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A *soxRS*-Constitutive Mutation Contributing to Antibiotic Resistance in a Clinical Isolate of *Salmonella enterica* (Serovar Typhimurium)

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The *soxRS* regulon is activated by redox-cycling drugs such as paraquat and by nitric oxide. The >15 genes of this system provide resistance to both oxidants and multiple antibiotics. An association between clinical quinolone resistance and elevated expression of the *soxRS* regulon has been observed in *Escherichia coli*, but this association has not been explored for other enteropathogenic bacteria. Here we describe a *soxRS*-constitutive mutation in a clinical strain of *Salmonella enterica* (serovar Typhimurium) that arose with the development of resistance to quinolones during treatment. The elevated quinolone resistance in this strain derived from a point mutation in the *soxR* gene and could be suppressed in *trans* by multicopy wild-type *soxRS*. Multiple-antibiotic resistance was also transferred to a laboratory strain of *S. enterica* by introducing the cloned mutant *soxR* gene from the clinical strain. The results show that constitutive expression of *soxRS* can contribute to antibiotic resistance in clinically relevant *S. enterica*.

Antibiotic resistance is an increasing problem in clinical treatment of infectious disease (10, 13, 20). The acquisition of resistance has been linked to plasmid-borne genes that specify resistance to individual antibiotics (9), chromosomal mutations that alter the cellular targets (33), and activation of bacterial gene expression that confers resistance to diverse agents (1, 25, 27).

Two groups of coregulated genes (regulons) have been associated with chromosomally based resistance to multiple antibiotics in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium: the *marRAB* and *soxRS* regulons (1, 6, 24, 25, 34; E. A. Martins, P. J. Pomposiello, and B. Demple, unpublished data). In the *soxRS* system, SoxR protein is activated by oxidation (18) or nitrosylation (11) to trigger transcription of the *soxS* gene. In the *marRAB* system, MarR-mediated repression is relieved in response to environmental agents to activate synthesis of MarA (1, 25), a close homolog of SoxS. The SoxS and MarA proteins are the direct activators of genes for resistance to oxidants and antibiotics (1, 16). Recent studies indicate that SoxS controls more than 15 genes in *S. enterica* and 39 genes in *E. coli* (31; P. J. Pomposiello, M. H. J. Bennick, and B. Demple, unpublished data) and that MarA in *E. coli* controls as many as 60 genes (4). There is some overlap between the *soxRS* and *marRAB* regulons, but most of the newly discovered genes are regulated uniquely by one or the other system (4; Pomposiello et al., unpublished data).

Antibiotic resistance mediated by *soxRS* and *marRAB* depends both on down-regulation of the outer membrane porin OmpF, mediated by the antisense RNA *micF* gene (6, 7), and on activation of the *acrAB*-encoded efflux pump (30, 35). Some evidence links the expression of the *soxRS* or *marRAB* regulons and resistance to antibiotics in pathogenic bacteria. One study correlated clinical quinolone resistance in *E. coli* with constitutive expression of either *marA* or *soxS* mRNA (~15% of cases [29]). Mutations in the *marR* gene derepressed *marA* expression in these strains, but the mechanism of high *soxS* expression was not investigated. In the present work, we have investigated the contribution of *soxRS* to clinical quinolone resistance in *S. enterica* serovar Typhimurium. We show that constitutive expression of *soxRS* contributed significantly to the drug resistance of an *S. enterica* strain and that a constitutive mutation in *soxR* evidently arose during the treatment.

MATERIALS AND METHODS

The strains and plasmids used in these studies, along with their relevant properties and source descriptions, are listed in Table 1. Bacteria cultured overnight in Luria-Bertani broth (23) were diluted 1/100 in fresh Luria-Bertani broth and incubated with aeration for 120 min at 37°C. Inducing treatments with paraquat (PQ) were as described previously (31).

Standard procedures (3) were used to prepare cell-free extracts by sonication for electrophoresis using sodium dodecyl sulfate-polyacrylamide gels and for blotting to membrane filters. The anti-SoxS antiserum was prepared in New Zealand rabbits using purified, recombinant SoxS protein (21). The antibody titer and specificity were estimated by immunoblotting against known amounts of purified SoxS protein. The optimal serum was obtained at 2 weeks after the second booster inoculation and was partially immunopurified by depleting nonspecific antibodies using filters containing whole-cell extracts of strains not expressing SoxS protein (15).

To clone *soxRS* from the clinical strains of *S. enterica* serovar Typhimurium a 1-kb fragment corresponding to just the *soxRS* region was amplified from bacterial genomic DNA using PCR with a forward primer (5′-TCAGTATTGTCA GGGATGGCA-3′; base pairs 208 through 228) and a reverse primer (5′-GTA GAGAGAAAGACAAAGACC-3′ [the underlined region corresponding to *soxRS* base pairs 1,266 through 1,254]). The amplified products were purified by

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant properties[a]	Source or reference
Strain		
ATCC 14028	*S. enterica* serovar Typhimurium *soxRS*$^+$	American Type Culture Collection
EM1	(Δ*soxRS*)::*tet* derivative of ATCC 14028	Martins et al., unpublished data
PP120	Δ*soxRS* derivative of ATCC 14028	31
SL4213	*S. enterica*, restriction deficient/modification proficient	J. Mekalanos, Harvard Medical School
St45	*S. enterica* P185645, clinical isolate	19
St46	*S. enterica* P185646, quinolone-resistant clinical isolate	19
Plasmid		
pAK45	1-kb *S. enterica* St45 *soxRS* fragment in pACYC177 (Ap^R)	This work
pAK46	1-kb *S. enterica* St46 *soxRS* fragment in pACYC177 (Ap^R)	This work
pEM45	1-kb *S. enterica* St45 *soxRS* fragment in Bluescript (Ap^R)	This work
pEM46	1-kb *S. enterica* St46 *soxRS* fragment in Bluescript (Ap^R)	This work
pEM300	1-kb *S. enterica* LT2 *soxRS* fragment in Bluescript (Cm^R)	Martins et al., unpublished data

[a] Abbreviations, Ap^R, resistant to ampicillin; Cm^R, resistant to chloramphenicol.

agarose gel electrophoresis and blunt-ended using T4 DNA polymerase (3). The products from strains LT2, St45, and St46 were cloned into the *Eco*RV site of pBluescript (Stratagene) to yield plasmids pEM300, pEM45, and pEM46 (Table 1). The plasmid constructs were first selected in *E. coli* DH5α (3) and then passed through *S. enterica* SL4213, which is a restriction-deficient, modification-proficient strain (26), before transfer into *S. enterica*.

For testing the multiple-antibiotic-resistance phenotype associated with *soxR*-constitutive mutations, a 1-kb fragment corresponding to just the *soxRS* region was amplified from the genomic DNA of St45 and St46 (Table 1) using PCR. The forward primer was 5′-CGCGGATCCGCGTCAGTATTGTCAGGGATGGCA-3′ and the reverse primer 5′-CCATCGATGGGTAGAGAGAAAGACAAAGACC-3′ (regions corresponding to *soxRS* base pairs 208 through 228 and 1,266 through 1,254, respectively, are underlined) and contained *Bam*HI (forward primer) and *Cla*I (reverse primer) restriction sites. The PCR products were purified using the QiaQuick kit (Qiagen), cut with *Bam*HI and *Cla*I, repurified by agarose gel electrophoresis and cloned into the *Bam*HI-*Cla*I sites of the low-copy-number pACYC177 plasmid to yield plasmids pAK45 and pAK46 (Table 1). After selection in *E. coli* DH5α and passage through *S. enterica* SL4213, these plasmids or the empty vector was transferred into the *S. enterica* Δ*soxRS* strain PP120 (Table 1).

Northern blotting and antibiotic gradient plate assays were performed as described elsewhere (Martins et al., unpublished data). The degree of resistance to each antibiotic was determined by scoring for growth along an antibiotic gradient after 12 to 24 h of incubation at 37°C (6, 14).

RESULTS

Laboratory strains of *S. enterica* serovar Typhimurium have a *soxRS* regulatory mechanism that is essentially identical to that of *E. coli*. Exposure to PQ (for example) induces Mn-containing superoxide dismutase (SOD), the *sodA* gene product, and confers resistance to multiple antibiotics (Martins et al., unpublished data). In examining samples of various pathogenic *Enterobacteriaceae* expressing multiple-antibiotic resistance, we discovered one *S. enterica* strain, St46, with high expression of an M_r ~25,000 protein corresponding to SodA even without PQ treatment (Fig. 1A). This strain also showed high basal SOD activity (18 U/mg in St46 versus 6 U/mg in ATCC 14028). PQ treatment elicited only a small further increase in the SOD activity of St46 (to 21 U/mg) compared to a threefold increase in laboratory strain ATCC 14028 (to 18 U/mg). Elevated Mn-containing SOD expression could reflect the activation of any one of at least six different regulatory pathways, including *marRAB* and *soxRS* (8, 16). However, Northern analysis (data not shown) did not indicate elevated *marRAB* expression in strain St46. Instead, St46 constitutively expressed high levels of a 13-kDa protein that cross-reacted with antiserum against *E. coli* SoxS, and PQ treatment of the cells produced only a small further increase in the level of this SoxS-cross-reactive protein (Fig. 1B). The slight mobility difference between SoxS in *S. enterica* cell extracts and the purified, recombinant *E. coli* protein may have arisen from the

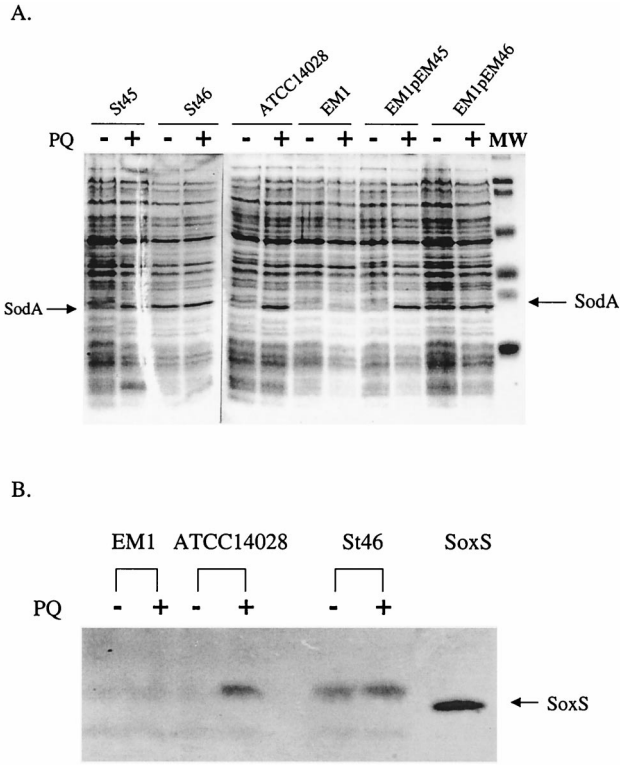

FIG. 1. Expression of SodA and SoxS proteins in antibiotic-resistant *S. enterica*. Laboratory strains ATCC 14028, EM1, EM1(pEM45), and EM1(pEM46) and clinical isolates St45 and St46 of *S. enterica* were cultured and treated for 60 min with 100 μM PQ where indicated (+). Cell-free extracts were prepared and analyzed by gel electrophoresis and Coomassie staining (A) or by Western blotting with anti-*E. coli* SoxS antiserum (B). For each type of analysis, 10 μg of total protein was loaded per lane. For panel B, 0.5 μg of purified *E. coli* SoxS protein (21) was loaded. The mobility difference between purified SoxS and the protein in crude cell extracts is likely related to the relatively high-salt lysis buffer present in the latter samples (100 mM HEPES and 100 mM NaCl). The position of SodA protein indicated in panel A was determined from separate experiments using an *E. coli sodA* strain.

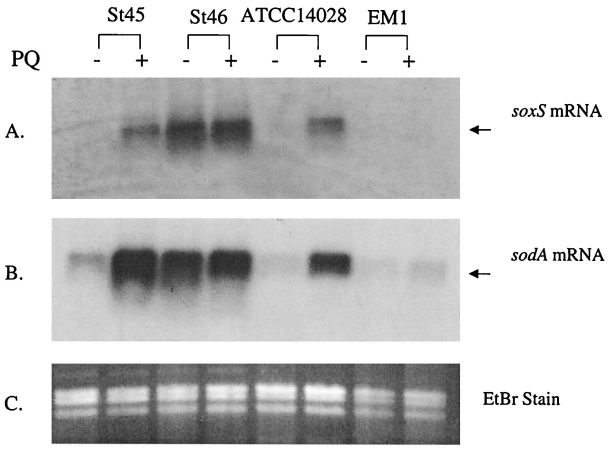

FIG. 2. Levels of *soxS* and *sodA* mRNA in antibiotic-resistant *S. enterica*. Total RNA was extracted using the RNeasy Mini Kit (Qiagen), electrophoresed (2 μg per lane), blotted, and probed with an *S. enterica*-specific probes for *soxS* (A) or an *E. coli sodA* probe (B). The ethidium bromide-stained (EtBr) gel prior to blotting is also shown (C) to demonstrate consistent loading in all lanes.

composition of the cell lysis buffer (see Fig. 1 legend), perhaps in combination with the five amino acid differences between the *S. enterica* and *E. coli* proteins. Consistent with the apparent expression of SoxS and SodA proteins, St46 expressed exceptionally high levels of both *soxS* mRNA and *sodA* mRNA (Fig. 2).

Strain St45 was isolated from a patient with salmonellosis, and St46 was obtained from the same patient after increased quinolone resistance had developed (19). Resistance to two quinolones and to chloramphenicol was PQ inducible in St45 (Fig. 3), as it is in laboratory strains of *S. enterica* (Martins et al., unpublished data). In contrast, St46 exhibited considerably higher constitutive resistance than St45 to all of these drugs (Fig. 3). The elevated chloramphenicol resistance of St46 was further increased in the presence of PQ (Fig. 3). The resistance of St46 to the quinolones was so high that a possible PQ-mediated increase in resistance could not be determined in this assay. In another set of experiments, inclusion of 50 μM PQ in the gradient plates with much higher levels of nalidixic acid (up to 4,000 μg/ml) or ciprofloxacin (up to 4 μg/ml) gave no significant increase in quinolone resistance in St46 (data not shown). Strain St46 also displayed elevated resistance to PQ compared to both St45 and laboratory strain ATCC 14028 (data not shown). This latter observation was also consistent with the possibility of constitutive activation of the *soxRS* regulon in St46.

Collectively, the foregoing results point to constitutive activation of *soxRS* in St46. To test this possibility directly, we transferred the *soxRS* region from St46 into EM1 (Δ*soxRS*) and examined whether the St46 phenotype was transferred with it. The *soxRS* regions from St46 and the nonresistant strain St45 were cloned via PCR to generate plasmids pEM46 and pEM45, respectively (Table 1). In fact, SodA protein was expressed to a high constitutive level in EM1 carrying plasmid pEM46 but not in EM1 carrying pEM45 (Fig. 1A). PQ treatment induced SodA significantly in the EM1(pEM45) strain,

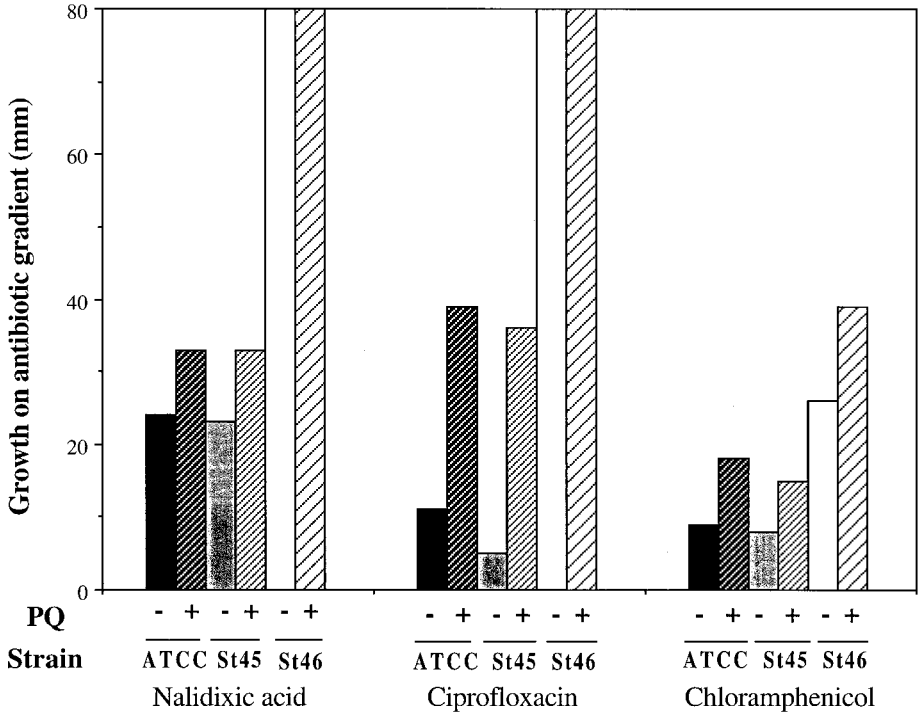

FIG. 3. PQ-inducible antibiotic resistance in *S. enterica*. Overnight cultures of laboratory *S. enterica* ATCC 14028 (ATCC) and clinical isolates St45 and St46 were plated on antibiotic gradient plates (14) in the presence (+) or absence (−) of 50 μM PQ. Growth was measured after 18 to 24 h of incubation at 37°C. The maximum antibiotic concentrations in the gradients were nalidixic acid, 15 μg/ml; ciprofloxacin, 0.25 μg/ml; and chloramphenicol, 40 μg/ml. The results shown are representative of four independent determinations.

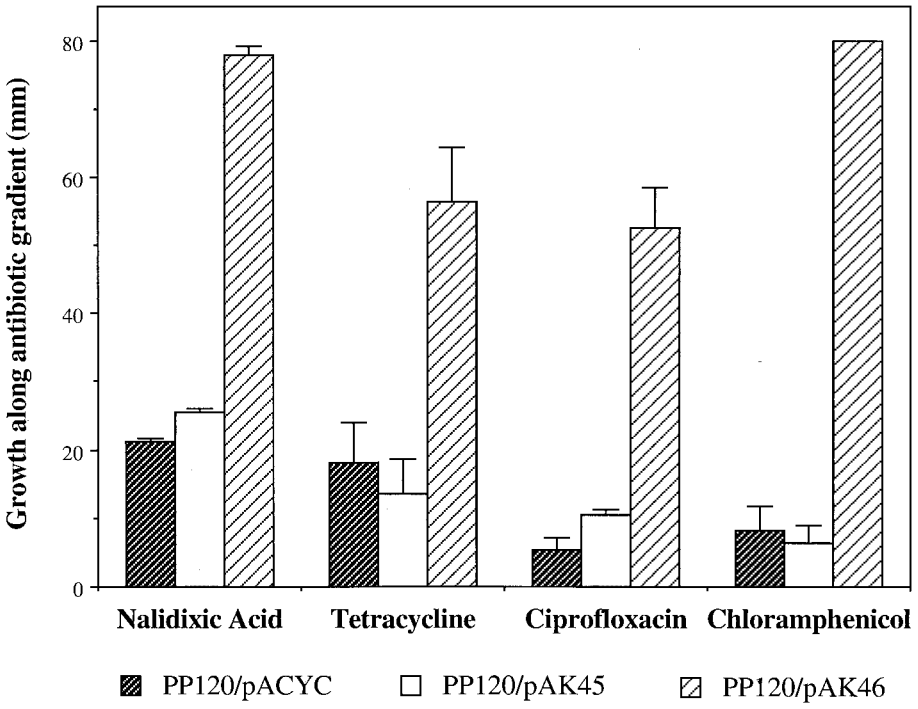

FIG. 4. Multiple antibiotic resistance conferred by the St46 *soxRS* locus. The Δ*soxRS S. enterica* strain PP120 (31) containing pAK45 (*soxRS* region from St45), pAK46 (*soxRS* region from St46), or the empty vector pACYC177 was tested for resistance using antibiotic gradient plates containing 100 μg of ampicillin per ml to select for the plasmids. Growth was measured after 18 to 24 h of incubation at 37°C. The maximum antibiotic concentrations in the gradients were nalidixic acid, 15 μg/ml; ciprofloxacin, 0.25 μg/ml; tetracycline, 15 μg/ml; and chloramphenicol, 40 μg/ml. The bars represent the means and standard deviations of six determinations.

exactly as it did in St45 itself and in the laboratory wild-type strain ATCC 14028 (Fig. 1A). However, PQ treatment did not further increase the already high expression of SodA in either St46 or EM1(pEM46) (Fig. 1A). Thus, the *soxRS* region from St46 determines the constitutive expression of SodA.

We also showed that increased resistance to multiple antibiotics was directed by the *soxRS* region from St46. For this purpose, we generated low-copy plasmids bearing the *soxRS* loci from St45 or St46 (plasmids pAK45 and pAK46, respectively). We introduced these plasmids into the *S. enterica* Δ*soxRS* strain PP120 (31) to assay for antibiotic resistance. In strain EM1, general antibiotic resistance was elevated due to the *tet* allele replacing *soxRS* (Martins et al., unpublished data) and prevented using this strain for resistance experiments. While pAK45 had no effect on basal antibiotic resistance in PP120, pAK46 strongly increased the resistance to tetracycline, chloramphenicol, nalidixic acid, and ciprofloxacin (Fig. 4). Inclusion of PQ in the plates strongly increased the resistance of PP120(pAK45) to all of the antibiotics, but it had only a small additional effect on PP120(pAK46) (data not shown). The St46 *soxRS* region therefore determined a multiple-antibiotic-resistance phenotype that does not require activation by oxidative stress.

Compared to the *soxRS* sequence of St45 (which was identical to that of the laboratory strain LT2; GenBank accession number U61147 and updated sequence [Martins et al., unpublished data]), the DNA sequence of the entire *soxRS* region from St46 revealed only a single mutation—a G-to-A mutation at position 1,092. This change would convert glycine-121, which is located within the cysteine cluster that anchors the [●●] centers to SoxR (5), to an aspartic acid. Several attempts to replace the mutant *soxRS* locus in St46 with a Δ*soxRS* allele were unsuccessful. We therefore pursued another approach, predicated on the ability of nonactivated wild-type SoxR to compete with the mutant-activated protein (12, 17). We transformed St45 and St46 with a multicopy plasmid (pEM300) carrying the wild-type *soxRS* region and assayed the antibiotic resistance in these strains. The multicopy plasmid bearing wild-type *soxRS* dramatically reduced the resistance of St46 to both nalidixic acid and ciprofloxacin, while the control plasmid (vector only) showed no significant effect (Fig. 5). Plasmid pEM300 had little effect on the already lower antibiotic resistance of strain St45 (Fig. 5). Thus, an important part of the antibiotic resistance of St46 is due to constitutive activation of the *soxRS* regulon caused by the mutant SoxR protein in this strain.

DISCUSSION

The occurrence of a *soxR*^{C} mutation in the antibiotic-resistant clinical *S. enterica* isolate St46 is noteworthy. This strain arose in a chronic-renal-failure patient with salmonellosis, and the treatment was complicated by the development of quinolone resistance during the infection (19). Strain St45, isolated from the same patient before the quinolone resistance developed, carried only plasmid determinants for resistance to tetracycline and ampicillin. The additional resistance of strain St46 to quinolones depends significantly on its *soxR*^{C} allele (Fig. 5).

The SoxRS- and MarRAB-regulated genes that contribute to

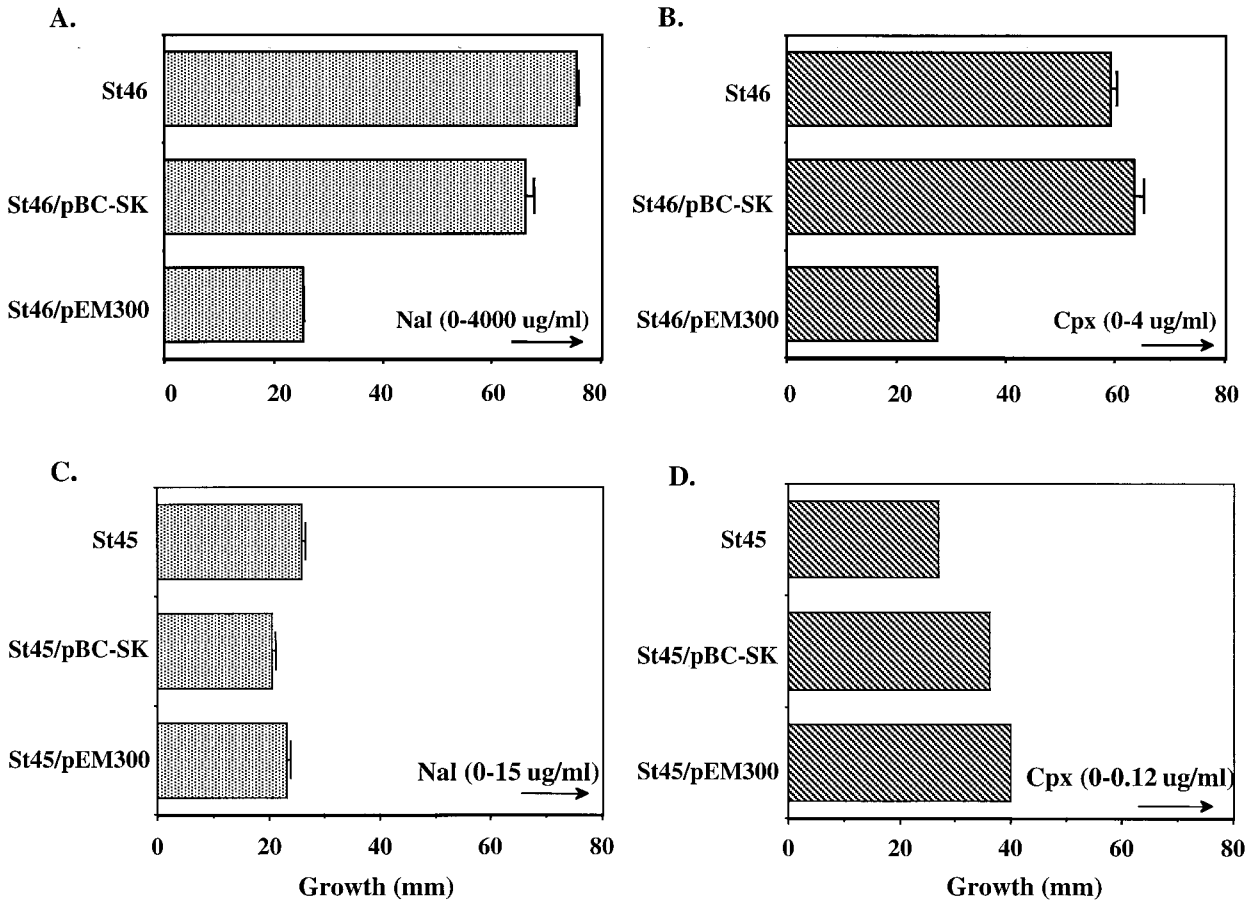

FIG. 5. Multicopy suppression of St46 antibiotic resistance by wild-type *soxRS*. (A and B) Specific suppression of quinolone resistance in St46. The multicopy *soxRS* plasmid pEM300 or the empty vector was transformed into *S. enterica* St46 (*soxR*^{C}), and the resulting strains were tested in gradient plates for resistance to nalidixic acid (Nal [panel A]) or ciprofloxacin (Cpx [panel B]). Growth was measured after incubation at 37°C for 18 to 24 h. (C and D) Multicopy *soxRS*$^{+}$ does not decrease quinolone resistance in St45. St45 was transformed with plasmids and tested for resistance as described for panels A and B. The maximum level of antibiotic in each plate is indicated above an arrow (μg, micrograms), indicating the direction of the gradient.

broad antibiotic resistance include the *acrAB* operon encoding a multidrug efflux pump (22, 25, 30) and the *micF* gene encoding an antisense RNA that inhibits synthesis of the OmpF outer membrane porin (6, 7, 32). The higher antibiotic resistance of St46, in the absence of PQ, than of either St45 or ATCC 14028 in the presence of PQ could reflect various differences. One possibility is that the constitutive SoxR protein encoded in St46 is simply more active than wild-type SoxR following PQ activation under our conditions. Alternatively, multiple cell divisions may be needed for some changes conferring resistance, such as the clearance of OmpF protein from the outer membrane. Other resistance components (such as AcrA and its efflux partner TolC [2]) may accumulate to higher levels due to the constant activity of the SoxR-constitutive protein. Indeed, the level of *soxS* mRNA was higher in untreated St46 than in St45 exposed to 250 μM PQ for 30 min, but the amount of *sodA* mRNA was about the same in these two cases (Fig. 2). Another possible difference is that additional time might be required for maximal expression of the *marRAB* operon, the *soxRS*-dependent expression of which produces an extra increment of resistance (1, 25). Finally, additional mutations in St46 might elevate resistance to specific antibiotics (e.g., *gyrA* mutations for quinolones).

Constitutive mutations in *soxRS* or in *marRAB* have been correlated with fluoroquinolone resistance in clinical *E. coli* infections (29). To our knowledge, this is the first report of a *soxR*^{C} mutation in an antibiotic-resistant *S. enterica* infection. Clearly, further studies are warranted to establish the frequency with which *soxR*^{C} mutations accompany antibiotic resistance in other cases of salmonellosis.

The *soxRS* and *marRAB* regulons may contribute significantly to antibiotic resistance for which specific plasmid- or transposon-borne genes do not exist, e.g., quinolones (1). Although resistance provided by these pathways is typically lower than that produced by highly specific resistance determinants (9), its general nature could contribute a first step in the development of higher-level resistance (1, 25). Transient *soxRS* or *marRAB* activation (as opposed to constitutive mutations as described here and elsewhere [29]) may aid in the spread of antibiotic resistance, but such activation would have gone undetected in most studies performed thus far. The activation of these systems by immune attack and inflammatory responses

(for *soxRS* [28]) or by antibiotics themselves (for *marRAB* [1, 25]) would provide multiple-antibiotic resistance similar to that observed for the constitutive strains, but this resistance would exist only while the regulons are activated. In this fashion, transient expression of *soxRS*- or *marRAB*-regulated resistance functions could allow for increased opportunities for the spread of other antibiotic-resistance determinants by increasing the probability of survival of cells lacking these determinants.

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