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Constitutive *soxR* Mutations Contribute to Multiple-Antibiotic Resistance in Clinical *Escherichia coli* Isolates

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The *soxRS* regulon of *Escherichia coli* and *Salmonella enterica* is induced by redox-cycling compounds or nitric oxide and provides resistance to superoxide-generating agents, macrophage-generated nitric oxide, antibiotics, and organic solvents. We have previously shown that constitutive expression of *soxRS* can contribute to quinolone resistance in clinically relevant *S. enterica*. In this work, we have carried out an analysis of the mechanism of constitutive *soxS* expression and its role in antibiotic resistance in *E. coli* clinical isolates. We show that constitutive *soxS* expression in three out of six strains was caused by single point mutations in the *soxR* gene. The mutant SoxR proteins contributed to the multiple-antibiotic resistance phenotypes of the clinical strains and were sufficient to confer multiple-antibiotic resistance in a fresh genetic background. In the other three clinical isolates, we observed, for the first time, that elevated *soxS* expression was not due to mutations in *soxR*. The mechanism of such increased *soxS* expression remains unclear. The same *E. coli* clinical isolates harbored polymorphic *soxR* and *soxS* DNA sequences, also seen for the first time.

Microbial antibiotic resistance has become a major clinical problem worldwide. Many hospital- and community-acquired infections that were easy to treat with the introduction of antibiotics have now become problematic because the bacteria causing them have developed resistance to the most commonly used antibiotics (5, 39). Understanding the mechanisms that underlie the development of antibiotic resistance will aid in designing strategies to overcome this problem.

Bacteria acquire antibiotic resistance in several different ways, many of them specific to individual antibiotics or classes of antibiotics. Plasmid- or transposon-borne genes confer high-level resistance to specific antibiotics (39) by expressing enzymes that inactivate the antibiotic or alter its target in the cell (e.g., β-lactamases or rRNA methylases, respectively) or antibiotic-specific efflux pumps that eliminate the antibiotic from the cell (e.g., the tetracycline efflux pump). Chromosomal mutations can alter the antibiotic's cellular target to render it insensitive to the antibiotic, as in *gyrA* or *parC* mutations encoding quinolone-resistant DNA gyrase subunit A or topoisomerase IV (39). Recognized more recently, regulatory mutations in chromosomal genes confer resistance to multiple, structurally unrelated antibiotics by alleviating the antibiotic burden in the cell through decreased influx combined with increased efflux of diverse drugs. This type of resistance is often called intrinsic antibiotic resistance (30).

In *Escherichia coli* and *Salmonella enterica*, one such chromosomally encoded genetic system that confers low-level multiple-antibiotic resistance is the *soxRS* regulon (3, 14, 28, 29). In this system, the SoxR protein is activated by oxidation (8–10, 12, 20, 25, 33) or nitrosylation (6) to trigger transcription of the *soxS* gene. Through increased expression, the SoxS protein is the direct activator of genes for resistance to both oxidants and antibiotics (37). SoxS is known to control several genes in *S. enterica* (36) and 65 to 80 genes in *E. coli* (26, 35, 42). The *micF* and *acrAB* genes are among these SoxS-regulated genes and are the main effectors of *soxRS*-mediated antibiotic resistance. Increased SoxS expression leads to increased expression of the antisense RNA *micF* (3, 29), down-regulation of the outer membrane porin OmpF, and a decrease in cell permeability. Increased SoxS expression also induces expression of the *acrAB*-encoded efflux pump (29, 30, 41) and a concurrent increase in efflux pump activity. This combination of increased efflux pump activity and decreased cell permeability results in *soxRS*-mediated antibiotic resistance.

In laboratory strains of *E. coli* and *S. enterica*, activation of the *soxRS* regulon with paraquat (PQ) treatment or by expression of constitutive SoxR (SoxR^c) proteins increases resistance to ampicillin, nalidixic acid, chloramphenicol, and tetracycline two- to fourfold (3). This moderate, multiple-antibiotic resistance is a hallmark of *soxRS*-mediated mechanisms.

The *soxRS* regulon is also connected to antibiotic resistance in clinical strains. We previously showed (24) that constitutive *soxS* expression contributed significantly to the quinolone resistance of an *S. enterica* clinical isolate, caused by a *soxR* mutation that evidently arose during clinical treatment. Also, Oethinger et al. (34) found that 16% of fluoroquinolone-resistant, organic solvent-resistant clinical *E. coli* isolates exhibited constitutive *soxS* expression. In a similar vein, Webber and Piddock (40) reported that 28% of fluoroquinolone-resistant clinical and veterinary *E. coli* isolates exhibited constitutively elevated *soxS* expression. However, the mechanism of increased *soxS* expression and its role in antibiotic resistance were not investigated in these two studies.

In the work presented here, we have analyzed the mechanism of constitutive *soxS* expression and its role in antibiotic resistance in a variety of *E. coli* clinical isolates. We show that constitutive *soxS* expression in three of six strains studied was

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
Strains		
GC4468	*E. coli* K-12 *soxRS*$^+$	32
JTG936	GC4468 *soxR*^{c}	14
JTG1052	GC4468 *soxR*^{c}	13
EH46	GC4468 *soxRS*$^-$ *soxS*::*lacZ*	19
TN402	GC4468 *soxR*$^-$ *soxS*$^+$	T. Nunoshiba, gift
E3	*E. coli* clinical isolate	34
E17	*E. coli* clinical isolate	34
E19	*E. coli* clinical isolate	34
M1	*E. coli* clinical isolate	34
I236	*E. coli* clinical isolate	40
I237	*E. coli* clinical isolate	40
I242	*E. coli* clinical isolate	40
I243	*E. coli* clinical isolate	40
I244	*E. coli* clinical isolate	40
I246	*E. coli* clinical isolate	40
I248	*E. coli* clinical isolate	40
I251	*E. coli* clinical isolate	40
I253	*E. coli* clinical isolate	40
I254	*E. coli* clinical isolate	40
Plasmids		
pACYC177	Vector (Ap^r)	New England Biolabs
pSE380	Vector (Ap^r)	Invitrogen
pSXR	*E. coli* wild-type *soxR* in pSE380 (Ap^r)	31
pAKWT	*E. coli* GC4468 *soxR* in pACYC177 (Ap^r)	This work
pAKE3	*E. coli* E3 *soxR* in pACYC177 (Ap^r)	This work
pAKE17	*E. coli* E17 *soxR* in pACYC177 (Ap^r)	This work
pAKE19	*E. coli* E19 *soxR* in pACYC177 (Ap^r)	This work
pAKM1	*E. coli* M1 *soxR* in pACYC177 (Ap^r)	This work
pAKI237	*E. coli* I237 *soxR* in pACYC177 (Ap^r)	This work

caused by point mutations in the *soxR* gene. In the other three isolates elevated *soxS* expression was not due to *soxR* mutations, and the mechanism of such increased *soxS* expression remains unclear. The mutant SoxR proteins contributed to the multiple-antibiotic resistance phenotypes of the clinical strains and were sufficient to confer multiple-antibiotic resistance when expressed in a fresh genetic background. We also show that the *soxRS* region in some of the *E. coli* clinical isolates is characterized by extensive DNA sequence polymorphisms.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in these studies, along with their relevant properties and source descriptions, are listed in Table 1. Bacteria were cultured overnight in Luria-Bertani (LB) broth (27) at 37°C, unless otherwise stated.

RNA extraction and Northern blotting. Overnight bacterial cultures were diluted 1/100 in fresh LB broth and grown with vigorous shaking (250 rpm) for 90 min at 37°C or for 105 min at 30°C, as indicated. At that point, the cultures were split, and the appropriate samples were treated with 1.3 mM PQ. Shaking at 37°C or 30°C and 250 rpm was continued for another 30 to 40 min, and RNA was harvested from 1.5-ml aliquots of culture using the RNeasy Mini kit from QIAGEN. RNA yield was estimated by measuring absorption at 260 nm and 280 nm. The appropriate amount of RNA was electrophoresed on 1.5% agarose gels containing 2% formaldehyde and 0.07 μg/ml ethidium bromide. The RNA was transferred overnight to a nylon membrane using the TurboBlotter downward transfer system (Schleicher & Schuell). The membranes were hybridized with appropriate ^{32}P-labeled probes, and gene expression was quantified on a phosphorimager (Bio-Rad). Hybridization with a ^{32}P-labeled 16S RNA probe was used to control for loading differences.

Sequencing of the *soxRS* regions from *E. coli* clinical isolates. A 1-kb fragment corresponding to the *soxRS* region was amplified by PCR from the genomic DNA of the *E. coli* clinical isolates. The forward primer was 5′-GGCGAAGCTTCCGCAGGTGTTTATGC-3′ and the reverse primer was 5′-CGTCGGGGGAAGCTTTCCTGTGTACC-3′ (regions corresponding to, respectively, bp 284 to 298 downstream of *soxS* in the GenBank deposited sequence and bp 1281 to 1271 downstream of *soxR* are underlined). The amplified fragments were gel purified, and both strands were sequenced at the High-Throughput Sequencing Facility of the Dana-Farber/Harvard Cancer Center.

Cloning of *soxR* from *E. coli* clinical isolates. A 600-bp fragment containing the *soxR* gene and its promoter was amplified by PCR from the genomic DNA of the *E. coli* clinical isolates. The forward primer was 5′-GCCGCTCGAGCTGAATAATTTTCTGATGGG-3′ (region corresponding to *soxS* bp 641 to 660 downstream of *soxR* is underlined) and contained an XhoI site. The reverse primer was the same one used for sequence analysis and contained a HindIII site. After digestion with these enzymes, the PCR fragments were cloned into vector pCR2.1-TOPO using the TOPO TA cloning kit from Invitrogen. Successful clones were confirmed by restriction digestion analysis. Several clones for each mutant were sequenced to verify that no mutations were introduced by the PCR procedure. The cloned *soxR* alleles were then subcloned under the control of their own promoters into pACYC177 (New England Biolabs) using restriction enzymes XhoI (New England Biolabs) and HindIII (New England Biolabs). The following plasmids were obtained: pAK-WT, which contains the wild-type *soxR* allele, and pAK-E3, pAK-E17, pAK-E19, pAK-M1, and pAK-I237, which contain, respectively, the *soxR* alleles from strains E3, E17, E19, M1, and I237 (Table 1). Subcloning was verified by restriction digestion analysis.

Transcriptional activity of cloned *soxR* alleles. Plasmids pACYC177, pAK-WT, pAK-E3, pAK-E17, pAK-E19, pAK-M1, and pAK-I237 were introduced into strain EH46 (Δ*soxR soxS*::*lacZ*) (Table 2), and the transcriptional activity of the expressed SoxR proteins was assayed by measuring expression of β-galactosidase directed by the reporter gene. The plasmid-bearing strains were grown overnight in LB broth containing 100 μg/ml ampicillin (LB-amp [100 μg/ml]) at 37°C with shaking (235 rpm). The next day, the cultures were subcultured 1/100 into fresh LB-amp (100 μg/ml) medium, grown for 105 min, and split into two cultures, PQ was added at a final concentration of 250 μM to one of each pair, and shaking (235 rpm) continued at 37°C for another 45 min. The cultures were chilled on ice for 15 min, and β-galactosidase activities were assayed in sodium dodecyl sulfate (SDS)-$CHCl_3$-treated cells as described by Miller (27).

Complementation of a Δ*soxR* strain with cloned *soxR* alleles for increased antibiotic resistance. Plasmids pACYC177, pAK-WT, pAK-E3, pAK-E17, pAK-E19, pAK-M1, and pAK-I237 were introduced into strain TN402 (Δ*soxR soxS*$^+$) (Table 1), and their contribution to the antibiotic resistance phenotype of the recipient strain was assayed on antibiotic gradient plates. Overnight cultures of TN402 (Δ*soxR*) (Table 1) bearing the appropriate plasmids were grown in LB-amp (100 μg/ml) at 37°C with shaking (235 rpm), and samples were applied to an antibiotic gradient plate. The resistance to the antibiotic was determined by measuring growth along the antibiotic gradient after 12 to 24 h of incubation at 37°C. The antibiotics tested were chloramphenicol up to 30 μg/ml, nalidixic acid up to 15 μg/ml, and ciprofloxacin up to 0.125 μg/ml. Antibiotic gradient plates were prepared as described by Cunningham et al. (4), except that each plate contained 60 ml of solid medium.

Multicopy suppression of antibiotic resistance with wild-type *soxR*. Clinical isolates E17, M1, and I242 and control laboratory strains GC4468, DJ901, JTG936, and JTG1052 were transformed with plasmid pSXR encoding isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible SoxR (Table 1) or the vector (pSE380). Overnight cultures of the transformed strains grown in LB-amp (100 μg/ml) at 37°C with shaking (235 rpm) were applied to antibiotic gradient plates containing 5 μM IPTG. Resistance was assessed as described in the previous section.

RESULTS

Expression of *soxS* mRNA in *E. coli* clinical isolates. We sought to determine the molecular basis of the previously reported constitutive *soxS* expression in antibiotic-resistant clinical *E. coli* isolates and its possible role in resistance. Strains E3, E17, and E19 (34) (Table 1) were isolated from blood samples from patients at cancer centers in Europe and the Middle East (34). The strains were all highly fluoroquinolone resistant (MIC of 64 μg/ml, compared to ≤0.25 μg/ml for the wild type [34]) and bore mutations in both *gyrA* and *parC* (34). Strain M1 (Table 1) was isolated from a urinary tract infection from a nonneutropenic patient (34). Strain M1 had modest fluoroquinolone resistance (MIC, 4 μg/ml [34]) and a *gyrA* mutation (30). Strains I236, I237, I242, I243, I244, I246, I248,

TABLE 2. Sequence analysis of *soxRS* in *E. coli* clinical isolates[a]

Strain	*soxR*/SoxR sequence		*soxS*/SoxS sequence		Basal *soxS* expression
	Base pair	Amino acid	Base pair	Amino acid	
E3	G_{1080} to A	A111 to T	wt	wt	wt
E17	$C_{1130}A_{1131}G_{1132}$ deletion	S128 deleted	G_{494} to A	Unchanged	Elevated
E19	$T_{1191}T_{1192}$ deletion	−1 frameshift at L148	wt		Elevated
M1	G_{808} to A	R20 to H	wt		Elevated
I236	G_{1079} to A	Unchanged	T_{341} to C	Unchanged	wt
			G_{494} to A	Unchanged	
I237 or I242 or I253	T_{848} to C	Unchanged	T_{341} to C	Unchanged	Elevated
	C_{862} to G	T38 to S	G_{365} to T	Unchanged	
	C_{875} to T	Unchanged	T_{485} to C	Unchanged	
	T_{920} to C	Unchanged	G_{512} to A	Unchanged	
	T_{926} to C	Unchanged	C_{518} to T	Unchanged	
	G_{969} to C	G74 to R	T_{545} to C	Unchanged	
	G_{977} to A	Unchanged	A_{553} to G	Unchanged	
	G_{1004} to A	Unchanged	T_{587} to G	Unchanged	
	G_{1142} to A	Unchanged			
	T_{1178} to C	Unchanged			
	C_{1181} to A	Unchanged			
I243 or I246	G_{1079} to A	Unchanged	G_{494} to A	Unchanged	wt
I251	G_{1080} to A	A111 to T	wt	wt	wt

[a] For DNA or protein sequence, wt indicates no difference compared to the published *soxRS* sequence.

I251, I253, and I254 (40) (Table 1) were obtained from hospitals in Spain. These strains were reported to have intermediate/high fluoroquinolone resistance, resistance to multiple antibiotics and a *gyrA* mutation (40). All of the above strains had been reported to exhibit high basal *soxS* mRNA expression (30, 36), but the mechanism(s) underlying this altered expression was unknown.

We first tested whether the strains retained elevated *soxS* expression under our experimental conditions. In our hands, compared to the wild-type laboratory strain GC4468, strains E17, E19, M1, I237, I242, and I253 all expressed *soxS* mRNA at elevated levels during normal ("unstressed") cell growth (Fig. 1). The absolute levels of basal *soxS* mRNA expression differed among the different strains. Most of the strains tested retained the ability to up-regulate *soxS* mRNA upon treatment with PQ (Fig. 1A, B, and C and data not shown). In strain E17, basal expression of *soxS* mRNA was already maximal, as it was not induced further by PQ treatment (Fig. 1A). In strain E19 (Fig. 1A), the high basal level of *soxS* mRNA was further elevated ~2-fold by PQ treatment. In strain M1 (Fig. 1B) the basal *soxS* expression was elevated but to a lesser extent than in strains E17 and E19. Strains I237, I242, and I253 expressed increased basal amounts of *soxS* mRNA compared to the laboratory strain GC4468 (Fig. 1C), but the absolute level of expression was low compared to the constitutive expression of strain JTG936 (14) (Table 1). None of the other strains expressed detectably increased amounts of *soxS* mRNA, in contrast to previous reports. However, most of the I-series strains did exhibit elevated basal *soxS* expression when cultured in Iso-Sensitest broth (M. Webber and L. J. Piddock, personal communication).

Mutations in the *soxRS* region of the *E. coli* clinical isolates. Theoretically, constitutive *soxS* expression could be due to mutations in the *soxR* gene that render the protein constitutively active, mutations in the *soxS* promoter that turn on transcription of the *soxS* gene constitutively (16), changes in the *soxS* gene that prolong the half-life of the *soxS* mRNA (6), or mutations in other genes that regulate the redox status of

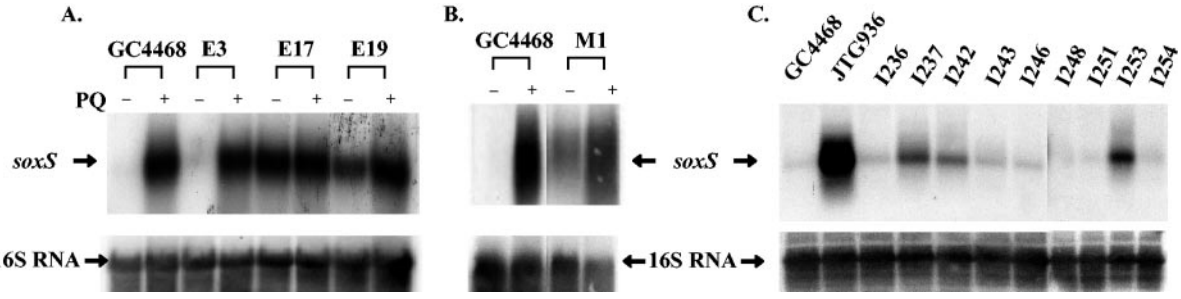

FIG. 1. Expression of *soxS* mRNA in clinical *E. coli* isolates. Overnight cultures were diluted 1/100 in fresh LB broth and grown with vigorous shaking (250 rpm) for 105 min at 30°C (A and C) or for 90 min at 37°C (B). At that point, the cultures were split, the appropriate samples were treated with 1.3 mM PQ, and shaking resumed at 250 rpm and 30°C or 37°C as appropriate for another 30 to 40 min. RNA was then harvested for Northern analysis. (A) Expression of *soxS* mRNA in *E. coli* strains GC4468 (*soxR*$^+$), E3, E17, E19, and M1, with or without 1.3 mM paraquat treatment. (B) Basal expression of *soxS* mRNA in *E. coli* strains GC4468, JTG936 (*soxR*^{c}), I236, I237, I242, I243, I244, I246, I248, I251, I253, and I254.

SoxR (8, 9, 11, 12, 22). To address some of these possibilities, we first sequenced the entire *soxRS* region of each of the clinical isolates.

Table 2 shows that strains E17, E19, M1, I237, I242, and I253, which exhibited increased *soxS* expression, all harbored missense mutations in the *soxR* gene. An in-frame 3-bp deletion ($C_{1130}A_{1131}G_{1132}$) in strain E17 resulted in deletion of serine-128 (S128) within the cysteine cluster of SoxR. A deletion of two consecutive thymines in strain E19 ($T_{1191}T_{1192}$) resulted in a −1 frameshift at leucine-148 and a termination three codons later, which truncated SoxR three residues from the C terminus. A G_{808}-to-A transition in M1 changed arginine-20 to histidine within the helix-turn-helix motif of the SoxR DNA binding domain. Strains I237, I242, and I253 all harbored the same two differences in the *soxR* gene compared to the reference laboratory strain: C_{862} to G changes threonine-38 to serine, and G_{969} to C changes glycine-74 to arginine. Both of these alterations lie within the second helix-turn-helix motif proposed to participate in DNA binding (1, 16).

The sequence analysis (Table 2) also showed that strains E3 and I251 harbor the same mutation (G_{1080} to A) that changes alanine-111 to threonine. However, the basal expression of *soxS* mRNA was not elevated in these strains (Fig. 1). The *soxS-soxR* intergenic region in all the strains was identical to that in the wild-type laboratory strain.

Various polymorphisms were observed in the clinical strains, either silent mutations or amino acid changes without an apparent phenotypic effect. Strains I237, I242, and I253 harbored nine additional silent point mutations in *soxR* and eight in *soxS* (Table 2). All the changes, missense and silent, were identical among the three strains, which suggested that I237, I242, and I253 may have arisen from the same clone. Strains I243 and I246 also shared a silent G_{1079}-to-A change in *soxR* and a silent G_{494}-to-A change in *soxS*. They shared both of these polymorphisms with strain I236, which harbored an additional silent point mutation in *soxS*: a T_{341}-to-C change. Interestingly, strain E17, which we obtained from a different source than strains I236, I243, and I246, also carried the silent G_{494}-to-A polymorphism in *soxS*.

Constitutive activity of cloned mutant *soxR* genes. To confirm that the increased basal *soxS* mRNA expression of the *E. coli* clinical isolates was actually due to constitutive SoxR activity, we cloned the mutant genes into expression vectors (see Materials and Methods). These plasmids (the empty vector pACYC177 and pAK-WT, pAK-E3, pAK-E17, pAK-E19, pAK-M1, and pAK-I237) were introduced into strain EH46 (Δ*soxR soxS*::*lacZ*) (Table 1). The in vivo activity of the mutant proteins was assayed by measuring β-galactosidase expression in the presence or absence of 250 μM PQ, a redox-cycling agent and inducer of the *soxRS* regulon (17). Figure 2 shows that the mutant *soxR* genes from *E. coli* clinical isolates E17, E19, and M1 expressed proteins that conferred high-level expression of the *soxS*::*lacZ* reporter, even in the absence of an activating signal. In contrast, the *soxR* gene from strain E3 did not appear to encode a constitutively active form of SoxR. These observations are in agreement with the results in Fig. 1A and B, which show expression of *soxS* mRNA in the clinical isolates. Unexpectedly, the SoxR protein expressed from pAK-I237 did not confer increased basal β-galactosidase expression in strain EH46, despite the elevated *soxS* expression in strain

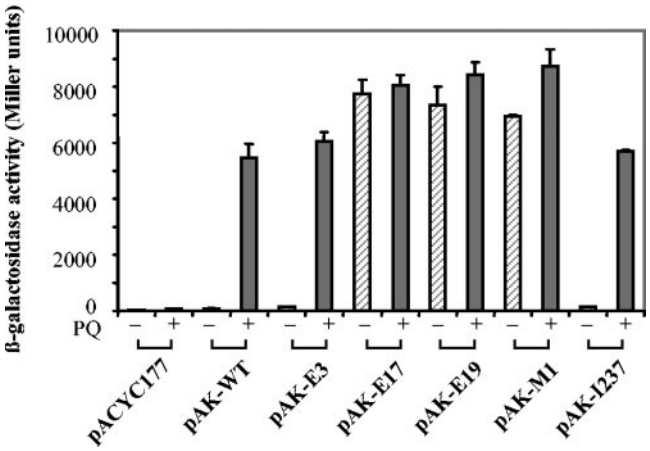

FIG. 2. Regulation of *soxS*::*lacZ* by mutant SoxR proteins. Plasmids pACYC177, pAK-WT, pAK-E3, pAK-E17, pAK-E19, pAK-M1, and pAK-I237 carrying no *soxR*, wild-type *soxR*, or mutant alleles of *soxR* were introduced into strain EH46 (Δ*soxR soxS*::*lacZ*) (Table 1), and the transcriptional activity of the expressed SoxR proteins was assayed by measuring expression of β-galactosidase in the presence or absence of 250 μM PQ. The data shown are the means and standard errors of two determinations.

I237 (Fig. 1C). Treatment with PQ induced β-galactosidase expression greatly and to the same extent (>20-fold) in strains harboring the wild-type (WT), E3, or I237 *soxR* genes. Paraquat treatment did not increase significantly the expression of β-galactosidase by the E17 and E19 SoxR proteins, while it slightly increased the β-galactosidase activity induced by the M1 protein (Fig. 2). For all three alleles—E17, E19, and M1—uninduced expression was already almost maximal (Fig. 2). PQ-induced expression was highest with pAK-E17, pAK-E19, and pAK-M1 (Fig. 2), while pAK-E3 and pAK-I237 paralleled the levels seen for wild-type SoxR (pAK-WT) (Fig. 2).

Antibiotic resistance conferred by cloned mutant *soxR* genes. If the SoxR^c proteins contributed to the increased antibiotic resistance phenotype of the *E. coli* clinical isolates, introduction of these proteins into a laboratory Δ*soxR* strain (TN402) (Table 1) would confer antibiotic resistance. Resistance was assayed on antibiotic gradient plates with chloramphenicol, nalidixic acid, or ciprofloxacin (Fig. 3). For all three antibiotics tested, introduction of the E3 (pAK-E3) and I237 (pAK-I237) mutant alleles gave the same basal antibiotic resistance as provided by the wild-type (pAK-WT) allele. The vectors encoding the constitutively active E17 (pAK-E17), E19 (pAK-E19), and M1 (pAK-M1) SoxR proteins conferred significantly higher basal resistance to all three antibiotics tested. For ciprofloxacin, these three mutant alleles increased the basal antibiotic resistance 12-, 8-, and 10-fold, respectively, much more than *soxRS*-mediated mechanisms alone usually provide (1, 20, 24). Treatment with PQ increased the antibiotic resistance of strains harboring the WT, E3, and I237 *soxR* alleles significantly and to the same extent for each antibiotic tested (Fig. 3). PQ treatment did not significantly affect the antibiotic resistance conferred by the E17 protein and only moderately increased the antibiotic resistance conferred by the E19 and M1 proteins. These results agree with the reporter gene data shown in Fig. 2.

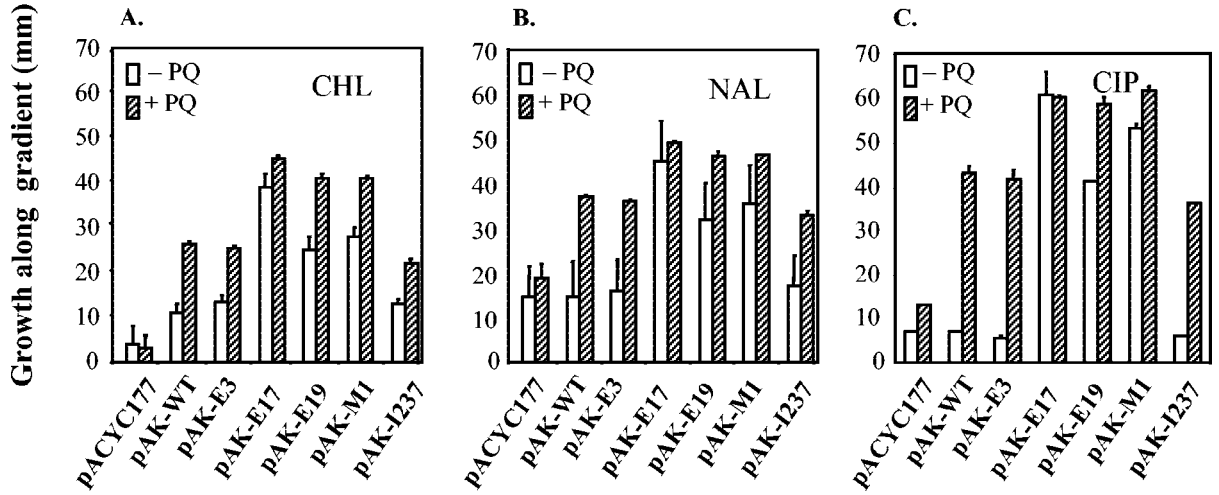

FIG. 3. Antibiotic resistance conferred by mutant *soxR* genes. Plasmids pACYC177, pAK-WT, pAK-E3, pAK-E17, pAK-E19, pAK-M1, and pAK-I237 encoding no *soxR*, wild-type *soxR*, or a mutant allele of *soxR* were introduced into strain TN402 (Δ*soxR*) (Table 2), and their contribution to the antibiotic resistance phenotype of the recipient strain was assayed on antibiotic gradient plates in the presence or absence of 50 μM PQ. (A) Chloramphenicol (CHL), 0 to 30 μg/ml; (B) nalidixic acid (NAL), 0 to 15 μg/ml; (C) ciprofloxacin (CIP), 0 to 0.125 μg/ml. The data shown are the means and standard errors of three determinations.

It is worth noting that, even in the absence of PQ, introduction of the wild-type *soxR* vector pAK-WT into strain TN402 (Δ*soxR*) (Table 1) increased its resistance to chloramphenicol about twofold compared to the vector-only control (Fig. 3A). The pAK-E3 vector had a similar effect, and neither pAK-WT nor pAK-E3 affected basal resistance to nalidixic acid or ciprofloxacin (Fig. 3). The *soxR*-dependent increase in chloramphenicol resistance might reflect activation of SoxR by this drug; however, such activation has not been reported. Chloramphenicol can activate MarA expression, but it was not tested whether this effect was *soxR* dependent (15).

Conversely, PQ treatment increased the antibiotic resistance of the vector-only control to ciprofloxacin about twofold (Fig. 3). This effect was specific to ciprofloxacin. PQ has been shown to be a weak activator of *marA* expression (35). This could account for the increased ciprofloxacin resistance in the absence of SoxR, particularly if ciprofloxacin is more sensitive than nalidixic acid and chloramphenicol to the *marRAB*-mediated resistance mechanisms.

Multicopy suppression of antibiotic resistance with wild-type *soxR*. Nonactivated wild-type SoxR competes with mutant activated protein (20), which allowed us to assess the contribution of mutant *soxR* alleles to the antibiotic resistance profile of the clinical strains. We introduced a multicopy vector with or without wild-type *soxR* into the clinical strains and assayed the basal antibiotic resistance. The *soxR* vector pSXR significantly reduced the basal antibiotic resistance of strains E17 and M1 to all the antibiotics tested (Fig. 4). The antibiotic resistance of the laboratory *soxR* constitutive strains JTG936 and JTG1052 was also diminished by the introduction of pSXR, while GC4468 (*soxR*$^+$) and DJ901 (Δ*soxRS*) were unaffected (Fig. 4). In contrast, the antibiotic resistance of strain I242 was not affected by pSXR (Fig. 4), consistent with the nonconstitutive nature of the *soxR* allele in this strain. Strain E19 was not included in this experiment because of its high resistance to all the available antibiotics. Strains I237 and I253 were not included because attempts to transform them with pSXR or pSE380 were unsuccessful.

DISCUSSION

Koutsolioutsou et al. (24) showed that a multiple-antibiotic-resistant *S. enterica* clinical isolate harbored a constitutive mutation in *soxR* that apparently arose during clinical treatment and contributed to the quinolone resistance profile of the strain. This was the first report of a *soxR*^{c} mutation in clinically antibiotic-resistant *S. enterica* and the first to test directly the *soxR*^{c} contribution to antibiotic resistance in a clinical strain. Previous studies (34, 40) had reported the occurrence of constitutive *soxS* expression in quinolone-resistant *E. coli* clinical isolates, but the mechanism of this expression or its contribution to antibiotic resistance had not been studied. As we have noted, an understanding of the mechanism of constitutive *soxS* expression will be important for establishing and eventually interfering with the contribution of the *soxRS* regulon to the antibiotic resistance phenotype.

Analysis of antibiotic-resistant strains may also provide information for elucidating the signal transduction pathways for *soxRS* activation. To date, elevated basal expression of *soxS* in laboratory strains of *E. coli* has been attributed to mutations in the *soxR* gene that render the SoxR protein active in the absence of oxidative stress (14, 21, 31, 38). In principle, constitutive *soxS* expression could also be due to mutations in the *soxS* promoter, such as the spacer mutations that have been engineered (19), mutations in other genes that function to maintain SoxR in its reduced and inactive state (22, 31), or an increase in the half-life of the *soxS* message (8).

In the present work, we focused on investigating the molecular mechanism(s) of elevated *soxS* expression in clinical *E. coli* isolates (34, 40). To our surprise, only 6 of the 14 reported strains (34, 40) exhibited elevated basal *soxS* expression under our experimental conditions. Recent experiments suggest that

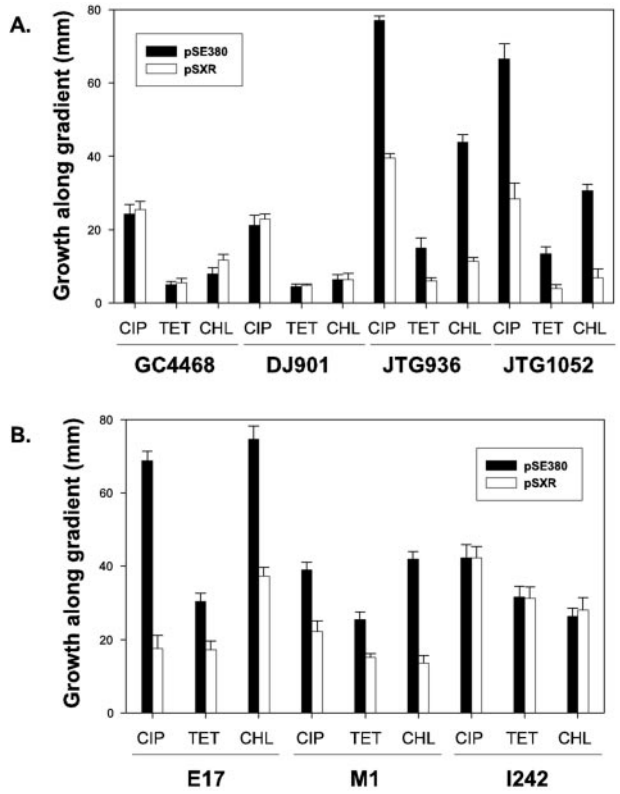

FIG. 4. Multicopy suppression of antibiotic resistance with wild-type *soxR*. Clinical isolates E17, M1, and I242, as well as control laboratory strains GC4468 (*soxR*$^+$), DJ901 (Δ*soxRS*), JTG936 (*soxR*^{c}), and JTG1052 (*soxR*^{c}), were transformed with the IPTG-inducible plasmid pSXR (Table 1) or the vector alone. The antibiotic resistance of the control and pSXR-containing strains was assayed on antibiotic gradient plates in the presence of 5 μM IPTG. The maximum antibiotic concentrations per plate were as follows: for strain E17, chloramphenicol (CHL) at 1.5 mg/ml, ciprofloxacin (CIP) at 250 μg/ml, and tetracycline (TET) at 750 μg/ml; for strain M1, chloramphenicol at 40 μg/ml, ciprofloxacin at 5 μg/ml, and tetracycline at 19 μg/ml; for strain I242, chloramphenicol at 20 μg/ml, ciprofloxacin at 1 μg/ml, and tetracycline at 300 μg/ml; for strains GC4468, DJ901, JTG936, and JTG1052, chloramphenicol at 20 μg/ml, ciprofloxacin at 0.05 μg/ml, and tetracycline at 15 μg/ml. The data shown are the means and standard deviations of three determinations.

some of this difference may be due to the bacterial culture conditions (Webber and Piddock, personal communication). In this context, it may be important that Webber and Piddock (40) used Iso-Sensitest broth from Oxoid, which contains menadione, a superoxide-generating agent and potent activator of SoxR, at a final concentration of nearly 6 μM, in the range where *soxRS* begins to be activated (13). Such observations call into question the use of this medium for testing antibiotic resistance.

The strains that exhibited elevated *soxS* expression in our hands (E17, E19, M1, I237, I242, and I253) (Table 2) all harbored mutations in the *soxR* gene that change the amino acid sequence of the SoxR protein. An in-frame 3-bp deletion ($C_{1130}A_{1131}G_{1132}$) in strain E17 resulted in deletion of serine-128 (S128) within the cysteine cluster of SoxR (Table 2). This cysteine cluster anchors the [2Fe-2S] center of the protein, which is the sensor of oxidative stress and critical for signal transduction and activation of the protein (7, 9, 17, 18). The S128 deletion may thus alter the structure of the Fe-S cluster so as to render SoxR active directly or by way of increased sensitivity to oxidation. A deletion of two consecutive thymines ($T_{1191}T_{1192}$) in strain E19 (Table 2) resulted in a −1 frameshift at leucine-148 (L148), a stop three codons later, and a truncation of the SoxR C terminus by three residues. Previous studies indicated that C-terminal truncations of the protein result in SoxR^c (31, 38). Apparently, the C terminus of the protein is important for keeping the protein in its reduced, inactive state, perhaps via interaction with an enzymatic function (8, 9, 11, 12, 22). A G_{808}-to-A transition in strain M1 changes arginine-20 (R20) to histidine (H), altering the same residue as a *soxR*^{c} mutation (arginine-20 to cysteine) isolated previously in laboratory *E. coli* (20, 31). Arginine-20 lies within the helix-turn-helix motif of the proposed DNA binding domain of SoxR, such that changes of this residue could affect activity.

The cloned *soxR* alleles from strains E17, E19, and M1 activated *soxS* transcription even in the absence of PQ and conferred elevated resistance to several antibiotics. Multicopy suppression of the mutant SoxR in strains E17 and M1 with wild-type SoxR resulted in a decrease of basal antibiotic resistance in these strains. These results support our hypothesis that the E17, E19, and M1 mutant *soxR* alleles are constitutively active, responsible for the increased basal *soxS* expression in the respective strains, and contribute significantly to the antibiotic resistance of the clinical strains.

Strains I237, I242, and I253 all harbor the same two alterations of the *soxR* gene: C_{862}-to-G and G_{969}-to-C transversion mutations changing threonine-38 (T38) to serine (S) and glycine-74 (G74) to arginine (R), respectively. Both of these mutations lie within the second helix-turn-helix motif of the polypeptide, which is thought to participate in DNA binding as suggested by comparison with other members of the MerR family (1, 2). However, this *soxR* allele did not confer constitutive *soxS* expression or antibiotic resistance when placed in laboratory strains, nor was the basal antibiotic resistance of strain I242 suppressed by multicopy wild-type *soxR* (Fig. 4B). This is the first instance of elevated *soxS* expression in a clinical strain not linked to a *soxR*^{c} and suggests another mechanism, such as a defect in the systems (such as the *rsx* operon and the *rseC* gene [23]) that maintain SoxR in its reduced and inactive state. However, an effort to complement strain I237 with the wild-type *rsxC* gene did not reduce its *soxS* mRNA expression (data not shown). Another possibility is that the eight sequence polymorphisms in the I-strain *soxS* gene (Table 2) change the stability of its mRNA.

Our analysis shows that constitutive mutations in the *soxR* gene can be significantly involved in antibiotic resistance in clinical *E. coli* infections. The observations with I237 and the related strains further indicate that alterations elsewhere in the SoxR regulatory pathway may have analogous clinical effects. It seems likely that many additional instances of *soxRS* involvement in the development of antibiotic resistance might also exist but go unnoticed because of transient and reversible (rather than constitutive) activation of SoxR. Identification of such cases poses a big experimental challenge but is a worthwhile goal.

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