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Activation of SoxR-Dependent Transcription in *Pseudomonas aeruginosa*

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The SoxR protein of *Escherichia coli* responds to redox signals by activating the transcription of *soxS*, which encodes another transcription activator that directly stimulates oxidative stress genes. In contrast, *Pseudomonas aeruginosa* has an open reading frame (ORF) encoding a putative protein homologous to *E. coli* SoxR, but not to SoxS. Instead of a *soxS* homolog, ORFs encoding an unknown hypothetical protein and *soxR* are arranged divergently with their 5′ ends separated by a 78 bp region containing a sequence homologous to the SoxR-binding *soxS* promoter. In this study, we report the overproduction and purification of SoxR from *P. aeruginosa* to investigate the mechanism of gene activation by SoxR. The spectroscopic properties of the purified SoxR protein indicate that it contains a redox active iron-sulfur [2Fe-2S] cluster. Redox titration of the SoxR protein revealed a midpoint potential of –290 mV. The SoxR protein specifically binds a fragment of the *SoxS* promoter–like region in a concentration-dependent fashion, as shown by both gel mobility shift and fluorescence polarization assays. The purified SoxR stimulates the *in vitro* transcription of the gene encoding the hypothetical protein in *P. aeruginosa*. This activity was lost following reduction of the SoxR [2Fe-2S] clusters. The levels of mRNA in the hypothetical protein increased in paraquat-treated cells. These results indicate that *P. aeruginosa* SoxR is a direct transcriptional activator of the hypothetical protein, and suggest that SoxR proteins may play multiple regulatory roles as a transcription factor in addition to its protective role in oxidative stress.

Key words: iron-sulfur cluster, *Pseudomonas aeruginosa*, redox signal, SoxR, transcription activator.

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; Mops, 3-(*N*-morpholino)propane sulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, G6PD, glucose-6-phosphate dehydrogenase; SOD, superoxide dismutase.

In *Escherichia coli*, the *SoxRS* regulon mediates an oxidative stress response that protects the cell against superoxide anions (*1–4*) or nitric oxide (*5–7*). The sensor for this regulon is the SoxR protein, a transcription factor that contains [2Fe-2S] centers (*8*, *9*). During normal growth *in vivo*, the [2Fe-2S] centers are in the reduced state; upon superoxide stress, the clusters are oxidized and the protein induces the transcription of its only known target, the *soxS* gene (*9–14*). Activated SoxR enhances the production of SoxS, a simple transcriptional activator of the AraC family, which in turn increases the transcription of other genes in the regulon. These inducible genes include *sod A* (manganese containing superoxide dismutase) (Mn-SOD), *zwf* (glucose 6-phophate dehydrogenase)(G6PD), *fldA* and *fldB* (two distinct flavodoxins), *fur* (another gene regulator that is mainly involved with iron metabolism), *nfo* (DNA repair endonuclease IV), *acrAB*(an efflux pump), and *micF* (down-regulates the expression of the porin OmpF) (*15*, *16*). Some *soxRS* regulon members are not clearly connected to oxidative stress (*e.g. micF*, *acrAB*) but instead mediate increased resistance to multiple antibiotics and organic solvents (*3*, *17–19*).

The *soxR* and *soxS* genes are arranged head-to-head in the *E. coli* genome (*2*). The regulatory region including the *soxS* promoter lies in the intergenic region (*3*, *4*, *20*). In terms of homologs, the *Salmonella enterica* serovar Typhimurium (*S. enterica*) genome (Microviral Genome Database) contains a sequence encoding predicted polypeptides that are 97% identical to SoxR and SoxS. In contrast, other bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*), *Vibrio cholera*, *Xanthomonas axonopodis* and *Chromobacterium violaceuace* have not been found to contain sequences encoding *soxS*-like gene products, although each has an open reading frame (ORF) encoding a putative protein homologous to the *E. coli* SoxR (Kyoto Encyclopedia of Gene and Genomes Gene Database). Figure 1 shows the sequences of the *soxR* homolog and surrounding regions in *P. aeruginosa* [obtained from the *P. aeruginosa* Genome Project web site (*21*)], *Xanthomonas axonopodis*, *Chromobacterium axonopoids*, and *Vibrio cholera*, as compared with *E. coli*. In *P. aeruginosa*, the homolog of the *soxR* gene (PA2273) and an unknown hypothetical protein (PA2274) are arranged divergently with their 5′ ends separated by a 78 bp intergenic region that contains a sequence homologous to the SoxR-binding *soxS* promoter of *E. coli*.

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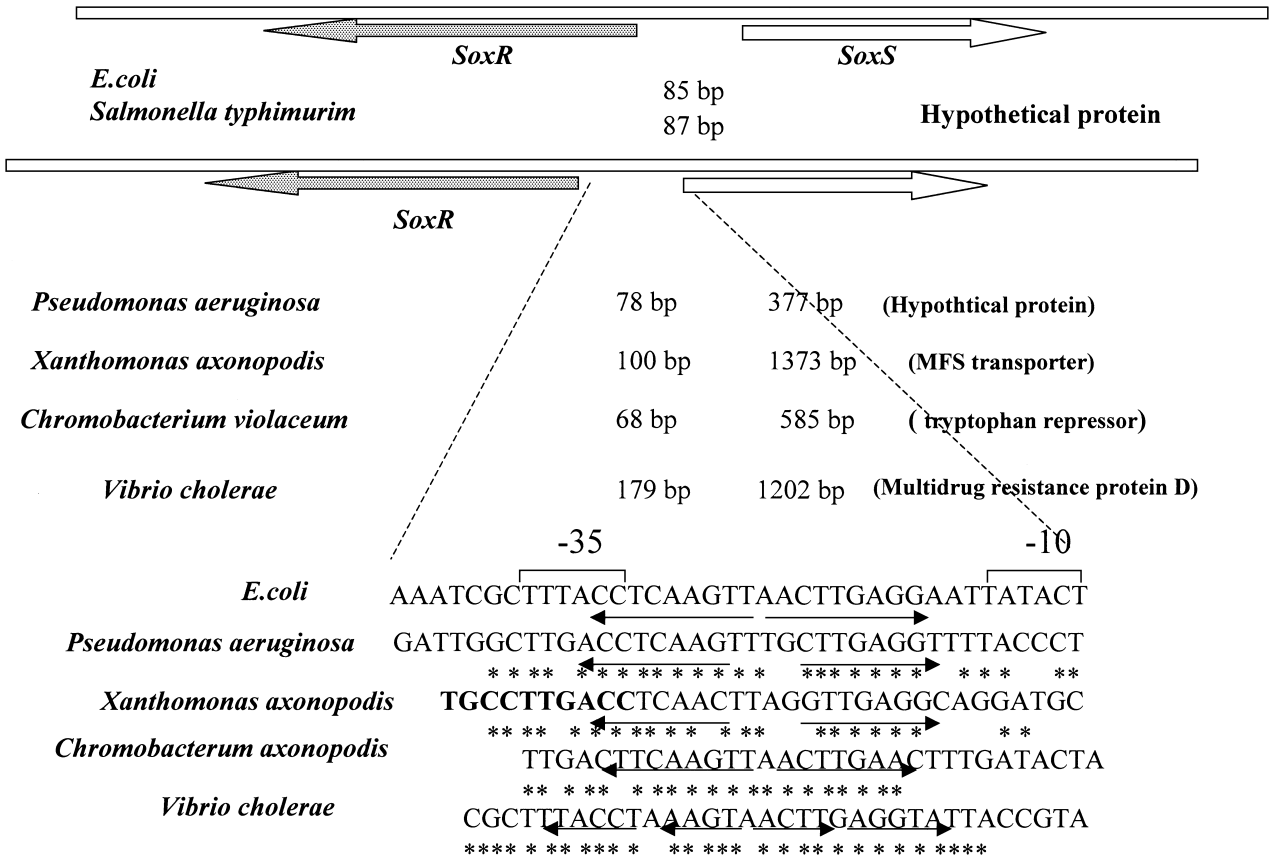

Fig. 1. **(A) Structures of the *SoxRS* regulon in *E. coli* and the ORFs of the SoxR homologs and hypothetical associated proteins in *P. aeruginosa*, *Xanthomonas axonopois*, *Chromobacterium axonopodis* and *Vibrio cholerae*. (B) Similarity of DNA sequences of the *E. coli* SoxS promoter and the SoxR downstream region in various bacteria.** The palindrome is indicated by arrows.

The mechanism of the activation of transcription by SoxR has been studied in *E. coli*. Although the observation that some bacteria have homologs for *soxR* but not *soxS* suggests that SoxR proteins may play alternative regulatory roles, there have been no previous reports on the role of SoxR in such organisms. Here, we report for the first time the overproduction and purification of SoxR from *P. aeruginosa* to investigate the gene activation by SoxR in this organism. We provide biochemical evidence that the SoxR protein is a direct transcriptional activator of the hypothetical protein encoded by PA2274.

MATERIALS AND METHODS

Plasmid Construction and Bacterial Strains—E. coli strain BL21 (λΔE3) and plasmid pET-3Xa were obtained from Novagen. *P. aeruginosa* strain 3080 was obtained from the National Institute of Technology and Evaluation in Japan.

Cloning of the P. aeruginosa SoxR—Chromosomal DNA was isolated from *P. aeruginosa* PA01 by the method of Chen and Kuo (*22*). The *soxR* gene was cloned from *P. aeruginosa* PA01 genomic DNA using specific primers (SoxR N-terminal, 5′-GCCACATATGAAGAATTCCTG-CGA-3′ and SoxR C-terminal 5′-CGCCGGATCCACG-GTGACCGCTGC-3′). All PCR products were sequenced to ensure that no errors were introduced during amplification.

The 502-bp PCR fragment was digested with *Nde*I and *BamH*I and inserted into pET-3Xa (Novagen) digested with the same enzymes. The resulting overexpression plasmid (pET3Xa-*SoxR*) was transformed into BL21 (DE3) cells (Novagen). A 100-ml saturated culture of *E. coli* BL21 (pET-3Xa-*SoxR*) was inoculated into 8 liters of medium containing ampicillin (50 μg/ml) and incubated at 37°C in a 10 liter fermentor (Oriental Biotechnological System LS-10). At an A_{600} = 0.7, the culture was cooled to 18°C and 5 ml of a 1 M IPTG solution was added. After 24 h incubation at 18°C, the cells were harvested and the cell paste was washed and resuspended in an equal volume of 10% sucrose, 10 mM Tris/ HCl buffer (pH 8.0).

Purification of SoxR in P. aeruginosa—The SoxR protein was purified as previously described for the *E.coli* SoxR (*8*, *12*, *23*, *24*). Briefly, the cell suspension was thawed and then incubated in standard buffer [20 mM MOPS/KOH (pH 7.6), 0.2 M KCl, 1 mM dithiothreitol and 10% glycerol] containing 0.2 M KCl, 0.0025% phenylmethanesulfonyl fluoride and 0.5 mg/ml egg white lysozyme. After 60 min at 4°C, the suspensions were sonicated for 5 min, and cell debris was removed by centrifugation at 33,000 × *g* for 90 min. An equal volume of saturated $(NH_4)_2SO_4$ solution was slowly added. The precipitate was dissolved in 200 ml of the standard

buffer, and applied to a P-11 phosphocellulose (Whatman) column previously equilibrated with the standard buffer containing 0.2 M KCl. Elution was performed with 100 ml of the standard buffer containing a linear gradient of 0.2 to 1.0 M KCl. The eluted fractions with a reddish color were collected and dialyzed against the standard buffer containing 0.3 M KCl. The sample was then applied to a heparin-agarose column (Amersham Pharmacia Biotech) and eluted with the standard buffer containing 0.5 M KCl. As a final purification step, the protein was loaded onto a High Load Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with a buffer solution containing 20 mM Tris/HCl (pH 7.6), 0.5 M KCl, and 1 mM dithiothreitol. The purity of the resulting SoxR protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of SoxR was determined spectrophotometrically using a millimolar extinction coefficient of 12.7 mM^{-1} cm^{-1} at 417 nm (*8*).

Redox Titration of SoxR—Spectrophotometric titrations were performed in a Hitachi U-3000 spectrophotometer equipped with a thermostated cell holder connected to a thermobath, a custom anaerobic cuvette (1 cm light path, 5 ml sample volume) equipped with a combined platinum and Ag/AgCl electrode (6860-10C, Horiba, Tokyo, Japan), and a screw-capped sidearm. A sample of SoxR in 50 mM Tris/HCl (pH 7.6) containing 0.5 M KCl and 1 mM dithiothreitol was mixed with 3 μM safranine O as a redox mediator. Before titration, solutions containing SoxR protein were equilibrated with argon. Reductive titration was performed at 20°C by the addition of small aliquots of sodium dithionite, which were introduced through a needle in the rubber septum on the side arm. The reduction of SoxR was measured at 415 nm. The redox potential was monitored directly with the electrode.

DNA Substrates—A 115 bp fragment containing the SoxR binding region was PCR amplified from *P. aeruginosa* chromosomal DNA for use in the electrophoretic mobility shift assay, using primers 5′-GGAATTCTTCATGATTGGCT-3′ and 5′-GCAGTAGGAGGAGGGGATGA-3′. Similarly, a 180 bp fragment containing the *soxS* promoter region in *E. coli* was PCR amlified and ^{32}P-labeled as described (*4*). The DNA template for assays of transcriptional activation by SoxR was a 194 bp PCR product encompassing the promoter region, amplified with primers 5′-GGAATTCTTCATGATTGGCT-3′ and 5′-TGGCGGGTTCCACCTCGATATCG ATG-3′. The obtained PCR products were cloned into pCRII vector (Invitrogen Co.) using the TA Cloning Kit (Invitrogen Co.) and digested with *Eco*RI for use in the transcriptional activation assay.

Electrophoretic Band Shift Assays—The PCR fragment containing the SoxR binding region was labeled at the 5′ end with [γ-^{32}P]ATP and purified on a small Sephadex G50 column. Prior to electrophoresis, the ^{32}P-labeled DNA fragments were incubated with 0 to 200 ng of SoxR protein for 20 min at 30°C in 15 μl of a solution containing 133 μg/ml poly(dI-dC), 300 μg/ml bovine serum albumin, 10% glycerol, 75 mM KCl, 2 mM dithiothreitol, 10 mM Tris/HCl buffer (pH 7.6) and 0.5–1 fmol of labeled DNA. The fragments were then subjected to electrophoresis in 5% polyacrylamide gels at 4°C.

Transcription Assays—The transcription assay was performed as previously described for the *E. coli* SoxR (*8*, *11*). Briefly, SoxR was diluted in a solution containing 20 mM Tris/HCl buffer (pH 8.0), 100 mM KCl, 1 mM dithiothreitol, and 50 μg/ml bovine serum albumin. SoxR (5–50 ng in 2 μl of diluent) was added to 18 μl of a solution containing 0.18 pmol of the DNA template and 0.16 mM each of ATP, GTP, and CTP, 40 mM Tris/HCl buffer (pH 8.0), 50 mM KCl, 1 mM dithiothreitol and 1 mg/ml bovine serum albumin. After the mixture was incubated for 10 min at 37°C, 0.3 U of *E. coli* RNA polymerase-σ^{70} holoenzyme (Sigma) was added in 3 μl. After an additional 10 min at 37°C, 3 μl of heparin sulfate (1 mg/ml) and 3 μl of 80 μM α-[^{32}P]UTP (3–30 Bq/mM) were simultaneously added. The mixtures were incubated at 37°C for 15 min and then treated with phenol/chloroform. The RNA products were precipitated with ethanol and analyzed by electrophoresis in an 8% polyacrylamide gel containing 7.7 M urea.

Under anaerobic conditions, the samples were deoxygenated in sealed vessels by repeated evacuation and flushing with argon. After the addition of small aliquots of sodium dithionite for reduction of SoxR, and then 1 μl of *E. coli* RNA polymerase-σ^{70} holoenzyme (Sigma) was added under anaerobic conditions. These reaction mixtures were incubated at 37°C as described above. For experiments using reoxidized SoxR, the sample was aerated prior to the addition of the RNA polymerase.

Fluorescence Polarization Assay—Polarization was monitored using a PanVera Beacon 2000 Fluorescence Polarization System (PanVera Corp.) (485–490 nm excitation, 525–535 nm emission) as described (*25*). Oligonucleotides (5′-fluorescence–labeled 5′-GGCTTGACCTCAAGTTTGCTTGAGGTTTTACCCTGGG-3′ and 5′-CCCAGGGTAAAACCTCAAGCAAACTTGAGGTCAAGCC-3′) were synthesized and purified by HPLC at Sigma Genosis Biotech Co., Ltd. (Japan). The strands were heated to 90°C for 5 min and then cooled slowly back to room temperature over a period of 1 h for annealing. The annealed labeled oligonucleotides (2.6 nM) were then mixed with 75 mM KCl, 2 mM dithiothreitol, 10 mM Tris/HCl buffer (pH 7.6), and 0 to 1.3 μM SoxR protein at 20°C in a 100 μl final volume. Similarly, oligonucleotides (5′-fluorescence-labeled 5′-TCCTCAAGTTAACTTGAGGTAAAGC and 5′-GCTTTACCTCAAGTTAACTTGAGGA) for SoxS promoter in *E. coli* were also employed as a control experiment. For each set of experiments SoxR protein was titrated into the DNA. After each addition of protein, the samples were incubated for 30 s to reach equilibrium before a measurement was taken. The increase in polarization of the fluorophore upon protein binding was measured. Each binding isotherm was fit to Eq. 1,

$$P = \{(P_{\text{bound}} - P_{\text{free}})[\text{protein}]/(K_d + [\text{protein}])\} + P_{\text{free}} \quad (1)$$

where P is the polarization measured at a given total protein concentration, P_{free} is the initial polarization of free fluorescein-labeled DNA, P_{bound} is the maximum polarization of specifically bound DNA.

Growth Conditions—Bacterial cells were grown in LB medium at 37°C. Overnight cultures were diluted (100-fold) into fresh LB medium and grown for 2 to 4 h at 37°C to an optical density at 600 nm of 0.3 to 0.4. Aliquots (100 ml) of the cultures were placed in 250-ml flasks, paraquat

(ICN) was added, and the incubation was continued for the indicated time intervals.

Assays for G6PD and SOD—Cells were harvested, washed three times in 50 mM potassium phosphate and, 0.1 mM EDTA (pH 7.5) resupended in the same buffer, and lysed with a French press. The extracts were clarified by centrifugation and then assayed for G6PD (26) and SOD (*27*).

Isolation and Analysis of PA2274 mRNA by Northern Blot Analysis—Bacteria cultures were grown overnight in LB broth at 37°C, diluted 1/100 in LB broth, and grown at 220 rpm to mid-log phase. At an optical density at 600 nm of ~0.5, growing cultures were exposed to different concentrations of paraquat for 30 min. Total RNA was extracted with an RNeasy Mini kit (Quiagen) and resupended in RNAase-free water. Samples of total RNA were loaded on 1.2% agarose gels containing formaldehyde and transferred to a nylon membrane. After the gels were stained with ethidium bromide to visualize 23S and 16S rRNA (as a loading control), the blots were hybridized with a PA-2274–specific probe previously labeled with a random primer system. The 350-bp PA-2274 probe was prepared by PCR amplifying the PA2274 gene from *P. aeruginosa* PA01 genomic DNA using primers (5′-AACCCGCCATGGTCATCCCCTCCTCCTA-3′ and 5′-AGACGGATCCTCGGCGCCGGCGGGC-3′.

Spectroscopic Measurements—Optical absorption spectra were measured with a Hitachi U-3000 spectrometer.

Protein Determination—Protein concentrations were determined by the method of Lowry *et al.* (*28*) using bovine serum albumin as a standard. N-terminal sequences were determined by Edman degradation using an Applied Biosystems model 491HT protein sequencer.

RESULTS

Induction by Paraquat—The *soxRS* regulon in *E. coli* provides a defense against oxidative stress imposed by redox-cycling agents such as paraquat (*16*, *17*). Among the identified members of the *soxRS* response are Mn-SOD and G6PD, and their levels are induced similarly by paraquat (*29*). Here, the effects of paraquat on Mn-SOD and G6PD were examined in *E. coli* and *P. aeruginosa*. As shown in Fig. 2, exposure of *E. coli* to paraquat caused a dose-dependent increase in the activities of G6PD and SOD, as previously reported (*29*). In contrast, exposure of *P. aeruginosa* to paraquat resulted in only slight increases in G6PD and SOD. This indicates that *P. aeruginosa* does not have the same *soxRS* regulon and redox-cycling–based response to oxidative stress as does *E. coli*.

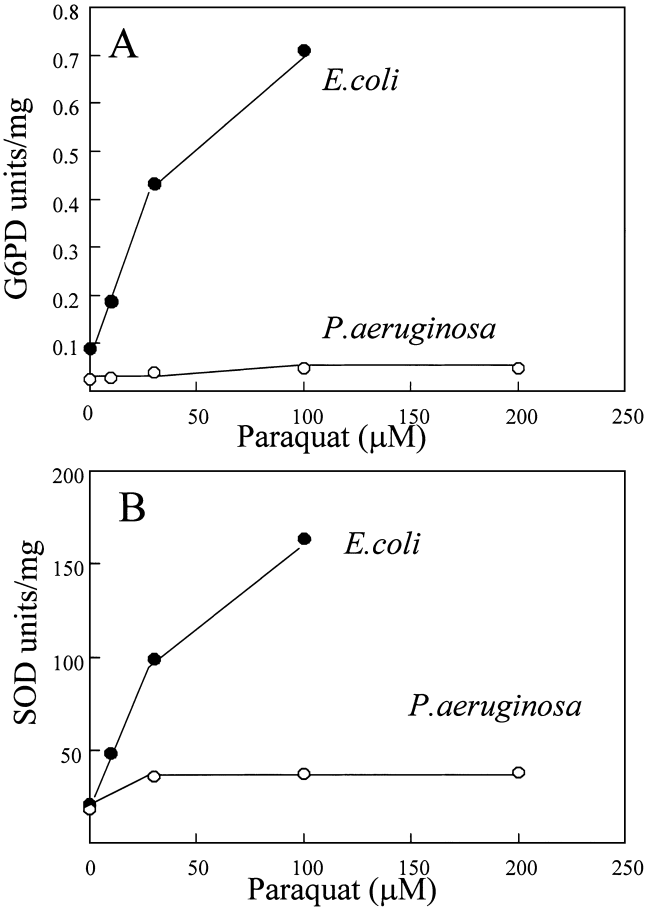

Fig. 2. **Paraquat-induced induction of glucose 6-phosphate reductase (G6PD) (A) and superoxide dismutase (SOD) (B) in *E. coli* and *P. aeruginosa*.** *E. coli* and *P. aeruginosa* were grown in LB medium containing the indicated concentrations of paraquat at 37°C. The cells were then collected by centrufugation, lysed in a French press, and the extracts were clarified by centrifugation. The soluble extracts were assayed for G6PD and SOD activities.

Gene Cloning and Sequence Analysis of SoxR in P. aeruginos—When we inspected the *P. aeruginosa* sequence

E. coli MEKKLPRIKALLTPGEVAKRSGVAVSALHFYESKGLITSIRNSGNQRRYKRDVLRYVAII

P. aeruginosa MKNSCASRELSVGELARRAGVAVSALHFYETKGLISSQRNAGNQRRFSRETLRRVVVI

KIAQRIGIPLATIGEAFGVLPEGHTLSAKEWKQLSSQWREELDRRIHTLVALRDELDGCI

KVAQRVGIPLAEIARALQTLPAGRSPSAADWARLSAQWKEDLTERIDKLLLLRDQLDGCI

GCGCLSRSDCPLRNPGDRLGEEGTGARLLEDEQN

GCGCLSLQACPLRNPGDQLSAEGPGAHWLDAEGREHDG

Fig. 3. **Protein sequences and homology of SoxR in *E. coli* and *P. aeruginosa*.**

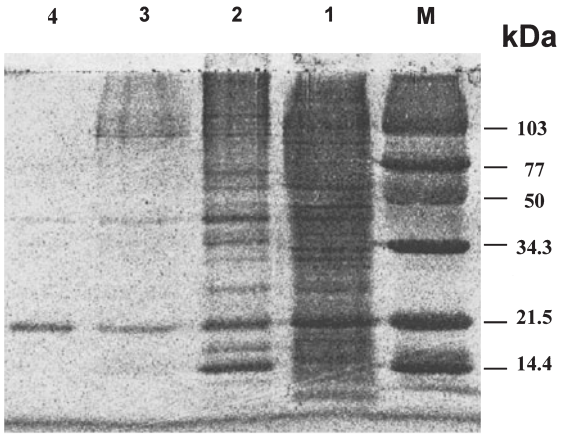

Fig. 4. **Electrophoretic analysis of SoxR fractions.** *E. coli* cells overexpressing *P. aeruginosa* SoxR were grown and the protein purified as described in "MATERIALS AND METHODS" with lanes 1–4. Samples of each purification fraction were analyzed by electrophoresis in an SDS–12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. Lanes: M, molecular weight markers; 1, sonicate supernatant; 2, phosphocellulose eluate pool; 3, heparin-agarose eluate; 4, purified protein.

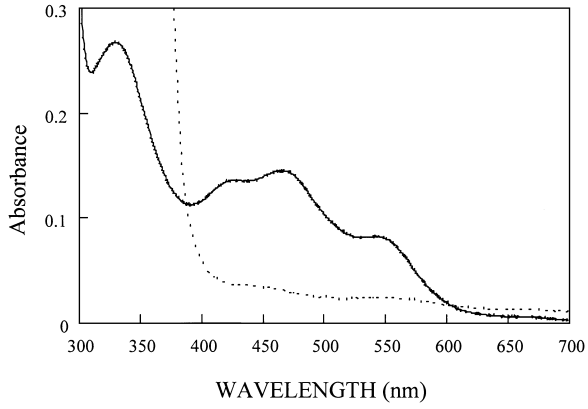

Fig. 5. **Optical absorption spectra of the oxidized and reduced forms of the purified *P. aeruginosa* SoxR protein.** The sample contained 47.2 μM SoxR in 20 mM Tris buffer (pH 7.6), 0.5 M KCl, and 1 mM dithiothreitol. The reduced form was obtained by the addition of dithionite. The strong absorption below 380 nm is due to the relatively large amount of dithionite used for reduction in this experiment.

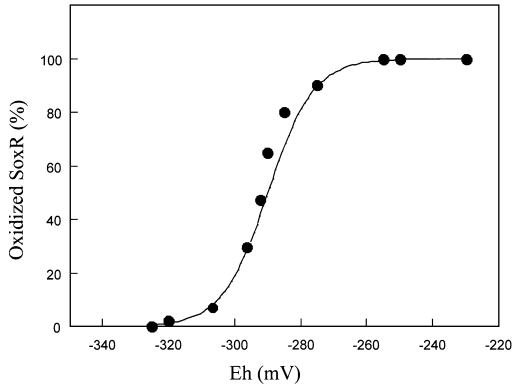

Fig. 6. **Redox titration of the SoxR [2Fe-2S] clusters.** SoxR protein (42 μM) in Mops buffer (pH 7.6) containing 500 mM KCl was supplemented with 3 μM sufranine O as a redox mediator, and the redox titration was performed in an anaerobic redox cuvette. The *x*-axis shows the redox potential measured with a microelectrode, and the *y*-axis shows the absorbance differences between 414 and 391 nm, normalized to 0 or 100% for fully reduced or oxidized SoxR, respectively. The redox potential was measured against a standard hydrogen electrode. The *solid line* drawn through the data points was generated from the Nernst equation with $n = 1$.

immediately upstream of the gene encoding the SoxR homolog (ORF PA2273), we found another putative ORF (PA2274) oriented in the opposite direction (Fig. 1). We cloned both genes and found that the SoxR homolog encodes a putative 156 amino acid protein having 62% identity and 78% similarity to the *E. coli soxR* gene product (Fig. 3). The sequence of the *P. aeruginosa* SoxR protein contains two predicted functional domains, an N-terminal helix-turn helix (DNA-binding) motif, and the C-terminal [2Fe-2S] centers anchored to four cysteine residues. In contrast, the predicted product of ORF PA2274 showed no significant homology to any known sequences in the GenBank database, including SoxS. However, a region homologous to a symmetric consensus DNA sequence found in the promoter region of *E. coli* SoxS was found in the DNA region between ORFs PA2273 and PA2274.

Purification of SoxR from P. aeruginosa—The inducible T7*lac* promoter of pET-3a provided tight control and good induction of *P. aeruginosa* SoxR expression. Following induction with IPTG for 24 h at 18°C, the cells were harvested. Under these conditions, almost all of the SoxR protein produced could be recovered from the soluble fraction, as was previously observed in *E. coli* (*8*). Figure 4 shows an SDS-PAGE gel of the different steps in the SoxR purification process; the final purified SoxR protein was detected at 17 kDa. When sequenced, the first five N-terminal amino acids of the purified protein matched those predicted from the DNA sequence of *soxR*. Although the molecular mass of the polypeptide was 17 kDa, the native protein had an elution volume between that of pancreatic DNase I (31 kDa) and ovalbumin (43 kDa) (data not shown). Thus, SoxR most likely exists as a globular homodimer in its active form. In *E. coli*, apo-SoxR, lacking the [2Fe-2S] clusters, was obtained when 2-mercaptoethanol is included during protein purification (*23*, *30*, *31*). In contrast, the apo form of *P. aeruginosa* SoxR was unstable and precipitated by treatment with 2-mercaptoethanol.

Figure 5 shows the optical absorption spectra of the purified *P. aeruginosa* SoxR. The spectrum of the oxidized form is characteristic of a [2Fe-2S] protein. Reduction by sodium dithionite decreased the absorbances at 414, 462 and 548 nm. This absorbance profile is essentially similar to that of *E. coli* SoxR (*8*, *9*).

A typical redox titration of the *P. aeruginosa* SoxR [2Fe-2S] cluster at pH 7.6 is shown in Fig. 6. The midpoint potential for the SoxR [2Fe-2S] clusters under the conditions used was estimated to be –290 mV, which is also close to that of the *E. coli* SoxR protein (280–285 mV) (*10*, *11*).

Promoter Binding by Purified SoxR—We investigated whether the purified SoxR protein could bind the DNA

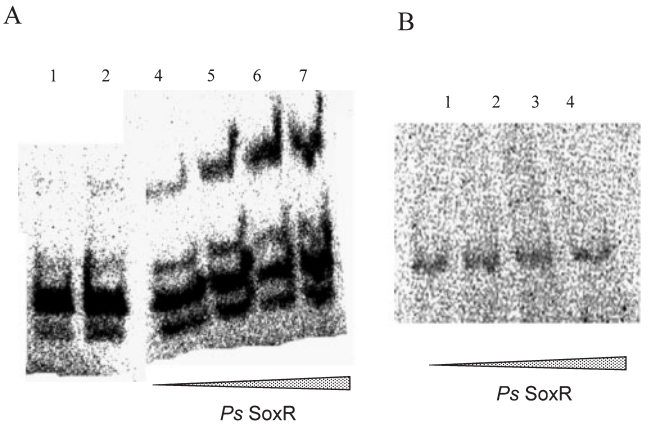

Fig. 7. **DNA binding by SoxR protein.** (A) Binding reactions contained the labeled fragment (115 bp) of the *P. aerginosa* SoxS promoter–like region with increasing amounts of the purified protein. Lanes 1 and 2 : no added protein; lane 3: 2.8 nM of SoxR; lane 4: 6.9 nM; lane 5: 13.8 nM; and lane 6: 55.8 nM. (B) Binding reactions contained the labeled fragment (180 bp) containing the *soxS* promoter region in *E. coli* with increasing amounts of purified protein. Lane 1: no added protein; lane 2: 36 nM; lane 3: 58.2 nM; lane 4: 106 nM.

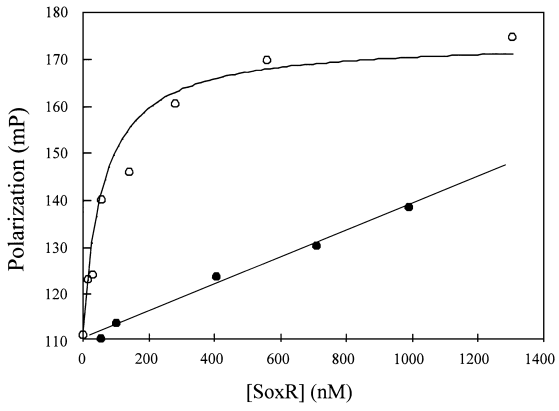

Fig. 8. **DNA binding to SoxR protein as determined by fluorescence polarization.** The sample contained 2.6 nM fluorescence labeled oligonucleotide containing the *P. aeruginosa* SoxS promoter–like region (open circles), *E. coli* SoxS promoter region (closed circles), 75 mM KCl, 2mM dithiothreitol, and 10 mM Tris/HCl (pH 7.6). The increase in polarization was monitored using a Beacon™ 2000 fluorescence polarization instrument (485–490 nm excitation, 525–535 nm emission) .

target. We performed gel band mobility shift assays after incubating increasing concentrations (2.8–56 nM) of purified SoxR with a ^{32}P-labeled 115 bp PCR product harboring the *P. aeruginosa* SoxS promoter-like region. The purified SoxR protein caused a gel band mobility shift of the PCR fragment in this assay (Fig. 7A). The amount of the retarded band increased with increased SoxR concentration. However, the free-DNA band was still seen in the presence of high levels of SoxR (55.6 nM), suggesting that the affinity of SoxR for its DNA target is not high.

The binding of SoxR to a fluorescene-labeled 38 bp oligonucleotide containing the SoxS promoter–like region was further examined by fluorescene polarization assay. Polarization increased with the addition of SoxR in a dose-dependent manner that reached saturation above 500 nM SoxR (Fig. 8A). The K_d value under the experimental conditions was calculated to be 56 nM. The low affinity of SoxR is consistent with the results of the gel shift mobility shift assay. In a control experiment, a purified *E. coli* SoxR protein was similarly bound to fluorescene- labeled 38 bp oligonucleotides containing the *E. coli* SoxS promoter. Polarization saturated at a concentration of 10 nM *E. coli* SoxR (data not shown). This result is consistent with the results of previously reported gel mobility shift assays (*23*).

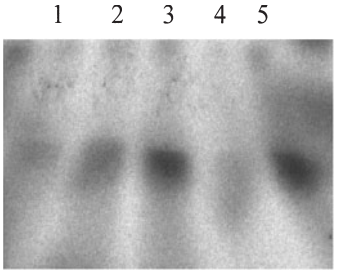

Fig. 9. ***In vitro* transcription of PA2274 by the SoxR protein as a function of its oxidation state.** A one-cycle transcription was performed using the ORF PA2274 DNA as the template in the presence of increasing amounts of SoxR protein. The autoradiograph shows the electrophoretically separated ^{32}P-labeled RNA products. Lanes: 1, no SoxR; 2, 8.7 ng SoxR; 3, 35 ng SoxR; 4, reduced SoxR; 5, reoxidized SoxR.

To test whether the SoxS promoter–like region is the authentic target of *P. aeruginosa* SoxR, we investigated whether the purified *P. aeruginosa* SoxR protein binds the *soxS* promoter in *E. coli*. In the gel band shift experiment, however, no retarded bands were observed in the presence of 100 nM of SoxR (Fig. 7B), and, furthermore, no increase in polarization was observed until SoxR in μM ranges was added (Fig. 8). These results strongly suggest that *P. aeruginosa* SoxR protein binds specifically to the SoxS promoter-like region, although the affinity is not high.

The purified *P. aeruginosa* SoxR was tested for its ability to activate the transcription of ORF PA2274. A 199-bp linear ORF DNA template was generated by PCR. A one-cycle transcription assay was used to produce homogenous run off transcripts that could be detected as discrete bands by electrophoresis. SoxR was incubated with the DNA template, *E. coli* RNA polymerase-σ^{70} holoenzyme was added in the presence of only three nucleoside triphosphates, and heparin was added to enable completion of the transcripts. The results (Fig. 9) indicated that transcription from the promoter is dependent on the addition of SoxR. In the presence of SoxR, a strong mRNA band was produced at the expected length of run off transcripts initiated from the promoter. The transcription was blocked when the SoxR was reduced either before or after the binding step, whereas this inactivation could be reversed by aeration, a treatment that produces the rapid reoxidation of SoxR.

Since the *in vitro* transcriptional assay provides an indirect measure of transcriptional activity, additional Northern blotting experiments were conducted to determine the PA2274 induction. The levels of PA2274 mRNA produced in paraquat-treated cells were measured as an

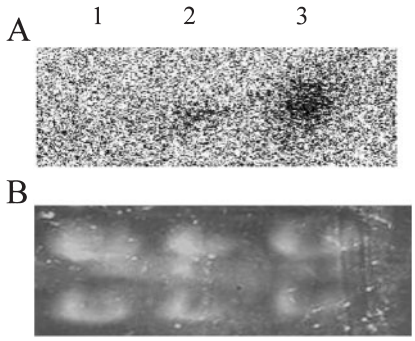

Fig. 10. **Northern blotting analysis of PA2274 expression.** (A) The Northern blot of the 350 bp fragment was hybridized with total RNA from *P. aeruginosa* treated with increasing concentrations of paraquat (lane 1, 0; 2, 10 μM; 3, 100 μM). (B) The corresponding ethidium bromide-stained gels show the 23S and 16S rRNAs that served as loading controls.

in vivo transcriptional assay. The presence of intracellular superoxide generated by paraquat was found to stimulate the expression of PA2274 protein, as shown in Fig. 10.

DISCUSSION

Here, we present the first characterization of *P. aeruginosa* SoxR and its interaction with DNA. The physicochemical properties of the *P. aeruginosa* SoxR protein are essentially similar to those of the *E. coli* protein. The *P. aeruginosa* SoxR protein is a homodimer that contains a pair of [2Fe-2S] clusters. The redox potential of the [2Fe-2S] clusters was found to be –290 mV, a value similar to that of *E. coli* (–285 mV) (*10*, *11*). In addition, oxidation and reduction of the [2Fe-2S] clusters can reversibly activate and inactivate SoxR transcriptional activity, respectively. However, the role of SoxR in the activation of transcription appears to be substantially different than that in *E. coli*. In the latter organism, selective oxidation or reduction of the [2Fe-2S] clusters of SoxR triggers the induction of SoxS expression, and the resulting increase in SoxS protein levels activates the regulon genes. In contrast, our results clearly show that *P. aeruginosa* SoxR is a direct transcriptional activator of the hypothetical protein PA2274 (MW 14.01 kDa). Although the function of this protein is presently unknown, it is unlikely that the protein is a transcription factor based on its predicted amino acid sequence. At present, however, we cannot exclude the possibility that the *P. aeruginosa* SoxR protein might bind to regions other than the promoter of PA2274. It is formally possible that a functional homolog of a SoxS or an unknown intermediate exists in *P. aeruginosa*, although no such homolog has been identified within the complete genome sequence of *P. aeruginosa*.

The functional differences between SoxR in *E. coli* and *P. aeruginosa* may further reflect the difference in their affinity constants for their individual target DNAs. The affinity of the *P. aeruginosa* SoxR protein ($K_d = 5.6 \times 10^{-8}$ M) for its target DNA is about two orders of magnitude lower than that of *E. coli* SoxR ($K_d = 4.5 \times 10^{-10}$ M) (*23*). The lower affinity requires higher SoxR concentrations for the activation of transcription of its target genes in *P. aeruginosa*. Gene activation by *P. aeruginosa* SoxR, therefore, seems to be a direct function of the concentration of the protein. In fact, it has been reported that the expression of SoxR in *P. aeruginosa* is inducible by treatment with paraquat (*32*). In contrast, the *E. coli* SoxR protein concentration *in vivo* is kept at a relatively low level that is unchanged by oxidative stress (*20*, *33*). This observation strongly suggests that the *soxRS* regulon in *E. coli* is regulated by the amount of SoxS protein, which is controlled at the transcriptional level *via* activation of the redox-sensitive SoxR (*4*, *23*). The regulatory system in *E. coli* seems ultimately to maximize the change in SoxS expression (*33*, *34*). In fact, the binding affinity of the SoxS protein for *soxRS* regulatory promoters is on the same order as that of the *P. aeruginosa* SoxR presented here, around 10^{-8} to 10^{-9} M (*34*).

Similar to the case in *P. aeruginosa*, an ORF homologous to SoxR and a non SoxS gene are arranged divergently with their 5′ ends separated by 68–179 bp in other bacteria such as *Vibrio cholera*, *Xanthomonas axonopodis*, and *Chromobacterium violaceuace*. Each of these intergenic regions similarly contains a region homologous to the *soxS* promoter of *E. coli*. Figure 1 shows a comparison among these sequences. In these bacteria, a SoxR dimer likely recognizes the repeated sequence in the DNA binding site, and may activate the transcription of the corresponding protein, as we postulate occurs in *P. aeruginosa*. The SoxR-associated genes in *Xanthomonas axonopodis*, *Chromobacterium axonopoids*, and *Vibrio cholera* are an MFS transporter, a tryptophan repressor, and a multidrug resistance homolog, respectively. In addition, the intergenic region contains a potential hairpin-forming sequence that could affect transcription at the DNA or RNA levels, or could affect the translation of the *soxR* message. The opposing transcription of these two genes and the potential for overlap between the transcripts could exert both transcriptional and posttranscriptional control (*33*).

It has been proposed that the iron-sulfur cluster of *E. coli* SoxR functions as a sensor of oxidative stress (*10*, *11*, *14*). Similarly, it is considered that the oxidation of the iron-sulfur cluster of *P. aeruginosa* SoxR *in vivo* regulates its activity as a transcription activator, because the midpoint potential of the SoxR [2Fe-2S] clusters in *P. aeruginosa* is close to that of *E. coli*. *P. aeruginosa* seems to increase the expression of a SoxR-controlled defense system, rather than playing a protective role in oxidative stress, in response to the cellular redox status. The present data strongly suggest that PA2274 is the primary target of the SoxR protein and plays an important role in the SoxR-dependent system. The PA2274 ORF encodes a putative protein of 126 amino acids that shows no significant homology to any sequence in the GenBank database. However, the PA2274 predicted protein sequence contains a short conserved region at the N-terminus, called the tonB-box motif (*36–38*), which is involved in interactions with the tonB protein (*38*). This raises the possibility that the PA2274 protein may act as a regulator of siderophore-mediated iron uptake by the ton B-box protein (*39*, *40*). Further work will be required to purify and further characterize the PA2274 protein.

In conclusion, our findings demonstrate that in *P. aeruginosa*, the SoxR protein acts as a direct transcriptional

activator of an upstream gene, and the oxidation and reduction of SoxR [2Fe-2S] clusters can reversibly activate and inactivate SoxR transcriptional activity. A similar gene arrangement has been found in several other bacteria, suggesting that SoxR proteins may be key regulators of responses to diverse environmental stresses acting in a manner beyond that already understood for the *E. coli* SoxR protein.

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