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The Transcriptional Regulator SoxS Is Required for Resistance of *Salmonella typhimurium* to Paraquat but Not for Virulence in Mice

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In *Escherichia coli*, the SoxRS regulon is required for resistance to redox-cycling agents which elevate cytosolic superoxide levels, as well as for resistance to nitric oxide-dependent macrophage killing. In *Salmonella typhimurium*, SoxS is also required for enhanced expression of Mn-superoxide dismutase and resistance to paraquat, but not for resistance to nitric oxide donor compounds in vitro, resistance to macrophage killing, or virulence in mice. Differences in other antioxidant defense systems or compensation by homologous regulons may account for species-specific differences in the role of SoxS.

A number of stress regulons which defend bacteria from oxidative injury have been identified. The divergently transcribed *soxR* and *soxS* genes encode components of an unusual two-component regulatory system which was first recognized to activate expression of more than 10 *Escherichia coli* genes in response to redox-cycling agents (e.g., paraquat), which generate elevated levels of superoxide ($O_2\cdot^-$) within the bacterial cytosol (56, 58). Generation of extracellular $O_2\cdot^-$ by xanthine oxidase-hypoxanthine does not induce the expression of SoxRS-regulated genes (49). However, induction of the SoxRS regulon is not specific for cytosolic $O_2\cdot^-$; dissolved nitric oxide (NO) gas, NO-producing macrophages, hypochlorous acid, copper sulfate, or diamide can also induce SoxRS-dependent gene activation (15, 19, 37, 41, 48).

Each SoxR monomer contains a [2Fe-2S] cluster which is required for functional activity but not for DNA binding at the *soxS* promoter region (17, 32). One-electron oxidation of the FeS center allows SoxR to activate transcription of the *soxS* gene (25). The SoxS protein in turn interacts with σ^{70}-RNA polymerase to promote transcription of multiple genes, including *zwf* (glucose-6-phosphate dehydrogenase), *sodA* (manganese-cofactored superoxide dismutase [SOD]), *micF* (antisense RNA for *ompF* mRNA), *acrAB* (inner membrane efflux pump), *nfo* (endonuclease IV), *rimK* (modifies ribosomal protein S6), *fumC* (oxidation-resistant fumarase), *acnA* (aconitase), *ribA* (GTP cyclohydrolase II), and *fpr* (ferredoxin-NADPH oxidoreductase) (9, 28, 29, 38, 41, 42, 56, 58, 59). Expression of SoxS alone can activate genes in the absence of SoxR or inducing agents such as paraquat (1).

The SoxR protein is homologous to the MerR family of transcriptional activators (1), while the SoxS protein has significant homology to the C-terminal DNA-binding region of the AraC family of transcriptional regulatory proteins (52). A subset of this family includes SoxS, MarA, Rob, TetD, and RamA from *Klebsiella pneumoniae*; PqrA from *Proteus vulgaris*; and AarP from *Providencia stuartii* (2, 10, 24, 26, 27, 33, 43, 54). These proteins have 37 to 52% amino acid identity with SoxS and, interestingly, appear to share some functional overlap as well. Overexpression of MarA, Rob, RamA, PqrA, or AarP results in the activation of at least some, though not all, SoxRS-regulated genes (2, 26, 33–35, 40, 43–45, 50). However, divergent environmental signals appear to induce expression of these homologous systems; for example, *marA* gene expression is induced by weak acids such as salicylate (11), while *rob* expression appears to be growth phase regulated (36).

E. coli strains carrying null mutations in *soxR* or *soxS* are hypersusceptible to redox-cycling agents (59). Of greater potential biological significance was an *E. coli* Δ*soxRS* mutant which was found to have a survival rate reduced by approximately fivefold in peptone-elicited peritoneal macrophages from CD1 mice (49). *E. coli* strains carrying mutations in SoxRS-regulated genes *nfo*, *micF*, *sodA*, and *zwf* were also observed to have decreased resistance to macrophage killing (48, 49). In related experiments, the hypersusceptibility of *soxS* mutant *E. coli* to macrophage killing was partially abrogated by the addition of N^G-monomethyl-L-arginine, an inhibitor of inducible NO synthase (48), suggesting that the SoxRS regulon functions to counteract NO-dependent antimicrobial systems of phagocytic cells. Other phenotypes associated with *soxRS* include resistance to multiple antimicrobial agents, tolerance to organic solvents, and resistance to heavy metals (3, 9, 44, 47).

Unlike *E. coli*, *Salmonella* species are intracellular pathogens with specific adaptations favoring survival within host macrophages (22, 23). Observations of experimental animals or of humans indicate that oxygen-dependent antimicrobial systems are important in host defense against *Salmonella* infection (7, 14, 46). Therefore, it has been suggested that the SoxRS regulon plays an important role in *Salmonella* virulence by mediating resistance to reactive oxygen and nitrogen intermediates produced by phagocytic cells (48). In the present study, we constructed a *Salmonella typhimurium* strain carrying a *soxS* mutation in order to assess the role of the SoxRS regulon in the resistance of *Salmonella* to oxidative and nitrosative stress in vitro, survival in phagocytic cells, and virulence in mice.

Primers 5′-ATCAAACTGCCGACGGAAAACGCGGGAGAA-3′ and 5′-TGACCAGCCGCTTAACATTGATGTAGTCGC-3′ were devised from the sequence of the *E. coli soxS* gene and used to amplify an internal 238-bp *soxS* fragment from *S. typhimurium* ATCC 14028s genomic DNA by reduced-stringency PCR (50°C). This fragment was ligated into the *Eco*RV site of pBLUESCRIPT (Stratagene, La Jolla, Calif.) in *E. coli* DH5α (30) and sequenced. Genetic manipulations and DNA sequence determination were performed by conven-

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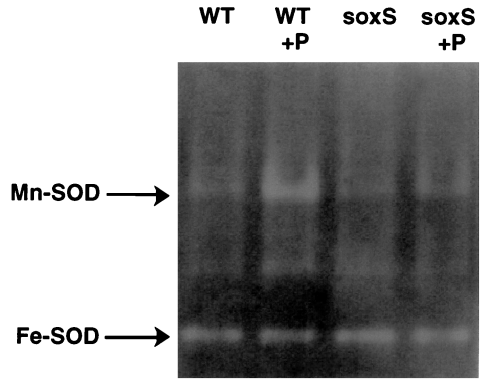

FIG. 1. Paraquat-inducible SOD activity of *soxS* mutant and wild-type (WT) *S. typhimurium* strains. Equivalent quantities of cell extracts from *soxS* mutant or wild-type *S. typhimurium* strains were electrophoresed through a 7% acrylamide nondenaturing gel and stained for visualization of SOD activity. The locations of Fe-cofactored (SodB) and Mn-cofactored (SodA) SODs are indicated. A 250 μM concentration of paraquat (+P) was used to induce the SoxRS regulon which controls *sodA* expression. Inducibility of *sodA* expression by paraquat is abrogated in the *soxS* mutant, but basal *sodA* expression is unchanged.

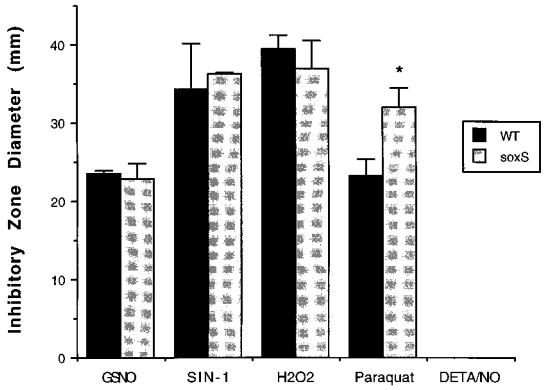

FIG. 2. Susceptibility of *soxS* mutant and wild-type (WT) *S. typhimurium* strains to reactive oxygen and nitrogen intermediates in vitro. Susceptibility was determined by a disk diffusion method (4, 13). Zone diameter (± standard deviation of the mean) is a measure of susceptibility. *soxS* mutant *S. typhimurium* has enhanced susceptibility to paraquat but not to *S*-nitrosoglutathione (GSNO), SIN-1, H_2O_2, or DETA/NO. DETA/NO failed to demonstrate detectable inhibition of either strain. *, $P < 0.01$.

tional methods (51). The cloned fragment was determined to be 81% identical to the corresponding region of the *E. coli soxS* gene. Subsequently, the entire sequence of the *S. typhimurium soxS* gene was deposited in GenBank (U61147) by other investigators and confirmed to correspond precisely with the cloned fragment. The *E. coli* and *S. typhimurium* predicted SoxS proteins are highly related, with 95% identity at the amino acid level.

The internal *S. typhimurium soxS* fragment was subcloned into the *Eco*RI and *Hin*dIII sites of suicide vector pRR10 (Δ*trfA*) (21) in *E. coli* S17-1 (55) and conjugated into *S. typhimurium* 14028s to create a *soxS* gene interruption linked to a β-lactam resistance marker. Integration of the suicide vector results in the deletion of the last 30 nucleotides of *soxS* gene, which were previously shown in *E. coli* to be essential for production of a functional SoxS protein (58). The *E. coli* donor strain was counterselected in M9 minimal medium (51) with 250 μg of penicillin (Sigma, St. Louis, Mo.) per ml, and *S. typhimurium* exconjugants on xylose-lysine-deoxycholate agar (Difco, Detroit, Mich.) were identified. Interruption of the *soxS* gene was confirmed by Southern blotting of genomic DNA (not shown) with the ^{32}P-labelled *soxS* internal fragment as a probe.

Expression of the SoxRS regulon was induced by treatment of 2×10^8 bacteria in 5 ml of Luria-Bertani (LB) broth with 250 μM paraquat (Sigma) for 4 h. Cells were subsequently lysed and analyzed by SOD activity gel electrophoresis (5) (Fig. 1). Bacteria were lysed by freeze-thawing with or without 4 h of pretreatment with 250 μM paraquat (Sigma). Extracts were separated by electrophoresis through a 7% acrylamide nondenaturing gel. The gel was stained with nitroblue tetrazolium in the presence of photoactivated riboflavin, revealing SOD activity as a negative image. Marked augmentation of Mn-SOD activity was seen in the wild type, but not in the *soxS* mutant *S. typhimurium* strain treated with paraquat.

Susceptibility to oxidant and NO donor compounds was determined by disk diffusion assaying as described previously (4, 13). Paper disks (diameter, 1/4 in.) were placed over a lawn of 10^6 bacteria. A 15-μl volume of paraquat (1.9%; Sigma), *S*-nitrosoglutathione (500 mM; prepared as described previously [13]), 3-morpholinosydnonimine (SIN-1; 500 mM; Sigma), hydrogen peroxide (3%; Sigma), or diethylenetriamine-NO adduct (500 mM DETA/NO; Research Biochemists International, Natick, Mass.) was applied to the disks as indicated elsewhere (13). Zones of inhibition were measured after overnight incubation at 37°C; the zone of inhibition is a measure of bacterial susceptibility. Each susceptibility experiment was performed at least twice. *S. typhimurium* carrying a *soxS* mutation was found to be hypersusceptible to paraquat (1.9%) compared with the wild type (Fig. 2), but not to *S*-nitrosoglutathione (500 mM), SIN-1 (500 mM), hydrogen peroxide (3%), or DETA/NO (500 mM).

Susceptibility to several conventional antimicrobial agents was also determined by a disk diffusion method (4). Mueller-Hinton agar (BBL, Cockeysville, Md.) was used for antimicrobial agent susceptibility testing. Commercially prepared disks (BBL) impregnated with tetracycline (30 μg), ofloxacin (5 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), or gentamicin (10 μg) were used as indicated elsewhere (4). Wild-type *S. typhimurium* and *soxS* mutant *S. typhimurium* did not differ in their susceptibilities to tetracycline, ofloxacin, trimethoprim-sulfamethoxazole, or gentamicin (data not shown). Thus, basal levels of resistance to multiple antimicrobial agents do not appear to be dependent on SoxRS, in contrast to observations regarding the role of MarA in *E. coli*.

Relative survival of *soxS* mutant or wild-type *S. typhimurium* in both J774.1 macrophage-like cells and gamma interferon (IFN-γ)-stimulated resident peritoneal macrophages (Fig. 3) from C57/BL6 mice was determined essentially as described by Buchmeier and Heffron (8). J774.1 cells or resident peritoneal macrophages from C57/BL6 mice (57) were cultivated in RPMI with 10% horse serum, plated to a density of 5×10^5/ml, and allowed to adhere for 4 h. IFN-γ (200 U/ml) was added to the resident peritoneal macrophages for 18 h. Bacteria were opsonized in normal mouse serum and spun onto macrophages at a multiplicity of infection of 10:1. After 15 min of incubation, gentamicin (6 μg/ml) was added to kill extracellular organisms. At specified time intervals, the macrophages were lysed in 0.5% deoxycholate and the bacteria were dilutionally plated onto LB agar for quantitation of CFU. Nitrite production was assayed by the Griess reaction (18). Each of these experiments was performed at least twice. No significant effect of the *soxS* mutation on survival in either J774.1 or peritoneal cells was evident. NO production by the peritoneal macrophages was confirmed by measurement of its end oxidation

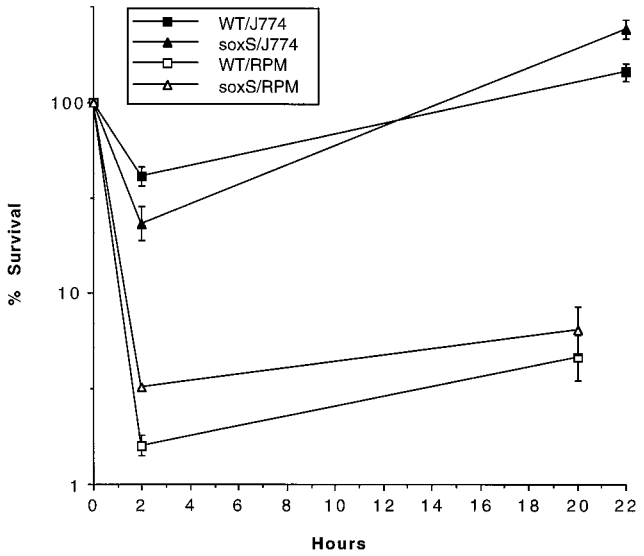

FIG. 3. Survival of *soxS* mutant and wild-type (WT) *S. typhimurium* strains in macrophages. Survival rates of *soxS* mutant and wild-type *S. typhimurium* strains in J774.1 macrophage-like cells or in IFN-γ-stimulated resident peritoneal macrophages (RPM) from C57/BL6 mice were quantitated. No significant effect of the *soxS* mutation on the ability of *Salmonella* to survive in macrophages was detected.

product nitrite, which was determined to be 800 μmol/10^6 cells/20 h following IFN-γ stimulation.

The mortality of *Ity* genetically susceptible (BALB/c and C57BL/6J) or *Ity*^{r} genetically resistant (C3H/HeN) mice was determined after intraperitoneal administration of wild-type or *soxS* mutant *S. typhimurium* to 6- to 8-week-old female BALB/c, C57BL/6J, or C3H/HeN mice (Jackson Laboratories, Bar Harbor, Maine). The intraperitoneal 50% lethal dose (LD_{50}) for wild-type *S. typhimurium* in BALB/c mice is <10 organisms (22). Therefore, an intraperitoneal inoculum of 10^3 organisms is routinely employed to detect significant attenuation of *Salmonella* virulence (22) in susceptible strains of mice. No significant effects of the *soxS* mutation on virulence were seen in any mouse model (Table 1), although a subtle effect on virulence cannot be excluded by these experiments. Mice challenged with *soxS* mutant *S. typhimurium* were necropsied to confirm the stability of the *soxS* insertion mutation in vivo; 100% of 100 individual bacterial colonies recovered from each mouse spleen were found to retain the β-lactamase marker after being picked onto selective medium.

Sequence analysis indicates that the transcriptional regulator SoxS has been highly conserved in *E. coli* and *S. typhimurium*. Elegant prior studies with *E. coli* have demonstrated that oxidative stresses which result in one-electron oxidation of the essential SoxS [2Fe-2S] cluster can activate the SoxS protein (17, 25) and ultimately result in the enhanced expression of more than 10 SoxS-activated genes which help to resist or repair oxidative cell damage. In this work, we report the mutagenesis and functional characterization of *soxS* in *S. typhimurium*. As demonstrated in *E. coli* (56), SoxS is required in *S. typhimurium* for paraquat-induced augmentation of Mn-SOD expression and resistance to the antimicrobial activity of paraquat.

However, despite earlier experiments with *E. coli* suggesting that the SoxRS regulon might allow bacteria to resist NO-related antimicrobial systems of phagocytic cells (48), *soxS* mutant *S. typhimurium* does not have enhanced susceptibility to NO donor compounds, enhanced susceptibility to killing by

TABLE 1. Virulence of *soxS* mutant and wild-type *S. typhimurium* strains in mice[a]

Mouse strain	Bacterial strain[b]	Mortality (no. of days to death)	Inoculum (i.p.)
BALB/c	WT	4/4 (7 days)	10^3
	soxS	4/4 (7 days)	10^3
	PBS only	0/4	
C57BL/6J	WT	3/3 (7 days)	1.4×10^3
	soxS	3/3 (7 days)	2×10^3
	rpoS	0/3	1.5×10^3
	Plasmid cured	1/3 (9 days)	1.4×10^3
	PBS only	0/3	
C3H/HeN	WT	3/4 (11 days)	1.6×10^3
	soxS	2/4 (13 days)	1.3×10^3
	PBS only	0/3	

[a] Mortality in BALB/c, C57BL/6J, or C3H/HeN mice following intraperitoneal (i.p.) inoculation with wild-type (WT) or mutant *S. typhimurium* was determined. The intraperitoneal *S. typhimurium* LD_{50} for BALB/c and C57BL/6J mice is <10 organisms (22), and the LD_{50} for C3H/HeN mice is 1×10^3 to 2×10^3 organisms (14). The *soxS* gene is not essential for *Salmonella* virulence in mice.

[b] PBS, phosphate-buffered saline.

NO-producing macrophages, or reduced virulence in mice, including both innately *Salmonella*-susceptible and -resistant mouse strains. These findings are somewhat unexpected, because several clinical and experimental observations have indicated that both the respiratory burst and NO production by phagocytic cells play important roles in host defense against *Salmonella* infection (7, 14, 46).

The specific reactive oxygen and nitrogen species responsible for microbial stasis or killing in vivo have not yet been precisely defined (20). Redox-cycling agents such as paraquat can induce expression of the SoxRS regulon by elevating cytosolic superoxide levels in vitro but are unlikely to represent a physiologic stimulus relevant to phagocyte-pathogen interactions, since the inner membranes of gram-negative bacteria do not appear to be permeable to superoxide anion (49, 53). Although NO radical (NO·) can penetrate membranes readily (16), it does not appear to be cytotoxic for *E. coli* or *Salmonella*, in contrast to other NO congeners (6, 13). Therefore, it is possible that the oxidants resisted by the SoxRS regulon are not the same reactants encountered by bacteria during phagocyte-pathogen interactions.

Alternatively, it is conceivable that the SoxRS regulon is nonessential for *Salmonella* virulence because of the presence of other antioxidant defenses. *Salmonella* is highly adapted to survival within macrophages (22, 23) and may have evolved additional systems capable of supplanting the role of SoxRS. Several SoxRS-activated genes are already known to be under the influence of other regulatory networks (e.g., *fur*, σ^S, *arcA*, IHF, and *fnr*) (12, 31, 39). Moreover, the complex interrelationships between SoxS and its homologs (e.g., MarR and Rob) have yet to be fully defined. Compensation for the loss of SoxS by a related transcriptional activator responding to a different environmental signal may account for the dispensability of SoxS for *Salmonella* virulence and survival in phagocytes. Additional important genetic regulatory systems controlling *Salmonella* resistance to oxygen-dependent antimicrobial systems remain to be elucidated.

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