Constitutive SoxS expression in a fluoroquinolone resistant strain with a truncated SoxR; identification of a new member of the marA/SoxS/rob regulon, mdtG

Anna Fàbrega¹, Robert G. Martin², Judah L. Rosner², M. Mar Tavio³, and Jordi Vila¹*

¹ Department of Microbiology, Hospital Clínic, School of Medicine, University of Barcelona, Spain

² Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-0560, USA

³ School of Health Science, University of Las Palmas de Gran Canaria, 35016, Spain

*Corresponding author. Mailing address: Servei de Microbiologia, Centre de Diagnòstic Biomèdic, Hospital Clínic, Facultat de Medicina, Universitat de Barcelona, Villarroel 170, 08036 Barcelona, Spain. Phone: (+34) 93 227 55 22. Fax: (+34) 93 227 93 72. Email: jvila@ub.edu

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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 using Western blots 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 RT-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 the loss of inducibility by MarA, SoxS and Rob. Thus, chromosomal mutations in parC and *soxR* are responsible for the increased antibiotic resistance of NorE5.
INTRODUCTION

*Escherichia coli* is the most frequent pathogen isolated from 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 values of >40% and >20%, respectively, in countries like 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 have been studied in detail in *E. coli* strains. 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 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 a 32- and 10-fold increase 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 the quinolone resistance levels (16). At this step, a multidrug resistance phenotype (MDR) 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 in Enterobacteriaceae which acts in conjunction with TolC (11, 32, 34). Five regulators have been described so far that exert a role on AcrAB expression (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, 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 mutations 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 bile salt, decanoate or 2,2’-dipyridyl (4, 36, 38). However, clinical significance has only been associated with 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 base pair (bp) asymmetric sequence with the degenerate consensus
sequence AYNGCACNNWNRYAAAYN (N = any base; R = A/G; W = A/T; Y = C/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 autoactivable) (21). SoxS and MarA bind to the marbox of different genes and activate them to different extents, such as the fpr promoter, which is more susceptible to activation by SoxS than 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 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 paraquat (PQ), 5 mM sodium salicylate (SAL) or 5 mM 2,2’-dipyridyl (DIP) and 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) in MH plates according to the manufacturer’s recommendations.
Microarray analysis. Total RNA from PS5 and NorE5 was extracted from a mid-exponential phase culture (OD600 of 0.6) using Qiagen 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 (Millipore, Billerica, MA), Cy-3- and Cy-5-labelled cDNA samples were mixed with SSC (2.5x final; 1x SSC=0.15M NaCl, 0.015M trisodium citrate, pH 7), sodium dodecyl sulphate (0.25%) and 40 µg of E. coli rRNA (Boehringer Mannheim, Ingelheim, Germany) in a final volume of 16 µL and hybridized with the DNA microarray for 5h at 65ºC. The DNA microarray contained 4058 open reading frames (ORFs) representing 95% of E. coli ORFs, 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 10 µm per pixel resolution. The resulting 16-bit TIFF images were analyzed using SCANALYZE software (http://rana.stanford.edu/software/). The reproducibility of the technique was assessed in two separate experiments. A normalized relative Cy5/Cy3 ratio >2 was considered as a significant increase in expression and a normalized relative Cy3/Cy5 ratio >2 was considered as 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 and aerated at 37ºC until strains reached OD600 values of 0.5-0.6. Three mL were 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 and pH 8.0) supplemented with 3 mg/mL lysozyme and vortexed, followed by an incubation at 32°C for 10 min with shaking. The RNA extraction was performed using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer’s recommendations. Samples were subsequently treated with DNA-free DNase (Ambion, Austin, TX, USA) 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, USA) according to the manufacturer’s recommendations and the following primers: gapA.1 (GTATCAACGGTTTGGCCG) and gapA.2 (AGCTTTAGCAGCACC GGTA) for gapA, and yceE.RT1 (GCCAGTTCCGCGGACATA C) and yceE.RT2 (CTGCGGGGCCTTTCTGTTACTTT) 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.

**Sequencing of the soxRS region.** The whole soxRS region of strains GC4468, PS5 and NorE5 was amplified by PCR using the same primers as previously described (soxRS.F GGCGAAGCTTCCGCAGGTGTATGC and soxRS.R CGTCGGGGGAAGCTTTCCCTGGTACCC) (19). The amplified fragments were directly used for sequencing and compared for detection of mutations.
Western blotting. Bacterial strains were grown overnight in 50 mL LB 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 each cycle of sonication followed by 30 s of rest) with an amplitude of 50%. Cell debris were removed by centrifugation for 20 min at 4ºC and 3500 rpm and the supernatant was collected and centrifuged again for 90 min at 4ºC and 16000 rpm. The final pellet was resuspended in 1x PBS (Roche, Mannheim, Germany). Protein quantification was performed using the RC DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) following the manufacturer’s indications.

Ten µg of each protein sample were loaded onto an 8% SDS-PAGE gel (Mini Protean II). Transfer from gel onto a nitrocellulose membrane was performed for 2 h at 60 V on ice. The membranes were blocked using 1x PBS containing Tween 20 diluted 1/2000 (PBS-T) and 5% skim milk for 1 h at RT, followed by an overnight incubation at 4ºC with the primary antibodies against AcrB and TolC proteins (Antibody Bcn, Barcelona, Spain) diluted 1/500 into PBS-T. The membranes were washed 3 times with PBS-T and once with PBS before secondary antibody, anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK), diluted 1/2000 in PBS-T, was added for 1 h incubation at RT. The membranes were washed as previously described and processed using EZ-ECL (Biological Industries, Kibbutz Beit Haemek, Israel) for chemiluminescence detection of bands in a Fuji LAS-3000 equipment.

DNA manipulations. Strains GC48-F, P5-F and N5-F were derived from parental strains GC4468, PS5 and NorE5 by transformation with a pRS551 plasmid (40)
harboring the *fpr*:lacZ transcriptional fusion, respectively. 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 template. The *mdtG* fragment was 165 bp long (from -159 to +6, relative to the ATG) and was amplified with primers

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\text{(AAAAAAGAATTCCGATGCTTCAGAATGGCATCCGGCATTACCACA, the EcoRI site is underlined) and (AAAAAGGATCCTGACATAGCAATCCGCTGTTGGTGCGCCA, the BamHI site is underlined). A shorter fusion was also constructed, containing the 104 bp fragment}
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\text{mdtG77 (from -98 to +6) using primers (AACCAAGAATTC TCTCTGGATTGCGCCCCCTGGAAGT, the EcoRI site is underlined) and (AACCAAGAATTCTCTCTGGATTGCGCCCCCTGGAAGT, the EcoRI site is underlined) and 1757. The amplified DNA fragments were digested with EcoRI and BamHI and ligated to the similarly cut vector pRS551. Recombinant plasmids were isolated in DH5α cells by selection for ampicillin resistance and 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.}
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**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 hour with PQ, SAL or DIP where indicated and at the above mentioned concentrations, were done...
as previously reported (38,39). All assays were carried out twice in duplicate and agreed to within 15%.

**DNA-binding assays.** SoxS was overexpressed using the soxS gene cloned in a pET15b vector (pRGM9948) by induction with IPTG of strain M9948 (21), purified to homogeneity, and the histidine tag 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, USA) according to the manufacturer’s recommendations, and annealed with a complementary 20 bp oligonucleotide. Trace quantities (approximately 10 fmoles/sample) of the radiolabeled oligonucleotide in 9 µl of buffer containing 1x TAE buffer in 25% glycerol, 50 fmoles 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 Figure 4. The samples were subjected to electrophoresis at 150V for 35 minutes. The amounts of unbound and SoxS-complexed DNA were quantified by analysis on a Molecular Dynamics Phosphoimager.

**RESULTS**

**Fluoroquinolone resistance acquisition and multidrug resistance (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 a significant increase 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 susceptibility levels of amikacin remained unchanged.

Changes in gene expression as determined by microarray and Western blot analyses. To determine the basis of this MDR, gene expression of strains PS5 and NorE5 was studied using microarrays of cDNA. In comparison to PS5, NorE5 overexpressed acrA and acrB by approximately 2.5-fold. Since these genes encode two components of the very important AcrAB-TolC drug efflux pump, this suggested a likely explanation for the MDR phenotype of NorE5. The ratios of increased expression for each gene, with 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 gels (Fig. 1). The AcrAB-TolC pump does not efflux aminoglycosides, consistent with the unchanged MIC for 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 was decreased 7.8-fold, in agreement with the previous study which 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 up-regulated by approximately 2-fold in NorE5 (Table 3). This gene has been reported to encode a putative transport protein and 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 region of GC4468, PS5 and NorE5 to detect any mutation that may have been acquired during the selection process 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 the nucleotide position 402, was only detected in NorE5. This second change in soxR encodes a frameshift starting at position Lys134 and generates a stop codon (TAG) seven codons later. This would remove the last 21 amino acids of the C-terminus of SoxR. No other amino acid change within SoxS or 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 a pRS551 plasmid carrying the SoxS-activable fpr::lacZ transcriptional fusion. These strains (GC48-F, P5-F and N5-F) were treated without or with PQ for 1 h and 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 in comparison to the values 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 as in the absence 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 strains GC4468, DJ901 (GC4468 Δ*soxRS*) and the *soxR* constitutive strain JTG936 (GC4468 *soxR*<sup>c</sup>) showed that *mdtG* is also up-regulated in the *soxR*<sup>c</sup> 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 lysogen containing the whole *mdtG* promoter (starting 159 bp upstream of the ATG), 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, obtaining maximum activity upon PQ induction (Table 5). Furthermore, a putative marbox sequence was found within the promoter region, 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 was made lacking two thirds of the identified marbox sequence (97 bp upstream the ATG) (Fig. 3), and the corresponding lysogen, strain 4452, was obtained. No significant
induction of \textit{mdtG} by PQ, SAL or DIP was observed (Table 5). In order to confirm the identity of the \textit{marbox} detected within the \textit{mdtG} promoter, binding of the SoxS protein to the 20 bp oligonucleotide of the presumed \textit{marbox} sequence was performed. As shown in Fig. 4, SoxS binds to this sequence with a dissociation constant of \(~100\) nM. Thus, \textit{mdtG} is a new member of the \textit{marA/soxS/rob} regulon. However, its role in stress-response is yet to be determined.

**DISCUSSION**

This study has focused on the mechanisms of fluoroquinolone resistance acquired by an \textit{E. coli} clinical strain after exposure to two selections of increasing concentrations of norfloxacin \textit{in vitro}. Two mutations acquired within the target genes were previously described (42). The clinical isolate, PS5, harbored a mutation within GyrA (Ser83L) associated with a nalidixic acid resistance phenotype (MIC of 1024 \(\mu\)g/mL) and decreased susceptibility to norfloxacin and ciprofloxacin (MICs of 0.5 \(\mu\)g/mL). The norfloxacin resistant strain selected \textit{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 MICs of 32 \(\mu\)g/mL and 8 \(\mu\)g/mL, respectively. Previous studies established an association between the MIC and the number of target gene mutations. MICs of ciprofloxacin of 1-4 \(\mu\)g/mL have been associated with two target gene mutations (one in \textit{gyrA} and one in \textit{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 in 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 performed in the previous study (42), the results revealed a 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 previously been reported (5,7,9). In terms of efflux pumps, the microarray results showed increased expression of acrAB of >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 whose overexpression contributes not only to increasing the levels of resistance to quinolones but also 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 an increase 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 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 operon, the tolC gene, as well as the antisense mRNA micF (which blocks ompF translation (28)), contain a marbox (21). The microarray results of this study revealed a significantly increased expression of soxS of >8-fold in NorE5 in addition to slightly increased expression of marA of >2-fold. Since the marRAB operon also contains a marbox in its promoter (23), the elevated SoxS activity could activate marRAB and increase expression of marA (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 present in NorE5 but not in PS5. This insertion should cause a frameshift at amino acid 134 and
transcription termination seven codons later leading to a truncated protein. Other mutations within the C-terminus 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 of 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 of PS5 were very similar to those of GC4468 in a Western blotting 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 up-regulation of the genes that belong to the regulon. However, due to the fact that soxR and soxS are divergently expressed from the soxRS regulon and the soxS promoter is within the intergenic region (47), the hypothesis was considered that the two nucleotide insertions within soxR may lead to a new promoter responsible for the constitutive expression of soxS. 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 in 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 fpr promoter was 5-fold greater in NorE5 than in the parental 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 the inducibility by all three compounds. The MdtG protein, also named YceE, appears to be a member of the major facilitator superfamily of transporters and, when overexpressed, it has been reported to increase fosfomycin and deoxycholate resistances by 4- and 2-fold, respectively (30). What other roles it may play in antibiotic resistance are not known.

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Figure Legends

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

FIG. 2. RT-PCR analysis to detect the levels of expression of \textit{mdtG} using RNA extracts from strains PS5 and NorE5 (A) and from GC4468, DJ901 and JTG936 (B). The \textit{gapA} gene was the internal control used to detect if similar amounts of RNA were added for each strain assays. Lane 1, PS5; Lane 2, NorE5; Lane 3, GC4468; Lane 4, DJ901; Lane 5, JTG936.

FIG. 3. Sequence of the \textit{mdtG} promoter showing the relationship of the inferred \textit{marbox} to the putative -35 and -10 RNP signals, the transcriptional start site (TTS) and the first amino acid of the coding sequence (aa1) (shown in bold). The backward orientation of the \textit{marbox} is indicated by the leftward heavy arrow inside the box. The distance between the -10 signal and the \textit{marbox} is indicated by the number in the middle of the small arrow. The underlined sequences represent the indicated primer binding sites.

FIG. 4. Radioautogram of a 6% acrylamide gel demonstrating the binding of SoxS to the $^{32}$P-labeled 20 bp fragment AGAGCTTTATCGCTAAATC (the \textit{marbox} of \textit{mdtG}). The multiple SoxS concentrations (nM), and the unbound DNA (free) or SoxS-complexed DNA (bound) are indicated.
**FIG. 3**

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mgtG
AAATCTCCCATGCCCAGATGGTTAGAAATGCACTGGGATTACACACGCAAATCCGGCTG
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**FIG. 4**

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1756 priming site
CTTATC TTTCTCTTTATGCTACGGGCGCTGGGGGACCACGACGCGATTGCATG TCA CCC
TSS
1757 priming site
ATTTAGCGATAAAGCTCTCTGATTTGCGCCGGGGAAATGCGGGGCTATAAT TAGTTG
```

<table>
<thead>
<tr>
<th>SoxS (nM)</th>
<th>0</th>
<th>14</th>
<th>23</th>
<th>39</th>
<th>65</th>
<th>108</th>
<th>180</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![SoxS Gel Electrophoresis](image-url)

- Bound
- Free
### TABLE 1. Bacteria, plasmids and phages.

<table>
<thead>
<tr>
<th>Bacteria, plasmid or phage</th>
<th>Parent strain</th>
<th>Relevant genotype and/or characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS5</td>
<td>E. coli</td>
<td>clinical isolate susceptible to fluoroquinolones</td>
<td>(42)</td>
</tr>
<tr>
<td>NorE5</td>
<td>Fluoroquinolone resistant mutant selected from PS5 with norfloxacin</td>
<td>(42)</td>
<td></td>
</tr>
<tr>
<td>GC4468</td>
<td>E. coli K-12 (ΔsoxRS+)</td>
<td>(12)</td>
<td></td>
</tr>
<tr>
<td>D3901</td>
<td>GC4468</td>
<td>ΔsoxRS</td>
<td>(12)</td>
</tr>
<tr>
<td>JTG936</td>
<td>GC4468</td>
<td>soxRe</td>
<td>(12)</td>
</tr>
<tr>
<td>GC48-F</td>
<td>GC4468</td>
<td>pRS551: fpr::lacZ</td>
<td>this study</td>
</tr>
<tr>
<td>P5-F</td>
<td>PS5</td>
<td>pRS551: fpr::lacZ</td>
<td>this study</td>
</tr>
<tr>
<td>N5-F</td>
<td>NorE5</td>
<td>pRS551: fpr::lacZ</td>
<td>this study</td>
</tr>
<tr>
<td>M4450</td>
<td>GC4468</td>
<td>mdtG67::lacZ (-159 to +6)</td>
<td>this study</td>
</tr>
<tr>
<td>M4452</td>
<td>GC4468</td>
<td>mdtG77::lacZ (-98 to +6)</td>
<td>this study</td>
</tr>
<tr>
<td>M9948</td>
<td>834(D3lysS)</td>
<td>pRGM9948</td>
<td>(21)</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pRS551</td>
<td>pBR322 derivative; AmpR</td>
<td>(40)</td>
<td></td>
</tr>
<tr>
<td>pRGM9948</td>
<td>pET15b carrying the soxS gene; AmpR</td>
<td>(21)</td>
<td></td>
</tr>
<tr>
<td>Phage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>λRS45</td>
<td>λimm21: KanR</td>
<td></td>
<td>(40)</td>
</tr>
</tbody>
</table>
# TABLE 2. Mutations detected within the quinolone resistance-determining regions and MIC determinations of the strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>AA Substitution</th>
<th>MIC (µg/mL)</th>
<th>GyrA</th>
<th>ParC</th>
<th>NOR</th>
<th>CIP</th>
<th>NAL</th>
<th>CHL</th>
<th>TET</th>
<th>AMX</th>
<th>ERY</th>
<th>TMP</th>
<th>AMK</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS5</td>
<td>S83L&lt;sup&gt;a&lt;/sup&gt;</td>
<td>---&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.5</td>
<td>0.5</td>
<td>1024</td>
<td>8</td>
<td>2</td>
<td>48</td>
<td>64</td>
<td>0.5</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NorE5</td>
<td>S83L S80R</td>
<td>32  8</td>
<td>2048</td>
<td>64</td>
<td>8</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>8</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> AA, Amino Acid.

<sup>b</sup> NOR, norfloxacin; CIP, ciprofloxacin; NAL, nalidixic acid; CHL, chloramphenicol; TET, tetracycline; AMX, amoxicillin; ERY, erythromycin; TMP, trimethoprim; AMK, amikacin.

<sup>c</sup> Data from reference 38.

<sup>d</sup> S, serine; L, leucine; R, arginine.

<sup>e</sup> ---, no mutation found.
### TABLE 3. Altered gene expression in NorE5 determined by microarrays

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Fold change&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Up-regulated</strong></td>
<td></td>
</tr>
<tr>
<td><em>soxS</em></td>
<td>Transcriptional activator of regulator of superoxide response regulon</td>
</tr>
<tr>
<td><em>marA</em></td>
<td>Transcriptional activator of multiple antibiotic resistance</td>
</tr>
<tr>
<td><em>acrA</em></td>
<td>MDR efflux membrane fusion protein</td>
</tr>
<tr>
<td><em>acrB</em></td>
<td>MDR efflux pump</td>
</tr>
<tr>
<td><em>mdtG</em></td>
<td>predicted drug efflux pump</td>
</tr>
<tr>
<td><strong>Down-regulated</strong></td>
<td></td>
</tr>
<tr>
<td><em>ompF</em></td>
<td>outer membrane porin 1a</td>
</tr>
</tbody>
</table>

<sup>a</sup> The ratio of expression in the NorE5 to PS5 strains from microarray analysis is shown for three independent experiments.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genetic background</th>
<th>β-galactosidase (Miller units)</th>
<th>Fold Induction&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>- PQ&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+ PQ&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GC4468</td>
<td>****</td>
<td>4.8</td>
<td>5.8</td>
</tr>
<tr>
<td>GC48-F</td>
<td><em>fpr::lacZ</em></td>
<td>2,149.1</td>
<td>19,744.6</td>
</tr>
<tr>
<td>PS5</td>
<td>****</td>
<td>5.3</td>
<td>6.0</td>
</tr>
<tr>
<td>P5-F</td>
<td><em>fpr::lacZ</em></td>
<td>3,460.8</td>
<td>26,360.7</td>
</tr>
<tr>
<td>NorE5</td>
<td>****</td>
<td>4.9</td>
<td>6.6</td>
</tr>
<tr>
<td>N5-F</td>
<td><em>fpr::lacZ</em></td>
<td>17,548.8</td>
<td>29,066.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> The fold induction is the β-galactosidase activity of the *fpr* fusion in the presence of PQ divided by the values obtained in the absence of PQ.

<sup>b</sup> – PQ, no PQ; + PQ, addition of 50 µM of PQ.
TABLE 5. Activation of two mdtG promoters measured by mdtG::lacZ expression.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Promoter length$^a$</th>
<th>β-galactosidase (Miller units)</th>
<th>Induction ratio$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Uninduced + PQ + SAL + DIP</td>
<td></td>
</tr>
<tr>
<td>M4450</td>
<td>159 bp</td>
<td>16.5</td>
<td>4.3  3.4  2.0</td>
</tr>
<tr>
<td>M4452</td>
<td>97 bp</td>
<td>10.5</td>
<td>1.1  2.2  1.3</td>
</tr>
</tbody>
</table>

$^a$ The length of the mdtG promoter sequence fused to the lacZ gene is the number of bp immediately upstream of the presumptive initiation codon (ATG).

$^b$ The induction ratio is the activity of the promoter in the presence of either PQ, SAL or DIP divided by the uninduced control.