Two-Stage Control of an Oxidative Stress Regulon: the *Escherichia coli* SoxR Protein Triggers Redox-Inducible Expression of the *soxS* Regulatory Gene

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Escherichia coli responds to the redox stress imposed by superoxide-generating agents such as paraquat by activating the synthesis of as many as 80 polypeptides. Expression of a key group of these inducible proteins is controlled at the transcriptional level by the *soxRS* locus (the *soxRS* regulon). A two-stage control system was hypothesized for *soxRS*, in which an intracellular redox signal would trigger the SoxR protein as a transcriptional activator of the *soxS* gene and the resulting increased levels of SoxS protein would activate transcription of the various *soxRS* regulon genes (B. Dimple and C. F. Amábile Cuevas, Cell 67:837–839, 1990). We have constructed operon fusions of the *E. coli lac* genes to the *soxS* promoter to monitor *soxS* transcription. Expression from the *soxS* promoter is strongly inducible by paraquat in a manner strictly dependent on a functional *soxR* gene. Several other superoxide-generating agents also trigger *soxR*⁺-dependent *soxS* expression, and the inductions by paraquat and phenazine methosulfate were dependent on the presence of oxygen. Numerous other oxidative stress agents (H₂O₂, gamma rays, heat shock, etc.) failed to induce *soxS*, while aerobic growth of superoxide dismutase-deficient bacteria triggered *soxR*-dependent *soxS* expression. These results indicate a specific redox signal for *soxS* induction. A direct role for SoxR protein in the activation of the *soxS* gene is indicated by band-shift and DNase I footprinting experiments that demonstrate specific binding of the SoxR protein in cell extracts to the *soxS* promoter. The mode of SoxR binding to DNA appears to be similar to that of its homolog MerR in that the SoxR footprint spans the –10 to –35 region of the *soxS* promoter.

Reactive oxygen species (e.g., superoxide and hydrogen peroxide) are potentially toxic agents that occur as mutagenic byproducts of aerobic metabolism (8, 13, 28) or mediate the effects of diverse environmental agents (26, 33). Enteric bacteria have evolved adaptive responses that adjust the expression of numerous genes to the stresses exerted by reactive oxygen (oxidative or redox stress [6]). In *Escherichia coli*, two oxidative stress regulons are triggered, respectively, by H₂O₂ (the *oxyR* regulon [13, 14, 29]) and by superoxide-generating (redox-cycling) agents (the *soxRS* regulon [1, 15, 32, 34]). Both systems achieve the coordinate transcriptional induction of dispersed genes but have no known regulatory overlap.

The *soxRS* regulon is positively controlled by a single locus and responds to agents (such as paraquat [PQ]) that divert electrons from NADH or NADPH to molecular oxygen to generate a flux of superoxide (11, 18). The *soxRS*-controlled genes include those encoding Mn-containing superoxide dismutase (Mn-SOD), DNA repair endonuclease IV, glucose-6-phosphate dehydrogenase, and the *micF* antisense RNA, which suppresses synthesis of the OmpF outer membrane porin (15, 32).

The functional *soxRS* locus includes two genes, *soxR* and *soxS*, arranged head to head (1, 34). The *soxS* promoter lies in the intergenic region, whereas the *soxR* promoter lies within the *soxS* gene (Fig. 1). The predicted SoxR (17-kDa)

and SoxS (13-kDa) proteins each have a predicted helix-turn-helix motif that may mediate specific DNA binding, and each protein is related to a known group of prokaryotic DNA-binding transcriptional regulators. The SoxR protein is homologous to the MerR family of proteins (1), which activate transcription of the *mer* operon in response to Hg²⁺ (2, 16, 23, 25). This homology includes both the predicted helix-turn-helix and the cysteine cluster of MerR; the latter residues have been implicated as the Hg²⁺-binding ligands involved in signalling (16, 25).

The predicted SoxS protein is homologous to the C-terminal regions of the AraC family of one-component regulators, again including a predicted helix-turn-helix motif (1, 34). Individual expression of the SoxS protein in the absence of SoxR switched on the *micF*, *sodA*, *zwf*, and *nfo* genes (and presumably the entire *soxRS* regulon), whether or not the cells were exposed to PQ (1). Moreover, the *soxS* mRNA, but not that of *soxR*, is strongly inducible by PQ (34). These and other considerations led to the hypothesis that a two-stage system controls the *soxRS* regulon (7): a cellular redox signal (not necessarily superoxide itself) activates preexisting SoxR protein, which then triggers expression of the *soxS* gene, and the elevated level of SoxS protein induces the various genes of the regulon.

Here we test a critical prediction of this model, namely, whether the *soxS* gene is inducible by redox-cycling agents in a *soxR*-dependent manner. We also provide biochemical evidence that SoxR protein interacts with the *soxS* promoter in a manner analogous to the interaction of MerR with the *mer* promoter.

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FIG. 1. Structures of the *soxS'*::*lacZ* operon fusion plasmids. The transcriptional start points and directions for *soxR* and *soxS* (half-arrows) are as reported by Wu and Weiss (34); the large open arrows depict the *soxR* and *soxS* structural genes and the orientations of their open reading frames (1, 34). The small numbered arrows in pBD100 indicate the primers used to amplify the *soxS* promoter region. The construction of the fusion plasmids is described in the text.

MATERIALS AND METHODS

Strains and plasmids. The following strains were used as host cells for the *soxS'*::*lacZ* operon fusion plasmids in this study: GC4468 (K-12 Δ *lacU169 rpsL soxRS*⁺), DJ901 (as GC4468 but Δ *soxRS901*), (1, 15), and QC909 [as GC4468 but (*sodA*::Mu dPR13)25 (*sodB*::*kan*) Δ 1-2], a gift from D. Touati, University of Paris (4). Strain XA90 [Δ (*lac pro*)*XIII ara naIA argE*(Am) *thi Rif*^r (F' *lacI*^{q1}*ZY proAB*)], from M. Ptashne via G. Verdine, Harvard University, was used for overproduction of the SoxR protein. The *lac* fusion plasmid pNK1415 (27) was a gift from N. Kleckner, Harvard University.

We constructed two kinds of *soxS'*::*lacZ* operon fusion plasmids (Fig. 1): pTN1520 (*soxR*⁺ *soxS'*::*lacZ*) and pTN1530 (Δ *soxR soxS'*::*lacZ*). To construct pTN1520, the 2.6-kb *EcoRI*-*EcoRV* fragment from pBD100 (1), containing the whole *soxR* gene and the 5' ~65% of the *soxS* gene, was inserted into *EcoRI*- and *SmaI*-digested pNK1415 (27). Deletion from pTN1520 of the 2.1-kb *EcoRI*-*BsmI* fragment containing the 3' ~60% of *soxR*, blunting of both restriction sites with T4 DNA polymerase, and religation resulted in pTN1530.

The expression plasmid for SoxR (pKOXR) was constructed by inserting a polymerase chain reaction (PCR)-generated fragment containing *soxR* (1) into *EcoRI*- and *HindIII*-digested plasmid pKEN2 (provided by G. Verdine, Harvard University), which positioned the *soxR* coding region behind a *tac* promoter. Since strain XA90 carries the *lacI*^{q1} gene, expression of SoxR from the pKOXR construct could be induced by isopropyl- β -D-thiogalactopyranoside (IPTG).

Induction experiments. The cells carrying either pTN1520 or pTN1530 used in the induction assay were inoculated into LB broth (21) with 100 μ g of ampicillin per ml (LB-ampicillin) and incubated at 37°C for ~16 h with gentle shaking (100 rpm). The saturated cultures were diluted 100-fold into fresh LB-ampicillin and incubated at 37°C for ~45 min to reach an optical density at 600 nm (OD₆₀₀) of ~0.1. For treatments with various agents (see Table 1) under aerobic conditions, 2 ml of culture was placed in a 25-ml flask, the agent was added, and the sample was shaken vigorously (300 rpm) at 37°C for the times indicated in the figure legends and then

placed on ice. For the anaerobic experiments, the cultures were transferred to an Atmos Bag (Aldrich) that had been flushed and filled with argon gas (Ar) and were bubbled with Ar at room temperature for 2 to 3 min. The agent was then added, and the tubes were sealed with serum caps and bubbling tubes and then transferred to a 37°C bath, where bubbling with Ar was continued. After 60 min of incubation, the tubes were returned to the Atmos Bag, which was flushed with Ar, and chloramphenicol was added to each sample to a final concentration of 100 μ g/ml in order to stop further protein synthesis. The samples were then placed on ice in air. When added at the end of the aerobic treatments, chloramphenicol (100 μ g/ml) did not significantly affect the β -galactosidase activity (data not shown). β -Galactosidase activity in sodium dodecyl sulfate (SDS)-CHCl₃-treated cells was determined as described by Miller (21). The OD₆₀₀ was used as a measure of cell density.

Protein expression and cell extracts. Plasmid pKOXR (for SoxR expression) and the corresponding vector pKEN2 were introduced into strain XA90. XA90/pKOXR and XA90/pKEN2 were grown at 37°C in 50 ml of LB-ampicillin at 37°C to an OD₆₀₀ of ~0.5. IPTG was added to a final concentration of 1 mM, and the incubation was continued at 37°C for 45 min. The cells were then chilled, harvested by centrifugation at 5,000 $\times$ g for 15 min at 4°C, and washed with 50 ml of ice-cold M9 salts (21). The cell pellets, kept on ice, were resuspended in 1 ml of lysis buffer (50 mM HEPES [N-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.6], 0.2 M NaCl, 10% glycerol, 0.5 mM β -mercaptoethanol), and the cells were disrupted for 3 min at 4°C with glass beads in a Mini-bead beater (Biospec Products, Bartlesville, Okla.). The disrupted cell suspensions were centrifuged at 10,000 $\times$ g for 45 min at 4°C, and the supernatants were retained as cell extracts. The extracts were kept on ice for immediate assay or stored frozen at -80°C. Protein concentrations were determined by the Bradford method (3). The overexpression of SoxR protein was confirmed by SDS-polyacrylamide gel electrophoresis (PAGE) with Coomassie blue staining (1).

DNA-binding assay. Two synthetic oligonucleotides (primer 1: 5'-CTGAATAATTTTCTGATGGG-3', nucleotides 641 to 660; primer 2: 5'-GCCACACCGCTGCGTTTCGC-3', nucleotides 820 to 801) were labeled at the 5' end with [γ -³²P]ATP (3,000 Ci/mmol; Amersham) and T4 polynucleotide kinase (Promega) and used as PCR primers for the *soxS* gene (Fig. 1) from plasmid pBD100 (1). Protein extracts were obtained as described above. The DNA-binding reaction mixes (20 μ l) contained 10 mM Tris-HCl (pH 7.5), 75 mM KCl, 2 mM dithiothreitol, 10% (vol/vol) glycerol, 0.1 μ g of poly(dI)-poly(dC), 5 fmol of ³²P-labeled fragments, and the amounts of protein extracts indicated in the figures. Reaction mixes were incubated at 25°C for 15 min and subjected to electrophoresis in 5% polyacrylamide gels (in 20 mM Tris-HCl [pH 8.0], 3 mM sodium acetate [pH 7.9], 1 mM EDTA) at 200 V for 2 h. The gels were dried, and the DNA was visualized by autoradiography.

DNase I footprinting. The 180-bp fragment containing the *soxS* promoter was amplified by PCR as described above, with unlabeled primer 1 and ³²P-labeled primer 2. The PCR product was purified on a Sephadex G-50 column and by PAGE. The DNA-protein binding reactions were performed as described above. After incubation at 25°C for 15 min, MgCl₂, CaCl₂, bovine serum albumin, and calf thymus DNA were added to final concentrations of, respectively, 2.5 mM, 1 mM, 50 μ g/ml, and 2 μ g/ml. DNase I (25 ng in 5 μ l of 10 mM Tris-HCl [pH 8.0], 2.5 mM MgCl₂, 1 mM CaCl₂) was

FIG. 2. SoxR-dependent induction of *soxS'*::*lacZ* by PQ. Cell cultures were incubated at 37°C in the absence (open symbols) or presence (solid symbols) of 50 μ M PQ in air with vigorous shaking at 300 rpm. Samples were taken at 20-min intervals to measure β -galactosidase activity. Plasmids pTN1520 (circles) and pTN1530 (squares) were present in (A) DJ901 ($\Delta soxRS$) or (B) GC4468 ($soxRS^+$).

then added, and the reaction mixes were incubated at 25°C for 2 min. The reactions were stopped by addition of 5 μ g of yeast tRNA per ml–270 mM ammonium acetate–70% ethanol. After precipitation, the DNA pellet was resuspended in 5 μ l of formamide loading buffer (20), and the samples were subjected to electrophoresis through a denaturing sequencing gel (20), drying, and autoradiography.

RESULTS

***soxR*-dependent induction of the *soxS* gene.** The *soxS* message has been shown to be inducible by PQ treatment (34), but the possible dependence of this induction on the *soxR* gene was unknown. To clarify this point, we developed a system of two *soxS'*::*lacZ* fusion plasmids that differed in their *soxR* phenotype: pTN1520 (*soxR*⁺ *soxS'*::*lacZ*) and pTN1530 ($\Delta soxR$ *soxS'*::*lacZ*) (Fig. 1). The *lacZ* genes in these constructs carry ribosome-binding sites but lack a promoter, which is supplied by *soxS* (27). The expression of β -galactosidase from the *soxS'*::*lacZ* fusions was measured as a means of quantitating *soxS* transcription. The *soxR*⁺ construct directed PQ-inducible β -galactosidase expression in a $\Delta soxRS$ strain (DJ901), which increased with the time of exposure (Fig. 2A). In contrast, no increase in β -galactosidase activity was observed in DJ901 carrying the $\Delta soxR$ *soxS'*::*lacZ* plasmid pTN1530 (Fig. 2A). PQ-inducible β -galactosidase expression was restored when pTN1530 was introduced into strain GC4468, which carries *soxR*⁺ on the chromosome (Fig. 2B). Thus, we confirm the inducibility of *soxS* by PQ and conclude that this inducible expression

TABLE 1. Effect of various agents on expression of *soxS'*::*lacZ*^a

Treatment	Concn or temp (°C)	Relative β -galactosidase level	
		pTN1520 (<i>soxR</i> ⁺)	pTN1530 (Δ <i>soxR</i>)
Inducing ^b			
PQ	10 μ M	2.6	1.1
	50 μ M	3.8	1.3
	100 μ M	4.0	1.2
Menadione	200 μ M	2.8	1.2
Plumbagin	10 μ M	3.2	1.2
	30 μ M	5.4	1.3
Phenazine methosulfate	0.1 μ M	1.5	1.1
	0.5 μ M	3.1	1.1
	1.0 μ M	4.4	1.2
Noninducing ^c			
H ₂ O ₂	50–1,000 μ M	1.3–1.4	1.3–1.5
<i>t</i> -Butyl hydroperoxide	50–500 μ M	1.1–1.3	1.0–1.3
Formaldehyde	150–1,500 μ M	0.7–0.8	0.7–1.0
Bleomycin	0.5–5.0 μ g/ml	1.1–1.3	1.0–1.3
Methyl methanesulfonate	350–1,400 μ M	0.8–1.2	1.2–1.3
MNNG ^d	1–10 μ M	0.9–1.0	1.1–1.4
Chlorpromazine	50–200 μ M	0.8–0.9	0.8–1.3
Nalidixic acid	1–1,000 μ M	1.0–1.4	1.1–1.6
K ₂ Cr ₂ O ₇	10–500 μ M	1.0–1.4	0.9–1.3
CdCl ₂	1–1,000 μ M	1.1–1.4	1.0–1.6
Gamma irradiation	10 or 20 krad	1.3–1.4	1.4–1.5
Heat shock	30→37	1.1	0.8
	37→42	0.9	0.7
	37→45	1.1	0.6

^a Both plasmids were present in strain DJ901 ($\Delta soxRS$). β -Galactosidase activity, expressed as a ratio relative to the level in untreated cells, was determined after 60 min of exposure to most of the indicated agents. Control cultures were also incubated for 60 min prior to β -galactosidase determination. For heat shock, the cultures were first incubated at the higher temperature for 15 min. For gamma irradiation and heat shock, the cultures were incubated after the treatment for 60 min at the initial temperature. The uninduced level of β -galactosidase activity was 4,810 U for pTN1520 and 2,760 U for pTN1530.

^b No inhibition of cell growth was observed for these compounds at the concentrations tested.

^c The highest levels of these agents inhibited the growth of both strains.

^d MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

depends on a functional *soxR* gene. It is also noteworthy that, for both the *soxR*⁺ and $\Delta soxR$ constructs, the basal expression of *soxS'*::*lacZ* decreased slowly as the cells grew to mid-log phase (Fig. 2).

Specific induction of the *soxS* gene by redox-cycling agents. The inducibility of *soxS* by redox-cycling compounds and other agents was investigated by using the plasmid-borne fusions. Several well-characterized redox-cycling agents chemically unrelated to PQ also induce the *soxRS* regulon: phenazine methosulfate, menadione sodium bisulfite (menadione), and plumbagin. In addition to PQ, phenazine methosulfate, menadione, and plumbagin were all strong inducers of *soxS*, and in each case, the induction depended on a functional *soxR* gene (Table 1).

In contrast, a variety of other treatments failed to produce significant activation of *soxS* expression (Table 1). Some of these noninducing treatments may cause different types of oxidative stress: H₂O₂, *tert*-butyl hydroperoxide, formaldehyde, gamma irradiation, heat shock, chlorpromazine, and potassium dichromate (Table 1). Others have reported induction of the Mn-SOD enzyme by chlorpromazine (35) or heat shock (24). Evidently, these inductions are dependent

FIG. 3. Oxygen dependence of *soxS'::lacZ* induction by redox-cycling agents. Cultures of strain DJ901 (Δ *soxRS*) containing plasmid pTN1520 (*soxR*⁺ *soxS'::lacZ*) were maintained either aerobically (open bars) or anaerobically (hatched bars), with or without exposure to PQ (50 μ M) or phenazine methosulfate (PMS; 1 μ M). After 60 min at 37°C, β -galactosidase activity was determined as described in the text. This experiment was repeated three times with essentially identical results; a single experiment is shown.

on other transcriptional regulatory systems (6, 12) or on the posttranslational activation of the enzyme (31).

Oxygen dependence of *soxS* inductions. Redox-cycling agents require molecular oxygen to generate superoxide (18). We examined whether this requirement also applies to the induction of the *soxS* gene by these compounds. Significantly anaerobic conditions were achieved by incubating liquid cultures, previously bubbled with argon gas, in a sealed chamber flushed with argon (see Materials and Methods). The induction of *soxS'::lacZ* by PQ and phenazine methosulfate was almost totally eliminated when the cells were grown anaerobically (Fig. 3). Thus, oxygen is essential for *soxS* induction by the redox-cycling agents. It is noteworthy that the basal expression of β -galactosidase from the *soxR*⁺ *soxS'::lacZ* construct also decreased significantly in the cells incubated anaerobically (Fig. 3). Such a decrease was not observed for the Δ *soxR* *soxS'::lacZ* construct (data not shown), which suggests that SoxR might be partially activated even during normal aerobic growth.

Since endogenously generated superoxide is scavenged by superoxide dismutases (SODs), the intracellular flux of this radical can also be increased in *E. coli* by inactivating the SOD genes (*sodA* and *sodB* [4]). During aerobic growth, such SOD-deficient bacteria (which also carried chromosomal *soxR*⁺ and *soxS*⁺ genes) expressed the *soxS'::lacZ* fusion at higher levels than did their SOD-containing counterparts (Fig. 4). This difference increased from 1.5- to 4-fold-higher levels during continued aerobic growth, as *soxS* expression in the SOD-deficient strain increased modestly and expression in the SOD-containing strain diminished (Fig. 4), as seen earlier (Fig. 2). When the aeration was eliminated (by allowing the cultures to stand in air), *soxS* expression did not increase with time in the SOD-deficient strain (Fig. 4). However, the SOD-deficient cells grown without aeration did continue to express *soxS* to a higher level than did the SOD-containing cells grown without aeration, with a roughly constant difference (Fig. 4). Taken together, these experiments indicated that superoxide may trigger SoxR protein to activate *soxS* expression and that enough superoxide to give modest activation of SoxR may be formed even in poorly aerated cells lacking SOD.

SoxR binding to the *soxS* promoter region. We sought evidence that SoxR protein is the direct activator of the *soxS* gene. We therefore tested the ability of SoxR protein (in cell

FIG. 4. Induction of *soxS'::lacZ* during aerobic growth of an SOD-deficient strain. Overnight cultures of GC4468/pTN1530 (SOD producing; squares) and QC909/pTN1530 (SOD deficient; circles) were prepared at 37°C without shaking, diluted 100-fold, and grown at 37°C for about 60 min without shaking to reach an OD₆₀₀ of ~0.1. Cultures (10 ml) in 250-ml flasks were incubated in the absence of any inducers at 37°C with (solid symbols) or without (open symbols) vigorous shaking at 300 rpm. Samples were taken at 30-min intervals for determination of β -galactosidase activity.

extracts of an overproducing strain) to bind a small, PCR-amplified, ³²P-labeled DNA fragment that spanned the promoter region of the *soxS* gene. In gel mobility shift assays, SoxR-enriched extracts clearly produced a protein-DNA complex, the amount of which increased with increasing amounts of extract and which was not detected in assays of control extracts containing only the wild-type level of the SoxR protein (Fig. 5). The unlabeled *soxS* promoter fragment acted as a competitor that could be overcome at high protein levels (Fig. 5), while unrelated DNA fragments (e.g.,

FIG. 5. Specific binding of SoxR protein to DNA containing the *soxS* promoter. The 180-bp PCR fragment bearing the *soxS* promoter was incubated without (lane 1) or with the indicated quantities of protein extracts from strains carrying plasmid pKEN2 (vector alone) or pKEXR (for overexpression of SoxR). In some samples, a 10-fold molar excess of unlabeled competitor DNA was added to the binding reaction mix: lane 6, *Eco*RI-digested pBD100, bearing the *soxS* promoter; lane 7, *Bam*HI-digested Bluescript plasmid DNA, which does not contain *soxS*. Free DNA (D) and the DNA-protein complex (C) are indicated with arrows.

FIG. 6. DNase I footprint of the SoxR-*soxS* promoter complex. Lane 1, 180-bp PCR fragment containing the *soxS* promoter region labeled in the sense strand with primer 2, plus a protein extract of XA90/pKEN2 (1 μ g) as a negative control. Lanes 2 and 3, the same DNA plus 0.3 μ g of an extract of XA90/pKSOXR cells treated with IPTG to induce SoxR; for lane 3, the cells were also treated with 1 mM PQ for 40 min prior to extraction. Lanes A, C, G, and T, sequencing ladder of pBD100 DNA with primer 2. The -10 and -35 regions of the *soxS* promoter are indicated by vertical bars. The DNase-hypersensitive sites are marked with arrows.

Bluescript plasmid DNA) did not act as competitors (Fig. 5). A crude estimate of the stability of the SoxR-DNA complex can be calculated from the concentration of SoxR protein present in the cell extract (3 to 5% of the total protein, as judged from Coomassie blue-stained SDS-PAGE gels), the amount of DNA fragment present (as judged from ethidium bromide-stained agarose gels), and the amount of extract needed to bind 50% of the DNA fragment. This estimate yields a dissociation constant for SoxR binding to the *soxS* promoter of 10^{-8} to 10^{-10} M. Such stability is consistent with site-specific binding by SoxR. Thus, SoxR has the properties expected for a specific regulator of the *soxS* gene.

We used DNase I footprinting to define the site of the SoxR-DNA interaction. The results of a typical experiment are presented in Fig. 6, which shows that the SoxR protein binds specifically to a 27-nucleotide sequence in the sense strand of the *soxS* promoter. No other positions in the DNA fragment were protected by SoxR against DNase I cleavage. The protected area includes the -10 and -35 regions of the *soxS* promoter, and the binding of SoxR to this target site also leads to the formation of two DNase-hypersensitive sites (Fig. 6). Labeling of the antisense strand also revealed protection of the same region by the SoxR protein (data not shown).

Extracts of PQ-treated cells were also examined for DNA-

binding activity. Under our conditions, no binding activity was detected in PQ-treated cells that did not overexpress SoxR. For cells expressing high levels of SoxR, neither the extent of *soxS* promoter binding (data not shown) nor the extent of the SoxR footprint (Fig. 6, lane 3) was altered by this treatment. Thus, we cannot distinguish whether both inactive and activated forms of SoxR bind the *soxS* promoter or the protein extraction process itself converts SoxR to an active form.

DISCUSSION

We have used an operon fusion of the *lacZ* gene to the *E. coli soxS* promoter to monitor the regulation of *soxS*. These studies demonstrate that the induction of *soxS* by PQ and other redox-cycling agents is strictly dependent on a functional *soxR* gene. A direct role for the SoxR protein in transcriptional activation of *soxS* is supported by DNA-binding experiments, which show a strong and specific binding of SoxR within the *soxS* promoter. The *soxR*-dependent induction of *soxS*::*lacZ* by the redox-cycling agents was tightly dependent on the presence of oxygen, and the fusion was also induced by aerobic growth of an SOD-deficient strain. Other agents of oxidative stress (H_2O_2 , *t*-butyl hydroperoxide, heat shock, etc.) were unable to induce *soxS*, a pattern that parallels the induction of the *soxRS* regulon genes, such as *sodA* (31) and *nfo* (5).

These results support the hypothesis of two-stage regulation (7), in which the redox-activated SoxR protein binds the *soxS* promoter to activate transcription and the elevated levels of SoxS protein induce the various *soxRS* regulon genes. Thus, the SoxS protein may be merely a "slave" gene activator whose activity is controlled at the level of mRNA synthesis. In this scheme, the sole function of SoxR would be to trigger expression of *soxS*. The induction of *soxRS* regulon genes (*sodA*, *nfo*, *zwf*, and *micF*) in cells expressing high levels of SoxS and in the absence of SoxR protein or a redox stimulus (1) is consistent with this model, although direct activation of other genes (e.g., *soi-28*) by SoxR has not been ruled out. However, the possibility also has not been ruled out that lower (and perhaps more physiological) levels of SoxS could require the assistance of SoxR for activation of the regulon genes. Several preliminary experiments have now indicated some competition between the *sodA* and *nfo* promoters for SoxR binding to the *soxS* promoter, which could indicate joint regulation of the regulon genes. The effects of expressing *soxS* alone in a single copy per cell must also be examined.

The cellular signal that activates SoxR in response to PQ and other redox-cycling agents has not been established. An obvious candidate is the superoxide radical. This molecule is kept to very low steady-state levels in SOD-containing cells, but the level increases considerably in SOD-deficient bacteria (17). Thus, the induction of *soxS* during aerobic growth of SOD-deficient *E. coli* could be ascribed to metabolic production of superoxide. However, several reports cast doubt on this interpretation. Nettleton et al. first showed (22) that elevated levels of Fe-SOD (the *sodB* gene product) did not interfere with induction of Mn-SOD (the *sodA* gene product) by PQ. Touati (31) obtained a similar result by monitoring expression of a *sodA*::*lac* operon fusion in cells that overexpressed MnSOD. Neither of these studies distinguished *soxRS*-dependent induction from induction by other regulators of *sodA* (12, 30). More recently, Liochev and Fridovich (19) showed directly that Mn-SOD overproduction did not block the induction by PQ of another *soxRS*-regulated activ-

ity, fumarase C, and noted that the fumarase C induction by PQ was strongly enhanced in mutants deficient in glucose-6-phosphate dehydrogenase. They hypothesized that SoxR senses the ratio of NADP⁺ to NADPH in the cell. It is not known whether glucose-6-phosphate dehydrogenase affects the *saxRS* induction caused by aerobic growth of SOD-deficient cells.

It is difficult to imagine how a protein such as SoxR might sense the ratio of relatively abundant nucleotides, especially as many other proteins compete to bind these molecules. SoxR has a cluster of cysteine residues that might contribute to a redox-sensing center in the protein (1, 34). The location of an activation center in this region of SoxR is supported by two separate lines of evidence. At least three *saxR* constitutive mutations alter amino acids near this cluster but leave it intact (1, 22a, 34). Moreover, the only known homologs of SoxR, the MerR family of gene regulators, employ a similar cluster of cysteines to bind Hg²⁺ and stimulate transcriptional activation by MerR. Preliminary experiments indicate that SoxR is not activated by HgCl₂ (unpublished data). If the cysteines of SoxR are metal ligands, they would likely bind other redox-active metals, such as iron or copper.

The similarity between SoxR and MerR extends beyond linear comparisons, because the mode of DNA binding by SoxR seems remarkably like that of MerR. Both proteins protect the region between the -35 and -10 boxes of their target promoters and induce similar DNase-hypersensitive sites. Thus, activated SoxR could exert transcriptional activation by the same sort of torsional mechanism as seen for activated MerR (2), although the -35 to -10 spacing in the *saxS* promoter is only 18 bp (34). We are establishing an in vitro system to characterize transcriptional stimulation by SoxR, which may provide an assay for inducers that activate the protein. At the moment, we cannot say whether the SoxR protein extracted under our conditions is active or inactive for stimulation of RNA polymerase. In this connection, OxyR protein was activated by exposure to oxygen during extraction from cells (29).

The induced expression of *saxS'*::*lacZ* fusions seen in the experiments presented here seems to be lower than the induction of the *saxS* message reported by Wu and Weiss (34). This difference might be due to the use of high-copy-number plasmids carrying the fusions in the experiments shown here. Indeed, more recent experiments indicate that single-copy *saxS'*::*lacZ* fusions can be induced ~20-fold by 100 μM PQ (unpublished data). The source of the difference in expression between the single- and multicopy fusions is being explored. Further complications in this system will likely arise from the presence of the *saxR* promoter within the *saxS* structural gene. The overlapping mRNAs for *saxR* and *saxS* predicted by this arrangement might also play a special role that is not mimicked well by the fusions we have used. The purification of SoxR protein and its use in in vitro transcription experiments may help to answer such questions and will also be critical to the identification of agents that switch the protein to an active form.

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REFERENCES

- Amábile Cuevas, C. F., and B. Dimple. 1991. Molecular characterization of the *saxRS* genes of *Escherichia coli*: two genes

- control a superoxide stress regulon. *Nucleic Acids Res.* **19**: 4479-4484.
- Ansari, A. Z., M. L. Chael, and T. V. O'Halloran. 1992. Allosteric underwinding of DNA is a critical step in positive control of transcription by Hg-MerR. *Nature (London)* **355**:87-89.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
- Carliz, A., and D. Touati. 1986. Isolation of superoxide dismutase mutants in *E. coli*: is superoxide dismutase necessary for aerobic life? *EMBO J.* **5**:623-630.
- Chan, E., and B. Weiss. 1987. Endonuclease IV of *Escherichia coli* is induced by paraquat. *Proc. Natl. Acad. Sci. USA* **84**:3189-3193.
- Dimple, B. 1991. Regulation of bacterial oxidative stress genes. *Annu. Rev. Genet.* **25**:315-337.
- Dimple, B., and C. F. Amábile Cuevas. 1991. Redox redux: the control of oxidative stress responses. *Cell* **67**:837-839.
- Farr, S. B., R. D'Ari, and D. Touati. 1986. Oxygen-dependent mutagenesis in *Escherichia coli* lacking superoxide dismutase. *Proc. Natl. Acad. Sci. USA* **83**:8268-8272.
- Fee, J. A. 1991. Regulation of *sod* genes in *Escherichia coli*: relevance to superoxide dismutase function. *Mol. Microbiol.* **5**:2599-2610.
- Frantz, B., and T. V. O'Halloran. 1990. DNA distortion accompanies transcriptional activation by the metal-responsive gene-regulatory protein MerR. *Biochemistry* **29**:4747-4751.
- Fridovich, I. 1983. Superoxide radical: an endogenous toxicant. *Annu. Rev. Pharmacol. Toxicol.* **23**:239-257.
- Greenberg, J. T., J. H. Chou, P. A. Monach, and B. Dimple. 1991. Activation of oxidative stress genes by mutations at the *saxQ/cfxB/marA* locus of *Escherichia coli*. *J. Bacteriol.* **173**: 4433-4439.
- Greenberg, J. T., and B. Dimple. 1988. Overproduction of peroxide-scavenging enzymes in *Escherichia coli* suppresses spontaneous mutagenesis and sensitivity to redox-cycling agents in *oxyR*⁻ mutants. *EMBO J.* **7**:2611-2617.
- Greenberg, J. T., and B. Dimple. 1989. A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. *J. Bacteriol.* **171**:3933-3939.
- Greenberg, J. T., P. Monach, J. H. Chou, D. Josephy, and B. Dimple. 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **87**:6181-6185.
- Helmann, J. D., B. T. Ballard, and C. T. Walsh. 1990. The MerR metalloregulatory protein binds mercuric iron as a tricoordinate, metal-bridged dimer. *Science* **247**:946-948.
- Imlay, J. A., and I. Fridovich. 1991. Assay of metabolic superoxide production in *Escherichia coli*. *J. Biol. Chem.* **266**:6957-6965.
- Kappus, H., and H. Sies. 1981. Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. *Experientia* **37**:1233-1235.
- Liochev, S. I., and I. Fridovich. 1992. Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the *saxRS* regulon. *Proc. Natl. Acad. Sci. USA* **89**:5892-5896.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Nettleton, C. J., C. Bull, T. O. Baldwin, and J. A. Fee. 1984. Isolation of the *Escherichia coli* iron superoxide dismutase gene: evidence that intracellular superoxide concentration does not regulate oxygen-dependent synthesis of the manganese superoxide dismutase. *Proc. Natl. Acad. Sci. USA* **81**:4970-4973.
- Nunoshiba, T., and B. Dimple. Unpublished data.
- O'Halloran, T. V., B. Frantz, M. K. Shin, D. M. Ralston, and J. G. Wright. 1989. The MerR heavy metal receptor mediates positive activation in a topologically novel transcription com-

- plex. *Cell* **56**:119–129.
24. Privalle, C. T., and I. Fridovich. 1987. Induction of superoxide dismutase in *Escherichia coli* by heat shock. *Proc. Natl. Acad. Sci. USA* **84**:2723–2726.
  25. Ross, W., S. Park, and A. O. Summers. 1989. Genetic analysis of transcriptional activation and repression in the Tn21 *mer* operon. *J. Bacteriol.* **171**:4009–4018.
  26. Sies, H. 1991. *Oxidative stress: oxidants and antioxidants*. Academic Press, London.
  27. Simons, R. W., F. Houman, and N. Kleckner. 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**:85–96.
  28. Storz, G., M. F. Christman, H. Sies, and B. N. Ames. 1987. Spontaneous mutagenesis and oxidative damage to DNA in *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **84**:8917–8921.
  29. Storz, G., L. A. Tartaglia, and B. N. Ames. 1990. Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* **248**:189–194.
  30. Tardat, B., and D. Touati. 1991. Two global regulators repress the anaerobic expression of MnSOD in *Escherichia coli*: Fur (ferric uptake regulation) and Arc (aerobic respiration control). *Mol. Microbiol.* **5**:455–465.
  31. Touati, D. 1988. Transcriptional and posttranscriptional regulation of manganese superoxide dismutase biosynthesis in *Escherichia coli*, studied with operon and protein fusions. *J. Bacteriol.* **170**:2511–2520.
  32. Tsaneva, I. R., and B. Weiss. 1990. *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. *J. Bacteriol.* **172**:4197–4205.
  33. von Sonntag, C. 1987. *The chemical basis of radiation biology*. Taylor and Francis, London.
  34. Wu, J., and B. Weiss. 1991. Two divergently transcribed genes, *soxR* and *soxS*, control a superoxide response regulon of *Escherichia coli*. *J. Bacteriol.* **173**:2864–2871.
  35. Zhang, Q., and S. Yonei. 1991. Induction of manganese-superoxide dismutase by membrane-binding drugs in *Escherichia coli*. *J. Bacteriol.* **173**:3488–3491.