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Regulation of the *ribA* gene encoding GTP cyclohydrolase II by the *soxRS* locus in *Escherichia coli*

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Abstract We isolated a promoter that is inducible by paraquat, a superoxide-generating agent, from *Escherichia coli* using the promoter-probe plasmid pRS415. Sequence analysis revealed that the promoter derives from the *ribA* gene encoding GTP cyclohydrolase II, which is the first enzyme in the biosynthetic pathway of riboflavin. We fused the *lacZ* gene with the *ribA* promoter to monitor the expression of the gene in the single-copy state. LacZ expression from the *ribA* promoter was induced about eight-fold by 200 μM paraquat. Other known superoxide generators, menadione and plumbagin, also induced the expression of β-galactosidase in the fusion strain. On the other hand, no significant induction was observed following treatment with hydrogen peroxide, ethanol or heat shock. Induction of β-galactosidase was significantly reduced by the introduction of a Δ*sox-8*::cat or *soxS3*::Tn*10* mutation into the fusion strain, indicating that the *ribA* gene is a member of the *soxRS* regulon. The transcriptional start site was determined by primer extension analysis and putative binding sites for SoxS in both orientations were identified. GTP cyclohydrolase II activity in soluble extracts of *E. coli* increased more than three-fold on treatment with paraquat. This increase was dependent on the *soxRS* locus, and reflects the increase in transcript levels. However, flavin pools did not change significantly. A possible role for *ribA* induction during superoxide stress is discussed.

Key words *Escherichia coli* · *ribA* · GTP cyclohydrolase II · Oxidative stress · *soxRS* regulon

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Introduction

Living organisms are subject to various forms of environmental change and stress and have evolved specific adaptive mechanisms to cope with them, such as heat shock, nutrient starvation, oxidative stress, and SOS responses. Oxidative stress is of particular interest because aerobes are in continual contact with reactive oxygen species, formed as by-products of normal respiration due to incomplete reduction of oxygen, or by exposure to external sources, such as pollution, radiation, and redox-cycling agents, or released from macrophages in response to bacterial invasion (Fridovich 1987; Halliwell and Gutteridge 1989; Farr and Kogoma 1991; Sies 1991). *Escherichia coli* possesses two separate oxidative stress regulons, one for hydrogen peroxide (*oxyR* regulon; Christman et al. 1985, 1989) and the other for superoxide stress (*soxRS* regulon; Greenberg and Demple 1989; Greenberg et al. 1990; Tsaneva and Weiss 1990).

Redox-cycling compounds, such as paraquat, plumbagin, and menadione serve as a continuing source of superoxide as they undergo repeated cycles of oxidation and reduction (Hassan and Fridovich 1979). Upon exposure to redox-cycling compounds which are thought to generate superoxide in a cell, *E. coli* induces the synthesis of about 40 proteins (Greenberg and Demple 1989; Walkup and Kogoma 1989). A subset of these proteins are produced by a regulon controlled by two regulatory genes, *soxR* and *soxS*, constituting a *soxRS* regulon (Wu and Weiss 1991, 1992; Amábile-Cuevas and Demple 1991; Nunoshiba et al. 1992). The products of genes known to be regulated by *soxRS* include endonuclease IV (encoded by *nfo*), glucose-6-phosphate dehydrogenase (encoded by *zwf*), Mn-superoxide dismutase (encoded by *sodA*), fumarase C (encoded by *fumC*), *micF* which is an antisense inhibitor of *ompF*, aconitase (encoded by *acnA*), NADPH:ferredoxin oxidoreductase (encoded by *fpr*), and *inaA* and *pqi-5* which have unknown functions

Table 1 Bacterial strains, plasmids, and phages used in this study

Strain, plasmid or phage	Description	Source or reference
Strains		
MG1655	Wild-type *E. coli*	Bachman (1972)
GC4468	Δ(*argF-lac*)*169 rpsL* sup(Am)	Touati (1983)
BW829	GC4468 Δ*sox-8*::cat[a]	Tsaneva and Weiss (1990)
BW831	GC4468 *soxS3*::Tn*10*[b]	Tsaneva and Weiss (1990)
BW847	GC4468 *soxR4*::cat[c]	Tsaneva and Weiss (1990)
GSO18	*recD1014 lac gal rpsL* Δ*oxyR*::kan[d]	Altuvia et al. (1994)
JC101	GC4468 (λJC101)	This work
JC201	JC101 Δ*sox-8*::cat; P1(BW829) × JC101	This work
JC301	JC101 *soxS3*::Tn*10*; P1(BW831) × JC101	This work
JC401	JC101 *soxR4*::cat; P1(BW847) × JC101	This work
JC501	JC101 Δ*oxyR*::kan; P1(GSO18) × JC101	This work
Plasmids		
pRS415	*lacZYA* operon fusion vector, Amp[r]	Simons et al. (1987)
pJC101	*ribA* promoter cloned upstream of *lacZ* in pRS415	This work
Phages		
λRZ5	Φ(*bla'-'lacZ*) *lacY*$^+$	R. Zagursky
λJC101	Φ(*ribA-lacZ*) *lacY*$^+$ *bla*$^+$	This work
λ*c*I	Wild-type λ phage	Silhavy et al. (1984)
λ*vir*	Virulent derivative of λ phage	Silhavy et al. (1984)
P1*vir*	Virulent derivative of P1 phage	Silhavy et al. (1984)

[a] Δ*sox-8*::cat is a *soxRS* deletion
[b] *soxS3*::Tn*10* is an insertion mutation in *soxS*
[c] *soxR4*::cat is a *soxR* constitutive mutation
[d] Δ*oxyR*::kan is an insertion mutation in *oxyR*

(Liochev and Fridovich 1992; Chou et al. 1993; Gruer and Guest 1994; Liochev et al. 1994; Rosner and Slonczewski 1994; Koh and Roe 1995).

The multifaceted mechanism that cells employ upon exposure to highly oxidative conditions will be fully revealed only when the gene products induced or repressed during this process are fully characterized. We have previously used a multicopy promoter-probe plasmid, pJAC4, to isolate DNA fragments containing promoters inducible by paraquat, a superoxide-generating compound (Koh and Roe 1993, 1995). In this report, we demonstrate the isolation of the *ribA* promoter using another promoter-probe plasmid (pRS415) and present evidence for the regulation of this gene by the *soxRS* locus.

Materials and methods

Strains, phages, and plasmids

The strains used in this study are listed in Table 1. *E. coli* MG1655, a wild-type K-12 strain, was used for isolating chromosomal DNA and RNA. *E. coli* strain DH5α was used as the host for cloning recombinant DNA. Mutations in the *soxRS* and *oxyR* genes were introduced into the *ribA-lacZ* fusion strain (JC101) by P1 transduction, selecting for the antibiotic resistance associated with the insertion mutation.

Media and cell growth

Luria-Bertani (LB) media were used for routine bacterial growth. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added to the media as an indicator of lactose utilization (Silhavy et al. 1984). Antibacterial compounds were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 25 μg/ml; tetracycline, 20 μg/ml; chloramphenicol, 20 μg/ml. In order to determine the effect of various redox-cycling agents or oxidants, cells were grown in LB medium to an optical density of 0.2 at 600 nm and were treated with the agents at various concentrations for 1 h.

Enzymes and chemicals

Restriction enzymes, Klenow fragment of DNA polymerase I, and DNA ligase were obtained from New England Biolabs, Boehringer Mannheim Biochemicals, or Promega. 6,7-Dimethylpterin was from Dr. B. Schircks GTP, diacetyl, paraquat, riboflavin, FMN, and FAD were from Sigma. All the chemicals used were of reagent or molecular biology grade.

DNA manipulations

DNA purifications, ligations, restriction analyses and gel electrophoresis were carried out as described by Sambrook et al. (1989). DNA sequencing was done by the dideoxy chain termination method (Sanger et al. 1977) using Sequenase™ from United States Biochemicals.

Construction of a random promoter library

Chromosomal DNA of *E. coli* MG1655 was partially digested with *Alu*I under conditions where most fragments lie in the size range 500–1000 bp. The promoter-probe vector pRS415 was completely digested with *Sma*I and treated with calf intestinal alkaline phosphatase, in order to prevent self-ligation. The prepared plasmid was ligated with the chromosomal fragments at a molar ratio of 1 : 5 and was transformed into *E. coli* DH5α. The inserted fragment lies upstream of the *lacZ* reporter gene, and if it contains an intact promoter region the strain bearing it will express β-galactosidase. Transformants with promoter activity were selected on LB plates containing ampicillin and X-Gal by picking blue colonies. Since the vector expresses a basal level of β-galactosidase activity (5 units), the concentration of X-Gal was lowered to 20 μg/ml in order to discriminate between vectors with and without promoter regions.

β-Galactosidase assay

To screen a promoter library for paraquat-inducible clones, we used 96-well microtiter plates, in which β-galactosidase activity was assayed in whole cells by the addition of *o*-nitrophenyl-β-D-galactopyranoside, after permeabilization of the bacteria with SDS-chloroform (Miller 1992). Cells were grown in LB medium to an optical density of 0.2 at 600 nm and were distributed into two neighboring wells, one of which was treated with 100 μM paraquat for 1 h at 37° C. β-Galactosidase activity was measured using a microplate reader (Molecular Devices). Cells expressing more β-galactosidase in the presence of paraquat were again grown in 3 ml of LB medium and retested for paraquat inducibility.

Construction of *lacZ* operon fusions

In order to construct a strain containing a *ribA*-driven *lacZ* gene on the chromosome, we followed the procedure developed by Simons et al. (1987). The promoter-*lacZ* fusion in pJC101, in which relevant promoter region was cloned in front of the *lacZ* gene, was then transferred onto λRZ5 by homologous recombination in vivo. The recombinant phage was used to lysogenize strain GC4468. Amp^r Lac^+ colonies were selected and confirmed to be λ lysogens by cross-streaking against λ*c*I and λ*vir* (Silhavy et al. 1984). Single lysogens were further selected by measuring the level of β-galactosidase activities.

RNA isolation

Cellular RNA was extracted using RNAzol B (Biotecx Laboratories) according to manufacturer's recommendations, except that the cells were first treated with lysozyme (4 mg/ml) in 50 mM glucose, 25 mM TRIS-HCl (pH 8.0), and 10 mM EDTA for 5 min on ice.

Primer extension analysis

An oligonucleotide (5′-AACTGCGCGTCGCCGCTTTCATCG-3′) complementary to sequences at the *trp-lac* junction of pRS415 was synthesized and labeled at the 5′ end with [γ-^{32}P]ATP. The labeled probe was extended with avian myeloblastosis virus reverse transcriptase (Promega) as described by Sambrook et al. (1989). The resulting cDNAs were analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea.

GTP cyclohydrolase II and flavin assay

Assay methods for GTP cyclohydrolase II activity and flavin compounds were basically the same as the procedures previously described (Kozioł 1971; Foor and Brown 1975; Richter et al. 1993). Wild-type (GC4468) and BW829 (Δ*soxRS*) cells were grown in 100 ml of LB medium up to an optical density of 0.2 at 600 nm and were split into two 50-ml aliquots, one of which was treated with 200 μM paraquat for 1 h. Cells were then harvested and washed with 200 mM TRIS-phosphate buffer (pH 8.7). The washed cell pellet was resuspended in 200 mM TRIS-phosphate buffer (pH 8.7) and disrupted by abrasion with glass beads in a bead beater (Biospec). Cell debris was removed by centrifugation and the lysate was added to assay mixture containing 200 mM TRIS-phosphate (pH 8.7), 10 mM $MgCl_2$, 10 mM GTP, and 20 mM DTT in a total volume of 100 μl. They were incubated at 37° C for 30 min and then 100 μl of 11.5 mM diacetyl was added. The mixture was further incubated at 95° C for 30 min. The diacetyl treatment converted the enzyme product to 6,7-dimethylpterin, which was subsequently separated by reverse-phase high-performance liquid chromatography (HPLC) on a column of Deltapak C18-300 Å (Waters; 3.9 mm × 150 mm Spherical 5 μ), eluted with 100 mM ammonium formate and 40% methanol, and monitored fluorometrically (excitation, 365 nm; emission, 435 nm). One unit of enzyme activity was defined as the activity that catalyzes the formation of 1 nmol of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone 5′-phosphate per h at 37° C. The procedure of obtaining cell lysate for flavin assay was the same as for GTP cyclohydrolase II activity assay with the exception that cells were cultured in minimal media (M9 media containing 0.4% glucose) and disrupted in water instead of TRIS-phosphate buffer (pH 8.7). The lysate was incubated at 80° C for 10 min to extract the flavins and mixed with 0.1 volume of ice-cooled trichloroacetic acid aqueous solution to release the protein-bound flavins throughout the deproteinization. The mixture was kept at 0° C for 20 min to prevent the hydrolysis of FAD and rapidly centrifuged. The supernatant was subsequently separated by reverse-phase HPLC on the same column, eluted with 34% methanol, and the effluent was monitored fluorometrically (excitation, 450 nm; emission, 530 nm).

Results

Isolation of promoter clones inducible by paraquat, a superoxide generator

In order to isolate promoters that are specifically induced by paraquat, we constructed a random promoter library in a multicopy promoter-probe plasmid, pRS415, and screened clones for inducibility by paraquat. The pRS415 plasmid encodes the structural gene for β-galactosidase, *lacZ*, preceded by a ribosome binding site. Since the *lacZ* gene lacks its own promoter, β-galactosidase is expressed only when a promoter is inserted into the multicloning site. Thus, promoter-containing transformants were selected on the basis of β-galactosidase expression, screening for blue colonies on X-Gal selective plates. About one thousand transformants were selected and screened for the inducibility of β-galactosidase activities after treatment with paraquat, a redox-cycling agent. Two promoter clones were found to be induced more than 2.5-fold by treatment with 100 μM paraquat. We determined the nucleotide sequences of these promoter fragments and searched for homologous sequences in the DNA database

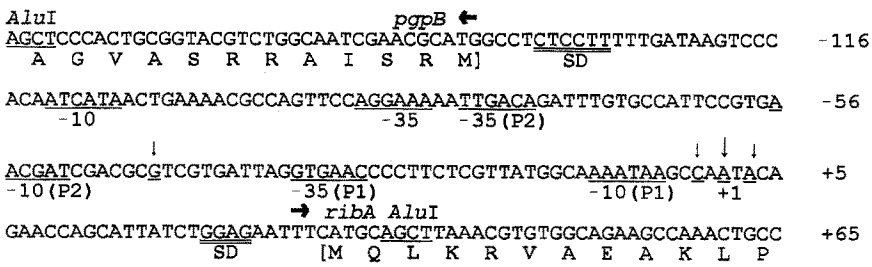

Fig. 1 Nucleotide sequence of promoter fragment of *ribA*. The nucleotide sequences as well as the deduced amino acid sequences of *AluI* fragments cloned into recombinant pRS415 plasmid are shown. The locations of the transcription start sites determined by primer extension analyses (Fig. 4) are indicated by *arrows*. The putative Shine-Dalgarno sequence (SD) as well as −35 and −10 promoter elements recognized by $E\sigma^{70}$ RNA polymerase are *underlined*. Numbering is with reference to the major transcription start site (+1)

(GenBank, EMBL; Altschul et al. 1990). Sequence analysis revealed that one promoter insert was identical to the upstream sequence of the *ribA* gene encoding GTP cyclohydrolase II in *E. coli* (Fig. 1).

Induction of the *ribA* gene by redox-cycling agents

The inducibility of the *ribA* gene by redox-cycling compounds and other agents was investigated using single-copy *lacZ* fusions (Fig. 2). For this purpose, a promoter-*lacZ* fusion was constructed as described in Materials and methods, and the lysogen (JC101) was treated with various redox-cycling agents known to produce superoxide within the cell, as well as with other agents. The *ribA* promoter was induced about eight-fold at 200 μM paraquat and up to 10-fold at 800 μM paraquat (Fig. 2A). This level of induction was comparable to the approximately 10-fold induction of *nfo*, *zwf*, *sodA*, and *micF* seen upon paraquat treatment in the single-copy state (Tsaneva and Weiss 1990; Chou et al. 1993). Treatment with plumbagin displayed a similar response, except that induction occurs at much lower concentrations and decreases at higher concentrations, probably due to the decrease in cell viability at these higher concentrations (Fig. 2B). Menadione was effective also (data not shown). On the other hand, treatment with hydrogen peroxide, ethanol, or a temperature shift from 30° C to 42° C did not cause significant induction of the promoter (Fig. 2C; data on ethanol and heat treatment not shown). From these results we were able to confirm that *ribA* is specifically induced by superoxide-generating agents in general.

Effect of *soxRS* and *oxyR* mutations on the inducibility of *ribA* by paraquat

Since the *ribA* gene was induced by various redox-cycling agents, we examined whether the induction was regulated by *soxRS*. The effect of mutations in *soxR* and *soxS* on the induction of the *ribA-lacZ* fusion by paraquat was examined (Fig. 3). Δ*sox-8*::cat, which is a *soxRS* deletion, and *soxS3*::Tn*10*, which is a null mutation in *soxS*, were transduced into the *ribA* fusion lysogen. Induction by paraquat at various concentrations was then measured. It is clearly demonstrated in Fig. 3 that *lacZ* expression is not induced by paraquat in *soxR* and *soxS* mutant strains, suggesting that *ribA* is positively regulated by *soxR* and *soxS*. When a *soxR* constitutive mutation (*soxR4*::cat) was transduced into the *lacZ* fusion strain, the basal level of β-galactosidase was about 4-fold higher than in wild type, again confirming the role of *soxR* as a positive regulator of *ribA*. The mutations in *oxyR* had no significant effect on the inducibility by paraquat. These genetic data are consistent with the observation that *ribA* was not induced by hydrogen peroxide. Therefore, we conclude that *ribA* gene is a member of the *soxRS* regulon.

Analysis of the *ribA* transcript

To locate precisely the 5′ end of the *ribA* transcript, primer extension analysis was performed as described above. RNA was isolated from cells harboring pJC101 with or without paraquat treatment. Several primer-extended products were observed. The most prominent one corresponds to a transcript that starts at an A 30 nucleotides upstream of the start codon for *ribA* and the longest one corresponds to a start site 74 nucleotides upstream of the start codon (Fig. 4). From the location of transcription start site we tentatively located −35 and −10 elements of both the upstream promoter (P2) with weak promoter activity, and the downstream promoter (P1) with stronger activity (see Fig. 1). The upstream transcript derived from P2 promoter was not induced by paraquat treatment whereas downstream transcripts derived from the P1 promoter were strongly induced. The degree of induction of downstream transcripts was estimated to be about seven-fold, similar to the value obtained by β-galactosidase assay (Fig. 2).

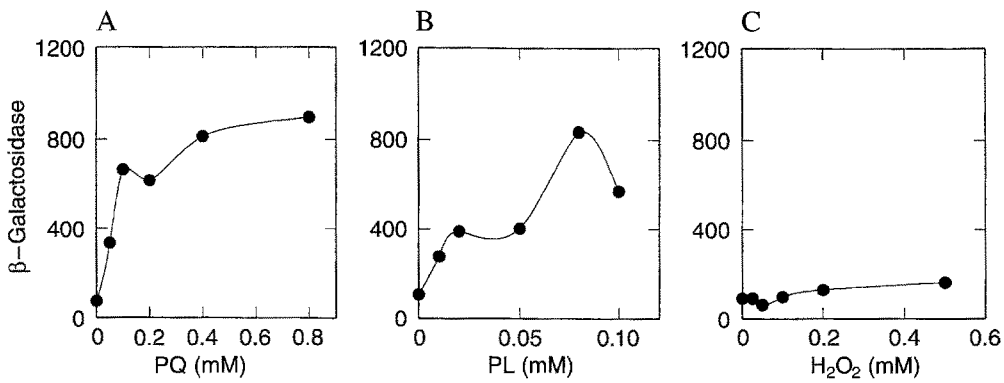

Fig. 2A–C Induction of β-galactosidase by various treatments in the *ribA-lacZ* fusion strain. Exponentially growing *E. coli* cells containing the *ribA-lacZ* fusion (JC101) were treated with various concentrations of (**A**) paraquat (PQ), (**B**) plumbagin (PL), or (**C**) hydrogen peroxide for 1 h. β-Galactosidase activity, expressed in Miller units, was assayed as described in Materials and methods

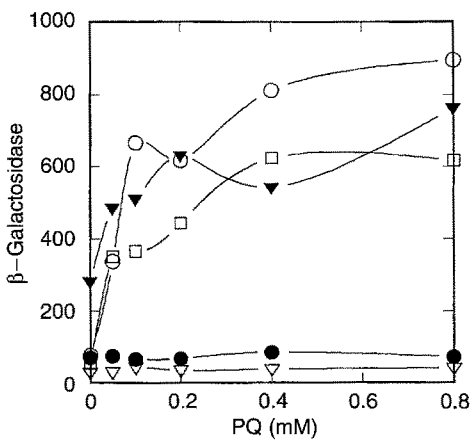

Fig. 3 Dependence of induction of β-galactosidase in the *ribA-lacZ* fusion strain by paraquat (PQ) on the *soxRS* locus. Various regulatory mutant alleles were transduced into the *ribA-lacZ* fusion strain (JC101; ○) by phage P1 to generate strains JC201 (Δ*sox-8*::cat; ●), JC301 (*soxS3*::Tn*10*; ▽), JC401 (*soxR4*::cat; ▼), and JC501 (Δ*oxyR*::kan; □), respectively. Following treatment for 1 h with various concentrations of paraquat, β-galactosidase activity was measured (in Miller units)

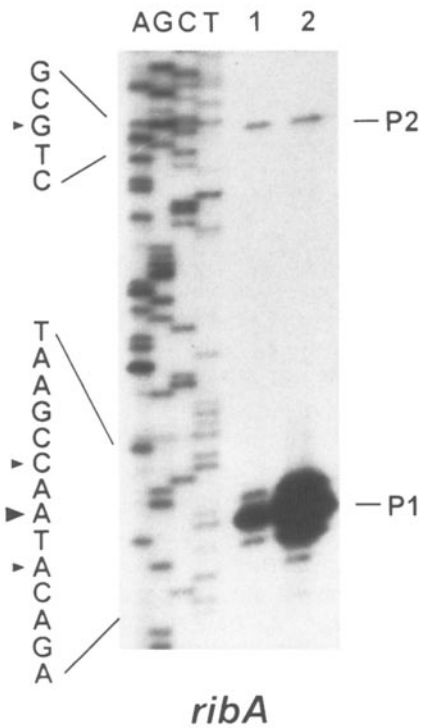

Fig. 4 Mapping 5′ ends of *ribA* transcripts by primer extension analysis. Radiolabelled primer was extended on the RNA samples (50 μg) isolated from cells with (lane 2) or without (lane 1) paraquat treatment. DNA sequencing ladders were generated from the purified recombinant pRS415 plasmid (pJC101) with the same primer (lanes A, G, C, and T). The nucleotide sequence of the coding strand is shown on the left, and the specific transcription initiation sites are indicated by *arrowheads* (*larger arrowhead* for major site)

Effect of paraquat treatment on GTP cyclohydrolase II activity and flavin content in *E. coli*

We determined whether the increased transcription of the *ribA* gene leads to an increase in the activity of GTP cyclohydrolase II and a consequent increase in flavin, which is the end product of the synthetic pathway in which GTP cyclohydrolase II is the first enzyme. GTP cyclohydrolase II enzyme activity in extracts from wild-type (GC4468) and Δ*soxRS* (BW829) cells, either untreated or treated with 0.2 mM paraquat, were measured as described in Materials and methods. *E. coli* GC4468 treated with paraquat contained about 3.2-fold more GTP cyclohydrolase II activity than untreated cells, whereas the Δ*soxRS* mutant exhibited no increase in enzyme activity on paraquat treatment (Table 2). The increase in enzyme activity was about 40% of that measured for β-galactosidase induction but was nevertheless substantial. The increase in enzyme activity following paraquat treatment was entirely dependent on the *soxRS* locus, confirming that regulation of *ribA* gene expression by SoxRS occurs mainly on the transcriptional level. We then measured the content of riboflavin and other flavin compounds (FMN, FAD), and found that it was not changed by paraquat treatment (data not shown). This is consistent with the finding by Richter et al. (1993) that overexpression of GTP cyclohydrolase II causes a 100-fold increase in

Table 2 Changes in GTP cyclohydrolase II activity induced by paraquat treatment in wild-type and *soxRS* deletion strains

		GTP cyclohydrolase II (U/μg)[b]			
β-Galactosidase[a]		Wild type (GC4468)		Δ*soxRS* (BW829)	
−Paraquat	+Paraquat	−Paraquat	+Paraquat	−Paraquat	+Paraquat
76 ± 7	642 ± 24	3.6 ± 1.4	11.4 ± 4.4	2.5 ± 0.8	2.3 ± 0.6

Exponentially growing cells were either treated or not with 0.2 mM paraquat for 1 h before preparing cell extracts for assay of enzyme activities

[a] β-Galactosidase in the *ribA-lacZ* fusion strain (JC101) was assayed as described in Materials and methods and expressed in Miller (1992) units. Values are means (± standard deviations) of three independent experiments

[b] GTP cyclohydrolase II activity in the wild-type (GC4468) and Δ*soxRS* (BW829) strains was assayed as described in Materials and methods and expressed in units per μg of crude protein extract. Values are means (± standard deviations) of five independent experiments

enzyme activity but does not increase the riboflavin content in *E. coli*.

Discussion

The function of GTP cyclohydrolase II in relation to oxidative stress has not been investigated so far. Constitutive expression of the *ribA* gene has been suggested in *E. coli* and *S. typhimurium* (Teslyar and Shavlovskii 1983; Wang 1991). Since it acts on the first step in the biosynthesis of riboflavin, which is used as a source of FMN and FAD, and considering the role of these cofactors in many electron-transfer flavoproteins, it is reasonable to suggest that GTP cyclohydrolase II may play an important role under oxidative stress conditions. However, even though the activity of GTP cyclohydrolase II was enhanced more than three-fold by paraquat treatment, we observed that the flavin content did not change. The lack of change in the content of riboflavin and other flavin compounds on paraquat treatment indicates that the induction of *ribA* is not a consequence of an increased demand for riboflavin or its derivatives. Indeed riboflavin acts as a photosensitizer and produces singlet oxygen photochemically. It also produces superoxide by an autooxidation reaction (Halliwell and Gutteridge 1989). It is therefore likely that an increase in riboflavin content might be harmful to cells. One possible reason for the need for *ribA* induction is that the immediate reaction product of GTP cyclohydrolase II or some intermediate reaction products along the pathway toward riboflavin synthesis may play some role in coping with the demands imposed on the cell by superoxide-mediated stress. The reaction products, ribosylamino pyrimidines and ribityllumazine, might be involved in radical scavenging or redox-active reactions. In this respect, the content and oxidation/reduction properties of these reaction products need be analyzed and their effect needs be investigated further using various mutants in the riboflavin synthetic pathway. Yet another possibility is that the cell requires overproduction of GTP cyclohydrolase II under oxidative conditions to ensure the constitutive or homeostatic production of riboflavin if some enzymes or metabolites in the synthetic pathway are labile under such conditions. The data in Table 2 seem to suggest that GTP cyclohydrolase II itself is not labile under superoxide stress, as shown by the insensitivity of enzyme activity to paraquat in noninducible (Δ*soxRS*) cells. However, since SoxR and SoxS are regulators that affect many components in the cell, more biochemical analysis needs be done to test whether GTP cyclohydrolase II becomes labile in the presence of superoxide radicals.

We analyzed the *ribA* promoter region for putative SoxS binding sequences upstream of transcription start sites. Sequences from −146 to −118 are similar to the proposed SoxS binding sequence (Fawcett and Wolf 1994; Li and Demple 1994) (Fig. 5). If SoxS binds to this site, it may interfere with the transcription of divergently transcribed *pgpB* gene, which encodes a membrane-bound phosphatase catalyzing the hydrolysis of at least three different phospholipid-type compounds (Icho 1988), as shown in Fig. 1. However, we were not able to observe any reduction in the expression of a *lacZ* gene driven by the *pgpB* promoter (data not shown). This implies either that SoxS does not bind to this site or that SoxS binding does not interfere with *pgpB* transcription. This putative site is unusual in that it is located far upstream of the −35 element, whereas other known binding sites of SoxS lie just upstream of and overlap the −35 region (Fawcett and Wolf 1994; Li and Demple 1994). If this upstream sequence is a binding site for SoxS, the activation may occur through the formation of DNA loops, similar to the regulatory mechanism of the AraC protein (Martin et al. 1986). Indeed, SoxS is homologous to the C-terminal domain of AraC (Wu and Weiss 1991; Amábile-Cuevas and Demple 1991), which constitutes a separable gene activator module (Bustos and Schleif 1986). There is another putative SoxS binding sequence around −66 on the noncoding strand. The sequence in this region matches the consensus Soxbox sequence

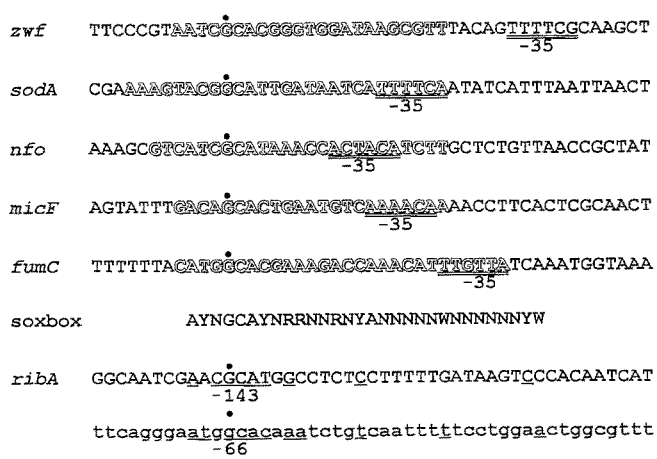

Fig. 5 Comparison of upstream promoter sequences of *ribA* with primary SoxS-binding sites located within the putative Soxbox of *soxRS* regulon promoters. MalE-SoxS binding sites identified in DNase I protection experiments (Fawcett and Wolf 1994) were aligned with the Soxbox consensus sequence. R = A or G, Y = T or C, W = A or T. A putative SoxS binding site on the non-coding strand is marked by the *lowercase letters*. Nucleotides identical to the Soxbox are indicated by underlining

better. So far, no other SoxS binding sequence has been localized on the noncoding strand. However, if SoxS binds as a dimer as AraC does, binding sites in both orientations may function equally well. Whether SoxS binds as a dimer has not been elucidated. There are reports of multiple SoxS binding sites in *micF*, *sodA*, and *zwf* promoters, where a secondary site further upstream of the −35 element is occupied in a concentration-dependent manner (Li and Demple 1994; Fawcett and Wolf 1994). Further investigation of the properties and activation mechanism of SoxS is therefore necessary.

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