

Identification of SoxS-Regulated Genes in *Salmonella enterica* Serovar Typhimurium

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***Salmonella enterica* serovar Typhimurium responds to superoxide-generating agents through *soxR*-mediated activation of the *soxS* gene, whose product, SoxS, is necessary for resistance to oxidative stress. The *S. enterica* serovar Typhimurium *soxRS* system also mediates redox-inducible resistance to diverse antibiotics, which may be relevant to clinical infections. In order to identify SoxS-regulated genes in *S. enterica* serovar Typhimurium, a *lacI*-regulated expression system for the *S. enterica* serovar Typhimurium *soxS* gene was developed. This system was used to demonstrate that *soxS* expression is sufficient for the induction of resistance to the superoxide-generating drug paraquat and for the transcriptional activation of the *sodA* and *micF* genes. In addition, a library of random *lacZ* insertions was generated and screened for clones displaying differential β -galactosidase activity in the presence or absence of SoxS. This selection yielded six independent chromosomal *lacZ* transcriptional fusions that were activated by either artificial expression of SoxS or exposure of wild-type cells to micromolar concentrations of paraquat. Moreover, disruption of the inducible genes by the insertions rendered *S. enterica* serovar Typhimurium hypersensitive to millimolar concentrations of paraquat. Nucleotide sequence determination identified the disrupted genes as *sodA* (Mn-containing superoxide dismutase), *fpr* (NADPH:ferredoxin oxidoreductase), and *ydbK* (a putative Fe-S-containing reductase).**

Aerobic organisms obtain energy by the oxidation of organic compounds, with oxygen as the final electron acceptor. As a by-product of this process, reactive oxygen species are generated, with potentially damaging consequences for the cell. *Escherichia coli* responds to the intracellular increase in reactive oxygen species by inducing sets of genes whose products either avert or repair the eventual oxidative damage (19). The response to increased levels of superoxide is regulated by the *soxRS* system, a pair of regulatory genes that, together with their downstream-regulated genes, define the *soxRS* regulon (3, 37).

The SoxR protein is expressed constitutively and is a homodimeric transcriptional regulator that contains redox-active iron-sulfur clusters (18, 38). The oxidation state of the iron-sulfur clusters in SoxR regulates the transcriptional activity of the protein (20): while reduced SoxR does not affect transcription, oxidized SoxR dramatically enhances the transcription rate of *soxS*, a gene that codes for a second transcriptional activator (22, 23). The SoxS protein is a member of the AraC/XylS family of transcriptional regulators, and enhanced expression of SoxS activates at least 15 genes (20, 24), including *sodA* (Mn-containing superoxide dismutase), *zwf* (glucose-6-phosphate dehydrogenase), *micF* (antisense RNA to the porin OmpF mRNA), *nfo* (DNA repair endonuclease IV), *fpr* (NADPH:ferredoxin oxidoreductase), *acrAB* (efflux pump), *acn* (aconitase), *fumC* (heat-resistant fumarase), and *nfsA* (nitroreductase A). The induction of SoxS also results in enhanced resistance to multiple antibiotics (3, 4), dependent on *micF* (11) and *acrAB* (25).

Recently, the *soxRS* genes from *Salmonella enterica* serovar Typhimurium were cloned (B. L. Martins, P. J. Pomposiello, Z. Li, and B. Dimple, unpublished data). Their nucleotide sequence (GenBank accession no. U61147) showed 97% identity

of the predicted polypeptides with SoxR and SoxS of *E. coli*. Moreover, the regulatory region between the two genes is nearly identical to that of *E. coli*. The cloned *soxRS* genes were used to generate *soxRS* deletion mutants by allelic exchange (Martins et al., unpublished data). These Δ *soxRS* strains are hypersensitive to the superoxide-generating agent paraquat (PQ), fail to induce the expression of *nfo* and *sodA*, and do not show increased resistance to antibiotics in response to treatment with PQ. Additionally, an independent study (16) showed that the *soxS* gene from *S. enterica* serovar Typhimurium is required for activation of *sodA* expression and resistance to redox-cycling agents.

Despite the similarity of the *soxRS* genes between *S. enterica* serovar Typhimurium and *E. coli*, enzyme activity measurements suggested that the *soxRS* regulons from *E. coli* and *S. enterica* serovar Typhimurium are different: while incubation with PQ induced the SodA protein and endonuclease IV, it failed to induce glucose-6-phosphate dehydrogenase or fumarase C in wild-type *S. enterica* serovar Typhimurium (Martins et al., unpublished data). The evolution of *E. coli* and *S. enterica* serovar Typhimurium under different environmental pressures might have led to differential recruitment of genes into the *soxRS* regulon. Therefore, a detailed description of the *S. enterica* serovar Typhimurium *soxRS* regulon might yield insights into the mechanisms of resistance to antibiotics and antimicrobial compounds generated by the immune system.

In order to characterize the *soxRS* regulon of *S. enterica* serovar Typhimurium, we built an inducible *soxS* expression system that is uncoupled from oxidative stress. We used this system both to measure the effect of the expression of SoxS on genes known to be under *soxRS* control in *E. coli* and to identify novel targets of *soxS* regulation in *S. enterica* serovar Typhimurium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Table 1 shows the bacterial strains and plasmids used in this work. Bacterial cultures were grown at 37°C

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TABLE 1. Strains and plasmids

Strain ^a or plasmid	Genotype or characteristic	Reference or source
Strains		
ATCC 14028	Wild type	American Type Culture Collection
SL4213	Restriction deficient	J. Mekalanos
EM1	ATCC 14028 Δ <i>soxRS</i> , Tc ^r	UD ^b
PP120	ATCC 14028 Δ <i>soxRS</i>	UD
TT10288	<i>hisD9953::MudJ</i> <i>hisA9944::MudI</i>	21
PP101	ATCC 14028, Sin1	This work
PP102	ATCC 14028, Sin2	This work
PP103	ATCC 14028, Sin3	This work
PP104	ATCC 14028, Sin4	This work
PP105	ATCC 14028, Sin5	This work
PP106	ATCC 14028, Sin6	This work
PP121	PP120, Sin1	This work
PP122	PP120, Sin2	This work
PP123	PP120, Sin3	This work
PP124	PP120, Sin4	This work
PP125	PP120, Sin5	This work
PP126	PP120, Sin6	This work
Plasmids		
pBR322	ColE1 Ap ^r Tc ^r	9
pSE380	ColE1 Amp <i>lacI</i>	Novagen
pET-21	ColE1 Amp <i>lacI</i>	Invitrogen
pRZ4004	ColE1 Amp <i>lacZ</i> promoter	W. Reznikoff
pJP103	pBR322 <i>lacI</i>	This work
pJP104	pJP103 <i>soxS</i>	This work
pJP105	pJP104 <i>lacZp::soxS_{St}</i>	This work
pDT1-5	<i>sodA</i> from <i>E. coli</i> Ap ^r	11
pBCKpn	<i>soxR</i> and <i>soxS</i> genes from <i>S. enterica</i> serovar Typhimurium Cm ^r	UD

^a All strains are from *S. enterica* serovar Typhimurium.

^b UD, B. L. Martins, P. J. Pomposiello, Z. Li, and B. Demple, unpublished data.

in Luria-Bertani (LB) broth (27) with vigorous aeration by shaking at 250 rpm. Ampicillin (100 μ g/ml) and kanamycin (50 μ g/ml) were added when necessary. The *lacZ* inducer isopropyl- β -D-thiogalactopyranoside (IPTG) and PQ were added at the final concentrations described in the figure legends. The indicator 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) was added to plates at a final concentration of 25 μ g/ml.

DNA manipulation and construction of pJP105. DNA purification, incubation with restriction enzymes, electrophoresis in agarose gels, ligation, and bacterial transformation were performed according to standard protocols (5). Phage P22 transductions were performed by standard methods (26). To construct plasmid pJP105, the *S. enterica* serovar Typhimurium *soxS* gene was subcloned from pBCKpn (Martins et al., unpublished data) as an *HpaI-SalI* fragment into pBR322 (9) digested with *EcoRV* and *SalI*, generating plasmid pJP103. The *lacI* gene from pSE380 (Novagen) was excised as a *PshAI-SalI* fragment and ligated to pJP103 previously digested with *AvaI*, followed by treatment with T4 DNA polymerase and *SalI*, to generate plasmid pJP104. The *lacZ* promoter from pRZ4004 (32) was liberated by digestion with *EcoRI*, filled in by Klenow fragment DNA polymerase, and digested with *HindIII*. Plasmid pJP104 was digested with *ClaI*, followed by incubation with T4 DNA polymerase and *HindIII*, and ligated to the *lacZ* promoter fragment from pRZ4004 to generate plasmid pJP105 (5.4 kbp). This construct was used to transform the intermediary *S. enterica* serovar Typhimurium strain SL4213 (restriction deficient), and plasmid DNA extracted from this transformant was used to transform strain EM1 (Δ *soxRS*) to ampicillin resistance.

Northern blot analysis. Bacterial cultures were grown overnight in LB broth at 37°C with the appropriate antibiotics, diluted to 1/100 in 2 ml of LB broth, and grown at 250 rpm in 18-mm-diameter tubes to mid-log phase. At an optical density at 600 nm of ~0.5, growing cultures were exposed to different concentrations of PQ or IPTG for 30 min. Total RNA was extracted with an RNeasy kit (Qiagen) and resuspended in RNase-free water. The concentration of the total RNA preparations was determined by measuring the absorbance at 260 nm, and 2 μ g of total RNA was run per lane in 1.25% agarose gels containing formaldehyde and transferred to Nytran membranes with a TurboBlotter setup

(Schleicher & Schuell). The RNA was cross-linked to the membrane by UV irradiation, and the membranes were then hybridized at 68°C with radioactively labeled DNA fragments in cylindrical tubes by using QuickHyb solution (Stratagene). The membranes were washed according to the instructions from the manufacturer. X-ray films were exposed to the membranes at -70°C and developed with a Fuji automatic developer. The radioactive signals were quantitated with an Applied Biosystems phosphorimager.

MudJ mutagenesis. The MudJ (promoterless *lacZ*, Kan^r) phage was delivered to the Δ *soxRS*/pJP105 strain by P22 transduction from strain TT10288 (*hisD::MudI hisA::MudI*) as described by Hughes and Roth (21). Kanamycin-resistant transductants were isolated on LB agar plates containing kanamycin and ampicillin. Each selection plate was replica plated onto LB agar plates containing kanamycin, ampicillin, and X-Gal. One of these plates also contained 1 mM IPTG to promote expression of SoxS from pJP105. Colonies displaying differences in blue coloration between the two plates were selected as candidates for harboring *lacZ* insertions in SoxS-regulated genes.

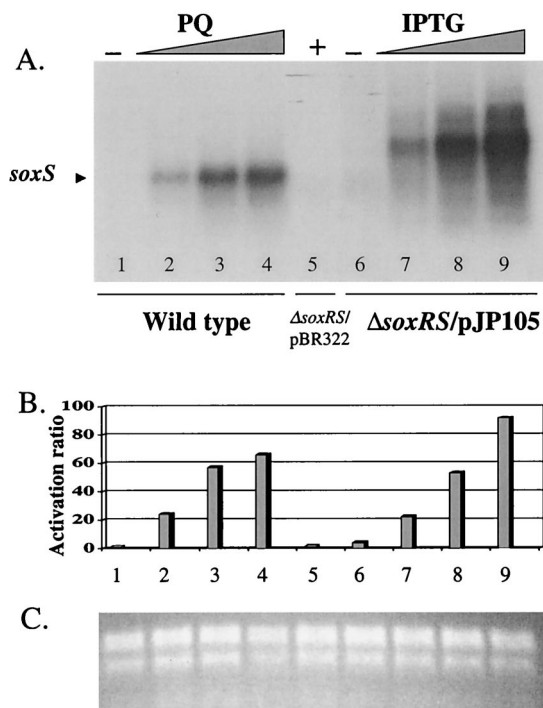
PQ sensitivity test on gradient plates. The sensitivities to PQ of different strains were determined by the extent of linear growth on LB agar plates containing a gradient of PQ (13). Briefly, overnight cultures of the different strains were diluted 1/100 and grown in LB broth with aeration for 8 h and stamped on gradient plates containing different maximal concentrations of PQ. The gradient plates were incubated at 37°C for 16 h, and the length of confluent growth along the gradient was measured.

β -Galactosidase assay. β -Galactosidase-specific activity in chloroform-treated cells was determined as described by Miller (27). Samples were taken from bacterial cultures grown overnight in LB broth, diluted 1/100, and grown with aeration for 2 h to an optical density at 600 nm of ~0.5. At that point, the cultures were either exposed to PQ or IPTG for 30 min or left untreated.

Nonspecific PCR amplification. Chromosomal DNA was extracted from each strain carrying a Mu insertion and used as a template in a PCR that included primer MuLEFT (5'-ATAATCCAATGTCCTCCCG-3'), derived from the sequence of the left end of phage Mu, and a primer of nonspecific sequence (5'-ATTGGAGCGCAGGTTAGTCG-3'). Conditions for these first-round PCRs were as follows: 1.5 mM MgCl₂, 0.5 U of *Taq* DNA polymerase (Sigma), 2 mM deoxynucleoside triphosphates (dNTPs), ~0.2 μ g of chromosomal DNA, and 50 pmol of each primer. Reaction mixtures were incubated for 10 cycles of 1 min at 40°C (annealing), 30 s at 72°C (extension), and 30 s at 95°C (denaturation), followed by 30 cycles of the same steps but with an annealing temperature of 55°C. A second PCR was conducted with 5 μ l of the first reaction mixture as the template, the nested primer MuEND (5'-TACTTCAAGTGAATCAATAC A-3'), derived from the sequence of the left end of phage Mu, and the same nonspecific primer used in the first reaction. Conditions for these second-round PCRs were as follows: 1.5 mM MgCl₂, 0.5 U of *Taq* DNA polymerase (Sigma), 2 mM dNTPs, ~0.2 μ g of chromosomal DNA, and 50 pmol of each primer. Reaction mixtures were incubated for 30 cycles of 1 min at 55°C (annealing), 30 s at 72°C (extension), and 30 s at 95°C (denaturation). The extension products from these reactions were isolated from agarose gels and sequenced at the Molecular Biology Core Facilities of the Dana-Farber Cancer Institute.

RESULTS

An engineered *soxS* expression system: SoxS is sufficient for enhanced resistance to PQ. In order to analyze the direct contribution of SoxS to the activation of antioxidant defenses, we built a *soxS* expression system that uncoupled *soxS* transcription from oxidative stress. In plasmid pJP105, the *lacZ* promoter (33) drives the transcription of the *soxS* cistron. As this plasmid also bears the *lacI* gene, the *lacZ* promoter is expected to be repressed in the absence of the inducer IPTG. When pJP105 was used to transform *S. enterica* serovar Typhimurium EM1 (Δ *soxRS*) and the expression of the plasmid-borne *soxS* gene was monitored by Northern analysis, *soxS* mRNA showed strong induction (up to 90-fold) by the gratuitous *lac* inducer IPTG (Fig. 1). As expected, the *soxS* message from pJP105 is longer than the wild-type message, due to the displaced transcriptional start site provided by the *lacZ* promoter and intervening cloning fragments, which add a total of 273 nucleotides. The induction level of the IPTG-regulated *soxS* transcript was comparable to that of the *soxS* transcript from wild-type strain ATCC 14028 treated with increasing concentrations of PQ (Fig. 1). In control experiments, untransformed strain EM1 showed no *soxS* message either in the presence or the absence of PQ (data not shown). We conclude from these results that the IPTG-induced expression of the *soxS* gene from plasmid pJP105 mimics the induction of wild-



type *soxS* by PQ and that this heterologous expression can be regulated by the concentration of inducer in the growth medium.

In order to test the correlation between the heterologous *soxS* expression from pJP105 and the cellular resistance to oxidative stress, we assayed the ability of plasmid pJP105 to complement the PQ-sensitive phenotype of a Δ *soxRS* strain. Figure 2 shows the growth of strains ATCC 14028 (wild-type), EM1 (Δ *soxRS*), and EM1/pJP105 on PQ gradient plates (maximum concentration, 2 mM PQ) at increasing concentrations of IPTG. While the growth of strains ATCC 14028 and EM1 was unaffected by the IPTG concentrations, the resistance to PQ of strain EM1/pJP105 was increased by IPTG. In the absence of IPTG, the resistance of EM1/pJP105 was similar to that of EM1, consistent with the low levels of *soxS* message (Fig. 1A). As the IPTG concentration in the plates was increased, the resistance to PQ of strain EM1/pJP105 increased in parallel, reaching the wild-type level at 0.2 mM IPTG (Fig. 2). These results demonstrate that the regulated expression of *soxS* from pJP105 directly controls the phenotypic resistance to PQ in *S. enterica* serovar Typhimurium; thus *soxS* expression is not only necessary, as shown previously (16; Martins et al., unpublished data), but also sufficient for full-scale resistance to PQ in this organism, as it is in *E. coli* (3).

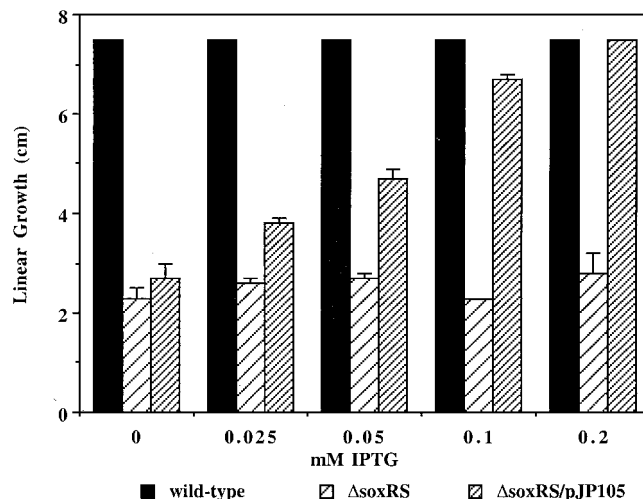


FIG. 2. Complementation of the sensitivity to PQ by pJP105. Cultures of strains ATCC 14028, EM1, and EM1/pJP105 were grown overnight in LB broth and stamped onto PQ gradient plates with increasing concentrations of IPTG (0 to 2 mM) as indicated. The plates were incubated at 37°C for 14 h, and the extent of growth of each strain was measured. The values are the averages of one representative experiment done in duplicate, and the error bars are the standard deviations.

Induction of *soxS* and transcriptional activation of *sodA* and *micF*. Enzyme assays have shown that SodA activity is induced in *S. enterica* serovar Typhimurium upon exposure to PQ in a *soxRS*-dependent manner (16; Martins et al., unpublished data). We probed the activation of the *sodA* gene in *S. enterica* serovar Typhimurium either by treatment of cells with PQ or by the regulated expression of SoxS in the absence of oxidative stress. Northern blotting shows a direct correlation between *sodA* induction (Fig. 3) and *soxS* expression (Fig. 1), either in response to PQ in the wild-type strain or by IPTG induction in EM1/pJP105. The maximal induction ratio for *sodA* expression in the wild-type strain after exposure to PQ was 13-fold, while the maximum in the Δ *soxRS*/pJP105 strain treated with IPTG was 8-fold (Fig. 3). Thus, other pathways such as the *marAB* system (1) may contribute to the PQ-induced expression of *sodA* in *S. enterica* serovar Typhimurium. In fact, the induction ratio for *sodA* in a Δ *soxRS* strain exposed to 0.25 mM PQ was threefold (data not shown). Nevertheless, transcriptional induction of *sodA* expression by SoxS is sufficient to account for most of the PQ-inducible SodA activity.

The periplasmic, Cu-Zn-containing superoxide dismutase encoded by the *sodC* gene protects *S. enterica* serovar Typhimurium from extracellular superoxide, and *sodC* mutants are less virulent than isogenic wild-type strains (14, 17). We tested whether *sodC* transcription is induced by oxidative stress or expression of *soxS* under normal growth. We performed Northern blotting of total RNA with a labeled fragment of the *S. enterica* serovar Typhimurium *sodC* gene (17). No consistent activation of *sodC* expression was observed, either in the wild-type strain treated with up to 0.25 mM PQ or in strain EM1/pJP105 treated with up to 1 mM IPTG (data not shown).

The *micF* gene is transcriptionally regulated by SoxS in *E. coli*. We tested for SoxS regulation of this gene in *S. enterica* serovar Typhimurium by analyzing the transcriptional activity of *micF* by Northern blotting. Preliminary studies showed that strain EM1, which we used in previous experiments, had an elevated endogenous level of *micF* RNA (data not shown). We hypothesized that this increase might be related to the *tet*

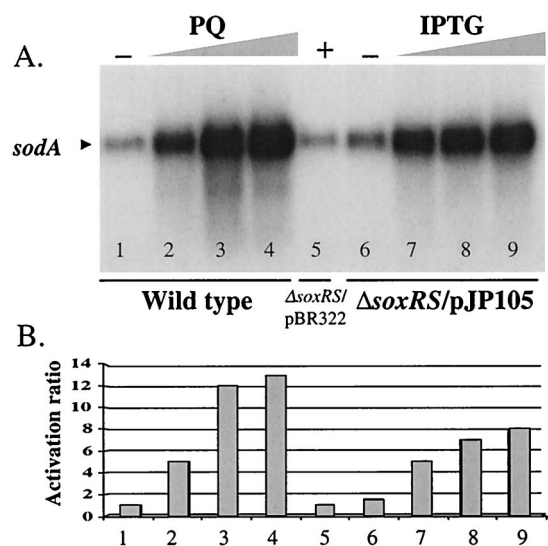


FIG. 3. Northern blotting analysis of *sodA* transcription. (A) Northern blotting. The membrane used for Fig. 1 was stripped and rehybridized with a ~1-kb *Ava*I fragment from plasmid pDT1.5 (12) containing the coding region of the *sodA* gene from *E. coli*. Strain ATCC 14028 (wild type) was treated with increasing concentrations of PQ (0, 25, 100, or 250 μ M; lanes 1 to 4, respectively), and strain EM1(Δ *soxRS*)/pJP105 was treated with increasing concentrations of IPTG (0, 0.125, 0.50, or 1.00 mM; lanes 6 to 9, respectively). The control strain, EM1/pBR322, was treated with 1.00 mM IPTG (lane 5). (B) Quantitation of the radioactive signals. The radioactivity for the *sodA* band in each lane was quantitated by phosphorimaging. The values are the relative activations for the treatments as described in the legend for Fig. 1. The lane numbers correspond to those used for panel A.

cassette used to replace the *soxRS* region in EM1. We therefore turned to PP120, a different Δ *soxRS* derivative of strain ATCC 14028 without a drug resistance cassette (Martins et al., unpublished data). This strain had a *micF* RNA level similar to that of the isogenic wild-type strain (Fig. 4, lane 1 versus lane 6). The transcription of *micF* increased dramatically with increasing concentration of PQ in a wild-type strain and with increasing IPTG concentration in strain PP120/pJP105. The maximal induction ratio for *micF* expression in the wild-type strain after exposure to PQ was 15-fold, and after treatment of PP120/pJP105 with IPTG it was also 15-fold (Fig. 4B). In control experiments, the activation ratio of *micF* expression in a Δ *soxRS* strain exposed to PQ was only twofold (data not shown), again consistent with a small contribution by some other regulatory system. This result shows that SoxS is sufficient to account for nearly all of the PQ-inducible *micF* expression.

MudJ mutagenesis and screening for SoxS-regulated genes.

In order to identify novel SoxS-regulated genes, we screened a library of random *lacZ* insertions for differential expression in the presence or absence of SoxS. A Δ *soxRS* strain was transformed with pJP105 and then mutagenized with MudJ, a bacteriophage Mu derivative with a promoterless *lacZ* operon for the generation of transcriptional fusions (10). Primary candidates for SoxS-regulated insertions were selected as described in Materials and Methods. From approximately 56,000 colonies, 114 candidates were picked, purified by single-colony isolation, and tested for β -galactosidase activity in liquid culture in either the absence or the presence of IPTG (1 mM). We disregarded any transductant showing less than twofold activation by IPTG. From the group of 114 candidates, 32 *Kan*^r transductants showed increased β -galactosidase activity after treatment with IPTG. In order to confirm regulation by SoxS in

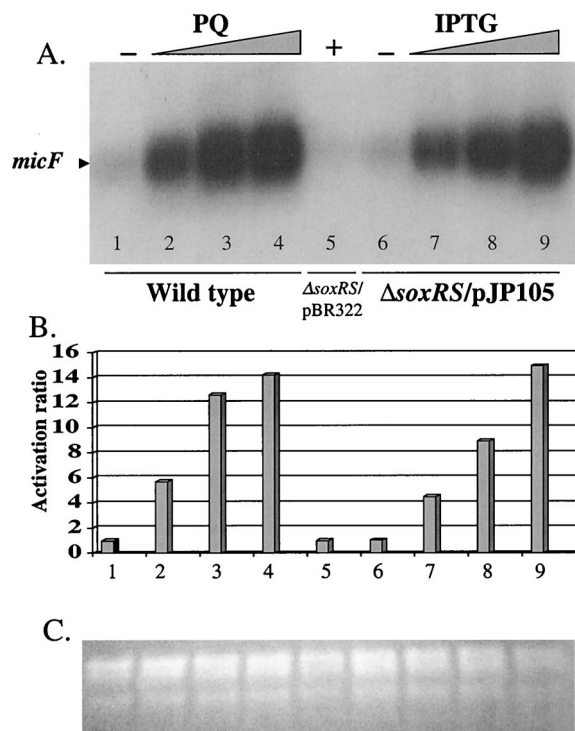


FIG. 4. Northern blotting analysis of *micF* transcription. (A) Northern blotting. A 51-mer synthetic oligonucleotide derived from the sequence of the *micF* gene from *S. enterica* serovar Typhimurium (15) was labeled with T4 polynucleotide kinase and [³²P]dATP. The probe was hybridized with total RNA from strain ATCC 14028 (wild type) treated with increasing concentrations of PQ (0, 25, 100, or 250 μ M; lanes 1 to 4, respectively) or strain PP120(Δ *soxRS*)/pJP105 treated with increasing concentrations of IPTG (0, 0.125, 0.50, or 1.00 mM; lanes 6 to 9, respectively). The control strain, PP120/pBR322, was treated with 1.00 mM IPTG (lane 5). (B) Quantitation of the radioactive signals. The radioactivity for the *micF* band in each lane was quantitated by phosphorimaging. The values are the relative activations for the treatments as described in the legend for Fig. 1. The lane numbers correspond to those used for panel A. (C) Total RNA loading. Shown is an ethidium bromide stain of a duplicate gel of total RNA.

a physiologically relevant setting, the MudJ (*Kan*^r) insertion from each of these 32 mutants was transduced into a wild-type *S. enterica* serovar Typhimurium strain and the β -galactosidase activity of each transductant was measured from LB cultures in the presence or absence of 0.25 mM PQ. Only six transductants showed increased β -galactosidase activity after treatment with PQ (Fig. 5A). The rest of the transduced insertions were confirmed as Mu transpositions onto plasmid pJP105, as evidenced by three observations: first, the cotransduction of the plasmid-encoded resistance to ampicillin together with the Mu-encoded resistance to kanamycin; second, the presence of plasmid DNA in the *Kan*^r transductants; third, the increased sizes of these plasmids with respect to pJP105, as revealed by restriction analysis, consistent with a MudJ insertion (data not shown).

The insertional fusions were named Sins, for SoxS induced. The transduction of these Sin fusions into a Δ *soxRS* strain abolished the PQ-induced activation of β -galactosidase activity (Fig. 5B). These results demonstrate the dependence on a functional *soxRS* system for the inducible activation of *lacZ* expression from all six Sin fusions.

To test the possibility that the MudJ insertions might have disrupted genes involved in resistance to oxidative stress, we measured the relative sensitivities of wild-type, Δ *soxRS*, and the six Sin strains with PQ gradient plates. The result of a

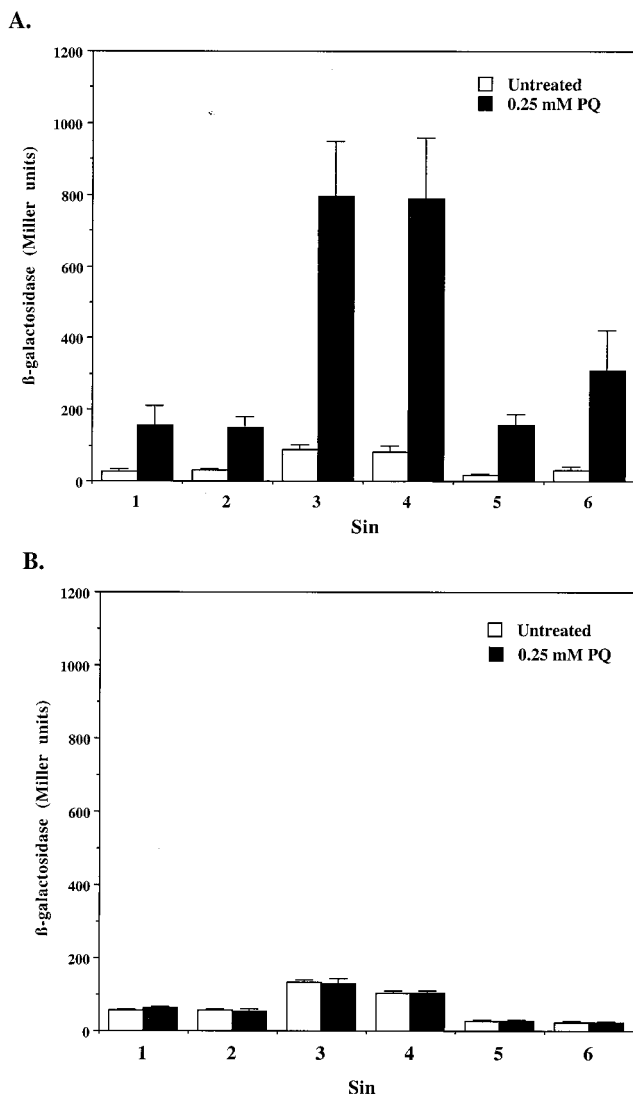


FIG. 5. Induction of β -galactosidase activity by PQ in six independent *lacZ* insertions. Liquid cultures of the mutant strains were assayed for β -galactosidase activity as explained in Materials and Methods. (A) Sin fusions in a *soxRS*⁺ background. (B) Sin fusions in a Δ *soxRS* background. The results are the averages of three independent experiments, and the error bars correspond to the standard deviations.

representative experiment (Fig. 6) showed that all six Sin strains were more sensitive to PQ than the wild-type strain. Thus, all the genes disrupted by the Sin insertions have roles in resistance to oxidative stress.

Molecular characterization of SoxS-regulated genes. To establish the identity of the *soxS*-regulated loci, we obtained the nucleotide sequence of the regions adjacent to the left end of the MudJ insertions by a nonspecific PCR method (see Materials and Methods). We then compared these nucleotide sequences with DNA databases by using the BLAST program (2). The Sin1 and Sin2 insertions were located in the *sodA* gene, which encodes a polypeptide with 97% identity to *E. coli* SodA (36). Both insertions were located at the 3'-untranslated end of the reported *sodA* sequence.

The Sin3, Sin4, and Sin5 insertions were located inside an open reading frame (ORF) homologous to that of the *fpr* gene of *E. coli*, which encodes the NADPH:ferredoxin oxidoreduc-

tase (7). The partial nucleotide sequence obtained from these insertions predicts a polypeptide 80% identical to the one of *E. coli* (data not shown).

The Sin6 insertion was located within an ORF homologous to that of *E. coli ydkB*. The *E. coli* ORF codes for a predicted polypeptide product that is 1,174 residues long; the product has no known function but has been annotated as a putative Fe-S-containing oxidoreductase (8). This predicted *E. coli* protein is homologous to the NifJ oxidoreductase from *Anabaena* spp., which shuttles electrons from pyruvate to reduce nitrogenase (6).

These three SoxS-regulated genes identified by the random-insertion approach have either proven or arguable roles in cellular defense against oxidative stress, confirming the pivotal role of SoxS as a transcriptional regulator of antioxidant defenses in *S. enterica* serovar Typhimurium.

DISCUSSION

The structural similarity between the *soxRS* genes from *E. coli* and those from *S. enterica* serovar Typhimurium (GenBank accession no., U61147) predicted that the *soxRS* regulon serves similar functions in both species. We constructed an IPTG-regulated expression system that uncouples *soxS* expression from oxidative stress in order to study the direct contribution of SoxS protein to the activation of antioxidant defenses. By using this expression system, the steady-state levels of *soxS* message could be titrated by increasing the concentration of IPTG, which mimicked the physiological range of *soxS* expression in response to oxidative stress. The PQ-induced expression of *soxS* from wild-type strain ATCC 14028 seemed to saturate at an induction ratio of \sim 70-fold, while IPTG-induced expression of *soxS* from pJP105 was linear up to \sim 100-fold. The somewhat higher level attained for the artificial construct could be due to the absence of *soxS* autorepression (30) in the IPTG-driven *lacZ* promoter.

The heterologous expression of SoxS in *S. enterica* serovar Typhimurium resulted in enhanced resistance to PQ (Fig. 2). This result demonstrates that, as in *E. coli*, an elevated level of SoxS protein is sufficient for activation of important antioxidant genes in *S. enterica* serovar Typhimurium. Expression of SoxS in the absence of oxidative stress was sufficient for transcriptional activation of *sodA* to a high level. Interestingly, exposure of a Δ *soxRS* strain to PQ elicited a small but reproducible increase in *sodA* transcription (data not shown). A modest, PQ-dependent, *soxRS*-independent induction of *sodA* was also observed by Martins et al. (unpublished data) and might be due to MarA, a transcriptional activator closely related to SoxS (1). The MarA protein activates *sodA* expression in *E. coli* (1) and may also do so in *S. enterica* serovar Typhimurium. The synthesis of MarA in *E. coli* was reported to be induced slightly by PQ (34), an effect that could underlie SoxS-independent induction of *sodA* in both organisms. The *S. enterica* serovar Typhimurium *marA* gene (35) codes for a predicted polypeptide 86% identical to *E. coli* MarA. Additionally, *sodA* in *E. coli* is regulated by ArcA, Fnr, and Fur (12). Of these, Fur is a reasonable candidate for contributing to PQ-induced *sodA* expression under aerobic growth. Expression of SoxS in Δ *soxRS* strains of *S. enterica* serovar Typhimurium in the absence of oxidative stress was sufficient for transcriptional activation of *micF*, which reached the same maximum level as that observed in wild-type cells exposed to PQ. This result demonstrates that SoxS is sufficient for full, PQ-induced expression of *micF*. The induction of *micF* has not been directly connected to oxidative stress resistance. However, induction of *micF* in *E. coli* is necessary for enhanced resistance to multiple

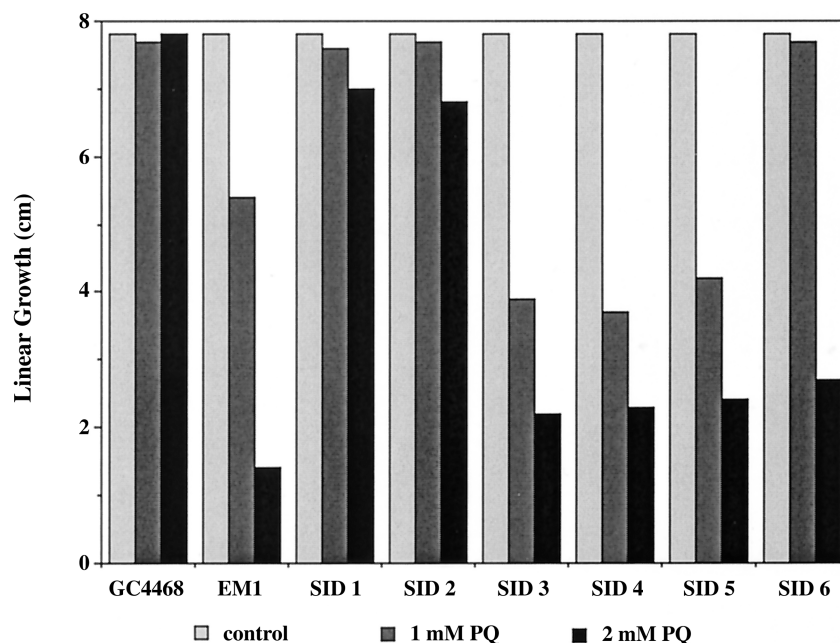


FIG. 6. Sensitivity to PQ of six independent *lacZ* insertions. Linear growth of different strains of *S. enterica* serovar Typhimurium on PQ gradient plates is shown. Maximum concentrations are shown below the graph. The maximum possible growth was 8 cm.

antibiotics. This phenotype depends on repression of the synthesis of porin OmpF, with a consequent reduction in permeability to small hydrophilic molecules (29). The apparent existence of the same mechanism in *S. enterica* serovar Typhimurium could be relevant in the development of antibiotic resistance during bacterial infection (see below).

SoxS also activates antibiotic resistance mechanisms in other species. In preliminary experiments, *Klebsiella aerogenes* transformed with pJP105 displayed increased resistance to chloramphenicol, nalidixic acid, tetracycline, and kanamycin in the presence of IPTG (P. J. Pomposiello and B. Demple, unpublished data). This result also suggests the existence of a *soxRS* regulon in *Klebsiella*. There is also evidence for a *soxRS* regulon in clinical strains of *Enterobacter agglomerans*, *Shigella flexneri*, and *Shigella dysenteriae* (B. L. Martins, A. Koutsolioutsou, and B. Demple, unpublished data).

Our screening for Sox-regulated genes with MudJ yielded *lacZ* insertions in three different genes. Of these, *sodA* was the only gene already characterized in *S. enterica* serovar Typhimurium (36), but its role in antioxidant defense is not completely understood. However, we have shown that the insertion of MudJ in *sodA* and the other two genes rendered the corresponding strains hypersensitive to oxidative stress in comparison to the isogenic wild-type strain. These results suggest strongly that all three genes have roles in protecting the cell against PQ-elicited oxidative stress. The relative PQ resistances of the Sin1 and Sin2 insertions (Fig. 6) are likely due to their locations in the 3'-untranslated region of *sodA*, which are expected to preserve substantial *sodA* function.

The remaining Mu insertions map to two uncharacterized *S. enterica* serovar Typhimurium ORFs. For Sin3, Sin4, and Sin5, the strong homology of the disrupted gene with the *E. coli fpr* gene and the high sensitivity of the Sin3, Sin4, and Sin5 strains to PQ are consistent with the identification of this locus as the *S. enterica* serovar Typhimurium *fpr* gene. The *E. coli fpr* gene was originally isolated in a screening for PQ-sensitive mutants (28). The *E. coli fpr* is a NADPH:ferredoxin oxidoreductase

required for anaerobic ribonucleotide reduction (7). Finally, Sin6 maps to a gene with a sequence similar to that of the *E. coli ydbK* ORF. The annotation of this ORF as a putative iron-sulfur-containing oxidoreductase, together with the hypersensitivity to PQ of the corresponding mutant, argues in favor of a role for this *S. enterica* serovar Typhimurium gene in protection against superoxide stress. Oxidoreductases contribute to resistance to oxidative damage by redirecting electron transfer, rereducing oxidized iron-sulfur centers, and replacing damaged components (20, 24).

There are 15 known genes in *E. coli* under *soxS* regulation (20, 24). This figure led us to expect a higher yield of SoxS-regulated genes for our mutagenesis and screening in *S. enterica* serovar Typhimurium. There are at least three possible reasons for this discrepancy. First, ~80% of the insertions identified as IPTG responsive were in plasmid pJP105 rather than in chromosomal sites. Thus, the MudJ mutagenesis was not saturating. Second, the strain used in the screening, EM1, has increased basal expression of *micF* for unknown reasons (unpublished data). If this effect extends to other genes in EM1, the difference between the basal and SoxS-activated levels of expression for various genes might be obscured. Third, it is possible that *S. enterica* serovar Typhimurium has fewer genes under *soxRS* control than does *E. coli*. As noted earlier, glucose-6-phosphate dehydrogenase and fumarate C are regulated by SoxS in *E. coli* but do not seem to be members of the *soxRS* regulon in *S. enterica* serovar Typhimurium (Martins et al., unpublished data).

SoxS does not seem to be a virulence factor in *S. enterica* serovar Typhimurium. Both $\Delta soxRS$ (Martins et al., unpublished data) and $\Delta soxS$ (16) strains were not attenuated in mouse infection experiments and had no disadvantage in survival inside activated macrophages. Of the *S. enterica* serovar Typhimurium genes under *soxRS* control, *sodA* confers some protection against early killing by macrophages, but a *sodA* strain of *S. enterica* serovar Typhimurium was not found to be attenuated in virulence assays (36). However, there is evidence

that the *soxRS* regulon could be contributing to the development of infection in humans through conferring elevated levels of multiple antibiotic resistance. Preliminary results show that expression of SoxS in *S. enterica* serovar Typhimurium is sufficient to enhance resistance to nalidixic acid and chloramphenicol (Pomposiello and Demple, unpublished data). The enhancement of antibiotic resistance via activation of the *soxRS* regulon could be a stepping-stone in the acquisition of clinically relevant antibiotic resistance, as has been proposed for the *marA* regulon (1). This hypothesis is supported by the isolation of *soxS*-constitutive *S. enterica* serovar Typhimurium (Martins et al., unpublished data) and pathogenic *E. coli* strains (31) from human patients with systemic infections. In one case of *S. enterica* serovar Typhimurium that developed increased antibiotic resistance during an infection, a mutation that contributes to the phenotype and that was mapped to a single-nucleotide change in the *soxR* gene arose (Martins et al., unpublished data). Thus, activation of the *soxRS* regulon by redox-active compounds generated by the immune system (19) could paradoxically enhance the antibiotic resistance of enteropathogenic bacteria and complicate the treatment of bacterial infections.

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