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

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\textit{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 soxS regulon genes (B. Demple and C. F. Amábile Cuevas, Cell 67:837–839, 1990). We have constructed operon fusions of the \textit{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 (H$_{2}$O$_{2}$, 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 \textit{Escherichia coli}, two oxidative stress regulons are triggered, respectively, by H$_{2}$O$_{2}$ (the soxR 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 Hg$^{2+}$ (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 Hg$^{2+}$-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.
CONTROL OF E. COLI saxS GENE BY saxR

FIG. 1. Structures of the saxS"::lacZ operon fusion plasmids. The transcriptional start points and directions for saxR and saxS (half-arrows) are as reported by Wu and Weiss (34); the open arrows depict the saxR and saxS structural genes and the orientations of their host cells in the (half-arrows) are as arrows

MATERIALS AND METHODS

Strains and plasmids. The following strains were used as host cells for the saxS"::lacZ operon fusion plasmids in this study: CC4468 (K-12 ΔlacU169 rpsL saxRS"), DJ901 (as CC4468 but ΔsaxRS901), 1, (1, 15), and Q2909 [as CC4468 but (sodA):Mu dPR1325 (sodB::kan')Δ-1], a gift from D. Touati, University of Paris (4). Strain XA90 [Δ(lac pro)III ara nalA argE(Am) thi Rif'] (F' lacF'Z Y proAB)], from M. Ptashne via G. Verdone, 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 saxS"::lacZ operon fusion plasmids (Fig. 1): pTN1520 (soxR" saxS"::lacZ) and pTN1530 (ΔsoxR saxS"::lacZ). To construct pTN1520, the 2.6-kb EcoRI-EcoRV fragment from pBD100 (1), containing the whole saxR gene and the 5' ~ 65% of the saxS gene, was inserted into EcoRI- and Smal-digested pNK1415 (27). Deletion from pTN1520 of the 2.1-kb EcoRI-BsmI fragment containing the 3' ~ 60% of saxR, 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. Verdone, Harvard University), which positioned the saxR coding region behind a tac promoter. Since strain XA90 carries the lacF" 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 µg 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 Atmom 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 Atmom Bag, which was flushed with Ar, and chloramphenicol was added to each sample to a final concentration of 100 µg/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 µg/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 plasmid 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 × g for 15 min at 4°C, and washed with 50 ml of ice-cold 9 M 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 × 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 (primers 1: 5'-CTGAATAATTTCGTGATGG-3', nucleotides 641 to 660; primer 2: 5'-GCCAACCGCTGCTTTCGCC-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 saxS gene (Fig. 1) from plasmid pBD100 (1). Protein extracts were obtained as described above. The DNA-binding reaction mixes (20 µl) contained 10 mM Tris-HCl (pH 7.5), 75 mM KCl, 2 mM dithiothreitol, 10% (vol/w) glycerol, 0.1 µg 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 saxS 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 then 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 µg/ml, and 2 µg/ml. DNase I (25 ng in 5 µl of 10 mM Tris-HCl [pH 8.0], 2.5 mM MgCl₂, 1 mM CaCl₂) was
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 (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 ΔsoxRS 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 ΔsoxR 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 depends on a functional soxR gene. It is also noteworthy that, for both the soxR+ and ΔsoxR 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 (1). Others have reported induction of the Mn-SOD enzyme by chlorpromazine (35) or heat shock (24). Evidently, these inductions are dependent

![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 μM 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 (ΔsoxRS) or (B) GC4468 (soxRS+).](http://jb.asm.org/)

![TABLE 1. Effect of various agents on expression of soxS\':::lacZ\(^a\)](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (μM)</th>
<th>Relative β-galactosidase level (pTN1520/soxRS+ vs. pTN1530/ΔsoxR)</th>
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<tbody>
<tr>
<td>Inducing</td>
<td></td>
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<tr>
<td>PQ</td>
<td>10</td>
<td>2.6/1.1</td>
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<td></td>
<td>50</td>
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<td></td>
<td>100</td>
<td>4.0/1.2</td>
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<tr>
<td>Menadione</td>
<td>200</td>
<td>2.8/1.2</td>
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<tr>
<td>Plumbagin</td>
<td>10</td>
<td>3.2/1.2</td>
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<tr>
<td></td>
<td>30</td>
<td>5.4/1.3</td>
</tr>
<tr>
<td>Phenazine methosulfate</td>
<td>0.1 μM</td>
<td>3.1/1.1</td>
</tr>
<tr>
<td></td>
<td>0.5 μM</td>
<td>4.2/1.1</td>
</tr>
</tbody>
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\(^a\) Both plasmids were present in strain DJ901 (ΔsoxRS). 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.

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

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\(^c\) MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.

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\(^d\) The highest levels of these agents inhibited the growth of both strains.

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\(^e\) The soxS\':::lacZ fusions decreased slowly as the cells grew to mid-log phase (Fig. 2).
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 soxS':::lacZ construct also decreased significantly in the cells incubated anaerobically (Fig. 3). Such a decrease was not observed for the ΔsoxR 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 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.,...
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 func-
tional 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-depen-
dent 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 (H₂O₂,
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 regula-
tion (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 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 bacte-
ria (17). Thus, the induction of soxS during aerobic growth
of SOD-deficient E. coli could be ascribed to metabolic produc-
tion 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 over-
expressed MnSOD. Neither of these studies distinguished
soxRS-dependent induction from induction by other regula-
tors 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 soxRS 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 soxR 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 soxS 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 soxS::lacZ fusions seen in the experiments presented here seems to be lower than the induction of the soxS 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 soxS::lacZ fusions can be induced ~20-fold by 10 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 soxR promoter within the soxS structural gene. The overlapping mRNAs for soxR and soxS 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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