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 accA/B 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 accAB-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*) 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
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 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 (235 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 M PO. 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 32p-labeled probes, and gene expression was quantified on a phosphorimager (Bio-Rad). Hybridization with a 32p-labeled 1.6 kb RNA probe was used to control for loading differences.

Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
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<tr>
<td>Strains</td>
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<tr>
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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 in 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,
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 \textit{gyrA} mutation (40). All of the above strains had been reported to exhibit high basal \textit{soxS} mRNA expression (30, 36), but the mechanism(s) underlying this altered expression was unknown.

We first tested whether the strains retained elevated \textit{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 \textit{soxS} mRNA at elevated levels during normal (“unstressed”) cell growth (Fig. 1). The absolute levels of basal \textit{soxS} mRNA expression differed among the different strains. Most of the strains tested retained the ability to up-regulate \textit{soxS} mRNA upon treatment with PQ (Fig. 1A, B, and C and data not shown). In strain E17, basal expression of \textit{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 \textit{soxS} mRNA was further elevated ~2-fold by PQ treatment. In strain M1 (Fig. 1B) the basal \textit{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 \textit{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 \textit{soxS} mRNA, in contrast to previous reports. However, most of the I-series strains did exhibit elevated basal \textit{soxS} expression when cultured in Iso-Sensitest broth (M. Webber and L. J. Piddock, personal communication).

\textbf{Mutations in the \textit{soxRS} region of the \textit{E. coli} clinical isolates.}

Theoretically, constitutive \textit{soxS} expression could be due to mutations in the \textit{soxR} gene that render the protein constitutively active, mutations in the \textit{soxS} promoter that turn on transcription of the \textit{soxS} gene constitutively (16), changes in the \textit{soxS} gene that prolong the half-life of the \textit{soxS} mRNA (6), or mutations in other genes that regulate the redox status of

\begin{figure}[h]
\includegraphics[width=\textwidth]{figure1.png}
\caption{Expression of \textit{soxS} mRNA in clinical \textit{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 \textit{soxS} mRNA in \textit{E. coli} strains GC4468 (\textit{soxR}'), E3, E17, E19, and M1, with or without 1.3 mM paraquat treatment. (B) Basal expression of \textit{soxS} mRNA in \textit{E. coli} strains GC4468, JTG936 (\textit{soxR}'), I236, I237, I242, I243, I244, I246, I248, I251, I253, and I254.

\begin{table}[h]
\centering
\caption{Sequence analysis of \textit{soxRS} in \textit{E. coli} clinical isolates$^a$
\begin{tabular}{lllllll}
\hline
Strain & \textit{soxR}/SoxR sequence & Amino acid & \textit{soxS}/SoxS sequence & Amino acid & Basal \textit{soxS} expression & Amino acid \\
\hline
E3 & G\_1086 to A & A111 to T & & wt & wt & wt \\
E17 & C\_1130A\_1131G\_1132 deletion & S128 deleted & T\_1191T\_1192 deletion & \_1 frameshift at L148 & \_ & \_ \\
E19 & & & & & & \_ \\
M1 & G\_808 to A & R20 to H & wt & wt & \_ \\
I236 & G\_1076 to A & Unchanged & T\_341 to C & Unchanged & E\_lavored & \_ \\
I237 or I242 or I253 & T\_48 to C & Unchanged & G\_111 to T & Unchanged & E\_lavored & \_ \\
C\_969 to G & T38 to S & Unchanged & & & \_ & \_ \\
C\_977 to A & Unchanged & & & & \_ & \_ \\
G\_1004 to A & Unchanged & & & & \_ & \_ \\
G\_1142 to A & Unchanged & & & & \_ & \_ \\
T\_1736 to C & Unchanged & & & & \_ & \_ \\
C\_1181 to A & Unchanged & & & & \_ & \_ \\
I243 or I246 & G\_1086 to A & A111 to T & & wt & wt & wt \\
I251 & & & & & & \_ \\
\hline
\end{tabular}
$^a$ For DNA or protein sequence, wt indicates no difference compared to the published \textit{soxRS} sequence.
\end{table}
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\textsubscript{1130}A\textsubscript{1131}G\textsubscript{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\textsubscript{1191}T\textsubscript{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\textsubscript{soxS}-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\textsubscript{962} to G changes threonine-38 to serine, and G\textsubscript{soxR} 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\textsubscript{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\textsubscript{1079}-to-A change in soxR and a silent G\textsubscript{soxR}-to-A change in soxS. They shared both of these polymorphisms with strain I256, which harbored an additional silent point mutation in soxS: a T\textsubscript{131}-to-C change. Interestingly, strain E17, which we obtained from a different source than strains I256, I243, and I246, also carried the silent G\textsubscript{soxR}-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, pAK-WT, pAK-E3, pAK-E17, pAK-E19, pAK-M1, and pAK-I237) were introduced into strain EH46 (ΔsoxR 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.

![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 (ΔsoxR 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.](http://aac.asm.org)
It is worth noting that, even in the absence of PQ, introduction of the wild-type soxR vector pAK-WT into strain TN402 (ΔsoxR) (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<sup>−</sup>) and DJ901 (ΔsoxRS) 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<sup>−</sup> mutation in clinically antibiotic-resistant *S. enterica* and the first to test directly the soxR<sup>−</sup> 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 (ΔsoxRS), JTG936 (soxR'), and JTG1052 (soxR'), 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.

SoxR regulatory pathway may have analogous clinical effects. In this context, it may be important that Webber and Piddock (40) used Iso-Sensititre 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 (C113G; A113/G113) 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 (T1191T1192) 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' (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 Gsox-to-A transition in strain M1 changes arginine-20 (R20) to histidine (H), altering the same residue as a soxR' 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: C862-to-G and G969-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' and suggests another mechanism, such as a defect in the systems (such as the rse 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 rscC 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.
ACKNOWLEDGMENTS

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