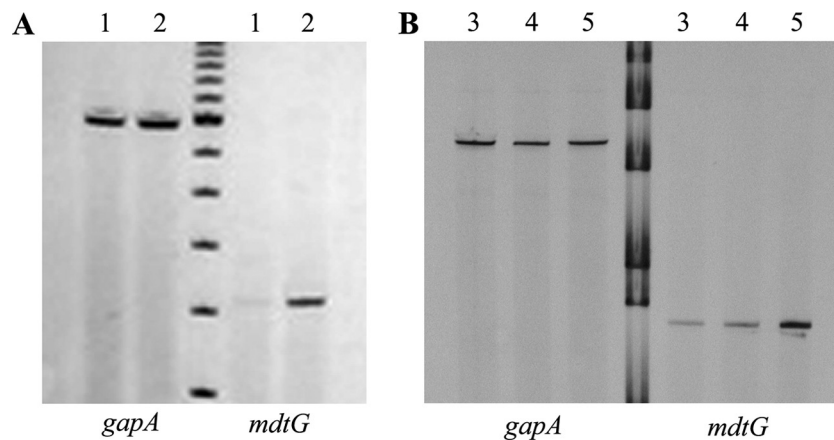
FIG. 2. RT-PCR analysis to detect the levels of expression of *mdtG* using RNA extracts from strains PS5 and NorE5 (A) and from GC4468, DJ901, and JTG936 (B). The *gapA* gene was the internal control used to detect if similar amounts of RNA were added for each strain assay. Lanes 1, PS5; lanes 2, NorE5; lanes 3, GC4468; lanes 4, DJ901; lanes 5, JTG936.

gion, in the backward orientation, 7 bp upstream of the −35 signal and 28 bp upstream of the −10 signal for RNA polymerase (RNP) (Fig. 3). This marbox is therefore a rare class I marbox like that found in the *acnA* promoter (21). A second *lacZ* fusion to the *mdtG* promoter, lacking two-thirds of the identified marbox sequence (97 bp upstream of the ATG codon), was made (Fig. 3), and the corresponding lysogen, strain 4452, was obtained. No significant induction of *mdtG* by PQ, SAL, or DIP was observed (Table 5). In order to confirm the identity of the marbox detected within the *mdtG* promoter, binding of the SoxS protein to the 20-bp oligonucleotide of the presumed marbox sequence was performed. As shown in Fig. 4, SoxS binds to this sequence with a dissociation constant of ~100 nM. Thus, *mdtG* is a new member of the *marA-soxS-rob* regulon. However, its role in stress response has yet to be determined.

DISCUSSION

This study has focused on the mechanisms of fluoroquinolone resistance acquired by an *E. coli* clinical strain after exposure to two selections with increasing concentrations of norfloxacin *in vitro*. Two mutations acquired within the target genes were described previously (42). The clinical isolate, PS5, harbored a mutation within GyrA (Ser83L) associated with a nalidixic acid resistance phenotype (MIC, 1,024 μg/ml) and decreased susceptibility to norfloxacin and ciprofloxacin (MICs,

TABLE 5. Activation of two *mdtG* promoters measured by *mdtG*::*lacZ* expression

Strain	Promoter length (bp)[a]	Uninduced β-galactosidase activity (Miller units)	Induction ratio[b]		
			+ PQ	+ SAL	+ DIP
M4450	159	16.5	4.3	3.4	2.0
M4452	97	10.5	1.1	2.2	1.3

[a] The length of the *mdtG* promoter sequence fused to the *lacZ* gene is the number of base pairs immediately upstream of the presumptive initiation codon (ATG).
[b] Calculated as the activity of the promoter in the presence of either PQ, SAL, or DIP divided by the activity for the uninduced control.

0.5 μg/ml). The norfloxacin-resistant strain selected *in vitro*, NorE5, acquired a second mutation, within ParC (Ser80Arg), during the two-stage selection process. The MICs of norfloxacin and ciprofloxacin showed 64- and 16-fold increases, reaching 32 μg/ml and 8 μg/ml, respectively. Previous studies established an association between the MIC and the number of target gene mutations. Ciprofloxacin MICs of 1 to 4 μg/ml have been associated with two target gene mutations (one in *gyrA* and one in *parC*) (35, 43). Since no other QRDR mutation was found in NorE5, we looked for mutations in other genes that could be responsible for the higher MIC of ciprofloxacin observed for this strain.

A comparative study of gene expression between PS5 and NorE5 was performed using microarrays of cDNA. In agreement with the outer membrane protein profile obtained in the previous study (42), the results revealed significantly decreased expression of *ompF* in NorE5. The role that reduced expression of porins, such as OmpF and OmpC, plays in conferring fluoroquinolone resistance has been reported previously (5, 7, 9). In terms of efflux pumps, the microarray results showed increased expression of *acrAB*, >2-fold. Furthermore, Western blotting corroborated this finding for AcrB and extended it to include increased expression of TolC. The AcrAB-TolC pump is the main efflux pump detected in *Enterobacteriaceae*; its overexpression contributes not only to increasing levels of resistance to quinolones but also to increasing levels of resistance to other, unrelated drugs, such as chloramphenicol, tetracycline, β-lactams, trimethoprim, and erythromycin, but not aminoglycosides (7, 10). Thus, the fluoroquinolone resistance and MDR phenotypes observed in NorE5, representing increases of ≥4- to 16-fold in the MICs of the affected antibiotics, are likely explained by these findings.

Regulatory mechanisms that decrease OmpF expression and increase AcrAB and TolC expression have been elucidated (10). Three members of the AraC/XlyS family of transcriptional activators, SoxS, MarA, and Rob, have been reported to activate those genes containing a marbox in their promoters (25). The marbox is the sequence where these activators bind to interact with RNA polymerase and activate the transcription of the genes of the regulon. The promoters of the *acrAB*
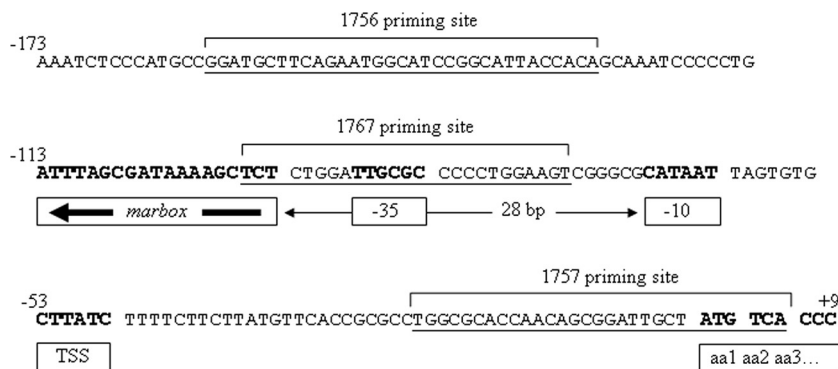
FIG. 3. Sequence of the *mdtG* promoter showing the relationship of the inferred marbox to the putative −35 and −10 RNP signals, the transcriptional start site (TTS), and the first amino acid of the coding sequence (aa1) (boldface). The backward orientation of the marbox is indicated by the leftward heavy arrow inside the box. The distance between the −10 signal and the marbox is given in the middle of the lightface arrow. The underlined sequences represent the indicated primer binding sites.

operon, the *tolC* gene, and the antisense mRNA *micF* (which blocks *ompF* translation [28]) contain marboxes (21). The microarray results of this study revealed significantly increased *soxS* expression, > 8-fold, in NorE5, in addition to slightly increased expression of *marA*, >2-fold. Since the *marRAB* operon also contains a marbox in its promoter (23), the elevated SoxS activity could activate *marRAB* and increase *marA* expression (15, 27).

To determine the basis of the elevated SoxS expression, the *soxRS* region was sequenced. The results showed an insertion of two adenines in *soxR* in NorE5 but not in PS5. This insertion should cause a frameshift at amino acid 134 and transcription termination 7 codons later, leading to a truncated protein. Other mutations within the C-terminal domain of SoxR have been shown to render SoxS constitutively active in the absence of the redox signals that are normally required to activate wild-type SoxR protein and lead to constitutive expression of *soxS* (19, 31, 47). Constitutive expression of SoxS due to an in-frame internal deletion of SoxR amino acids 136 to 144 (affecting the last 19 amino acids) has been described (31). A second mutation within SoxR, Gly74Arg, found in both PS5 and NorE5, has also been found in several *soxS*-overexpressing clinical isolates, but accompanied by a second mutation within the same locus, Thr38Ser (19). It seems likely that the

Gly74Arg change plays little or no role in the overexpression of SoxS, since the levels of AcrB expression in PS5 were very similar to those in GC4468 by Western blot analysis (data not shown).

The most likely explanation is that the frameshift is responsible for constitutive activity of SoxR leading to constitutive expression of *soxS* and hence an upregulation of the genes that belong to the regulon. However, due to the facts that *soxR* and *soxS* are divergently expressed from the *soxRS* regulon and the *soxS* promoter is within the intergenic region (47), the hypothesis that the two nucleotide insertions within *soxR* may lead to a new promoter responsible for the constitutive expression of *soxS* was considered. To test this hypothesis, the intergenic region, including the partial sequence of *soxR* where the nucleotide insertions were detected, was amplified from NorE5 and also from PS5. Both PCR products were digested and cloned into the pRS551 vector in order to assess its putative promoter activity. The corresponding assays of β-galactosidase activity revealed no significant difference in activity between the sequences (data not shown). Thus, the insertions did not create a new *soxS* promoter.

The constitutivity of the *soxS* expression was further demonstrated by monitoring the behavior of an *fpr*::*lacZ* transcriptional fusion in strains GC4468, PS5, and NorE5. As expected for a regulon promoter, expression of the *fpr* promoter was 5-fold greater in NorE5 than in the parental strain, PS5 (Table 4). Furthermore, when these cells were treated with PQ to activate SoxR, all three strains showed similar high levels of activity, strongly indicating that the SoxR protein is already in an activated state in NorE5.

This study has also revealed a new member of the *marA*-*soxS*-*rob* regulon, *mdtG*. *mdtG* is inducible by PQ, SAL, and DIP, and SoxS binds tightly to the marbox sequence reported within the *mdtG* promoter. Moreover, disruption of this marbox sequence was accompanied by a loss of inducibility by all three compounds. The MdtG protein, also named YceE, appears to be a member of the major facilitator superfamily of transporters, and it has been reported, when overexpressed, to increase fosfomycin and deoxycholate resistances by 4- and 2-fold, respectively (30). What other roles it may play in antibiotic resistance is not known.
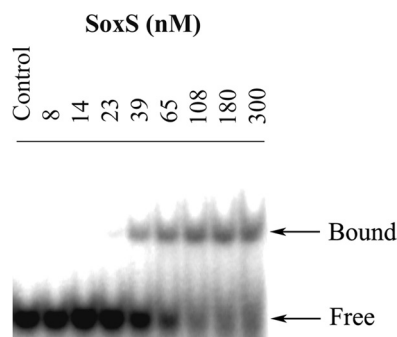

FIG. 4. Radioautogram of a 6% acrylamide gel demonstrating the binding of SoxS to the ^{32}P-labeled 20-bp fragment AGAGCTTTTAT CGCTAAATC (the marbox of *mdtG*). The multiple SoxS concentrations (nM), and the unbound DNA (free) and SoxS-complexed DNA (bound), are indicated.

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