

## Isolation and Characterization of Paraquat-inducible Promoters from *Escherichia coli*

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Promoters inducible by paraquat, a superoxide-generating agent, were isolated from *Escherichia coli* using a promoter-probing plasmid pRS415 with promoterless *lacZ* gene. Twenty one promoters induced by paraquat were selected and further characterized. From sequence analysis, thirteen of the promoters were mapped to their specific loci on the *Escherichia coli* chromosome. Several promoters were mapped to the upstream of known genes such as *usg1*, *katG*, and *mglB*, whose relationships with superoxide response have not been previously reported. Other promoters were mapped to the upstream region of unknown open reading frames. Downstream of HC96 promoter are uncharacterized ORFs whose sequences are homologous to ABC-transporter subunits. Downstream of HC84 promoter is an ORF encoding a transcriptional regulator-like protein, which contains a LysR family-specific HTH (helix-turn-helix) DNA binding motif. We investigated whether these promoters belong to the *soxRS* regulon. All promoters except HC96 were found to belong to the *soxRS* regulon. The HC96 promoter was significantly induced by paraquat in the *soxRS* deletion mutant strain. The basal transcription level of three promoters (HE43, HC71, HD94) significantly increased at the stationary phase, implying that they are regulated by RpoS. However, paraquat inducibility of all promoters disappeared in the stationary phase, suggesting that SoxRS regulatory system is active only in rapidly growing cells.

**Key words:** ABC-transporter, *Escherichia coli*, LysR family, paraquat-inducible promoter, *soxRS* regulon

All living organisms are subject to various forms of environmental changes and stresses and have evolved specific adaptive mechanisms, in response. The organisms respond to stress by sensing particular signals of the stresses and by controlling the expression of many associated genes. Oxidative stress is of particular interest because aerobes are in continual contact with reactive oxygen species which are formed as a byproduct of normal respiration due to incomplete reduction of oxygen, or by exposure to external sources such as pollution, radiation, and redox-cycling agents, or by release from macrophages in response to bacterial invasion (8, 9, 12, 14).

Reactive oxygen species or reactive oxygen intermediates include superoxide anion ( $O_2^{\cdot -}$ ), hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical ( $\cdot OH$ ), which are generated by the sequential reduction of molecular oxygen by single electron transfer (12). Because of their high reactivity, these molecules can damage many other intracellular molecules and cause serious cell damage and even cell death (14).

Both eukaryotic and prokaryotic cells have developed inducible defense systems to combat the oxidative stress. *Escherichia coli* possesses two separate oxidative stress regulons, one for hydrogen peroxide (*oxyR* regulon) (6) and the other for superoxide stress (*soxRS* regulon) (10, 11, 27). Each regulon consists of about 40 inducible proteins, most of which are of unknown function or control mechanism (10, 28). In the *oxyR* regulon, at least nine proteins, including catalase-peroxidase (encoded by *katG*), NADPH-dependent alkyl hydroperoxide reductase (encoded by *ahpCF*), glutathione oxidoreductase (encoded by *gorA*), a non-specific DNA binding protein (encoded by *dps*), and a small untranslated regulatory RNA (encoded by *oxyS*), are under the positive control of the *oxyR* gene (2, 16, 20). In the *soxRS* regulon, at least nine gene products are produced by a regulon controlled by two positive regulatory genes, *soxR* and *soxS* (7, 16, 20). The products of the genes known to be regulated by the *soxRS* gene 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*, acon-

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itase (encoded by *acnA*), NADPH: ferredoxin oxidoreductase (encoded by *fpr*), GTP-cyclohydrolase II (encoded by *ribA*), *inaA* and *pqi-5* with unknown functions (7, 16, 20). Although some of the proteins induced by H<sub>2</sub>O<sub>2</sub> and the superoxide radical overlap with each other and with those of the heat shock, carbon starvation, and SOS responses (8, 10, 22), these two regulons are for the most part distinct, do not develop cross resistance, and have no known regulatory overlap.

The multi-faceted mechanism that cells employ when exposed to highly oxidative conditions could be fully revealed if the gene products induced or repressed during this process are fully characterized. We have previously used multi-copy promoter-probing plasmids, pJAC4 and pRS415, to isolate DNA fragments containing promoters inducible by paraquat, a superoxide-generating compound (15, 17, 18). However, more extensive screening of paraquat-inducible promoters is required to unravel the cellular defense mechanism in more detail. In this paper, we report the isolation of about 20 more paraquat-inducible promoters using promoter-probing plasmid pRS415 and present evidence for the regulation of most of these genes 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, which originated from the wild-type *K-12* strain, was used as the source for isolating chromosomal DNA. *E. coli* strain DH5 (*supE* 44 *lacU169* (80 *lacZ* M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-relA1*) was used as the host for cloning recombinant DNAs. BW829 is a mutant strain with a *soxRS*-deletion mutation derived from *E. coli* wild type strain GC4468. BW829 and GC4468 were used as the hosts to investigate the SoxRS dependence of isolated promoters. The promoter probing vector pRS415, which contains promoterless *lacZ* gene and the ampicillin resistance marker, was used to screen

promoter inserts cloned into the *Sma*I site directing LacZ expression (25).

### Media and cell culture

LB media (1% trypton, 1% NaCl, and 0.5% yeast extracts) were used for routine bacterial growth. TB media (1% trypton and 0.5% NaCl) was used for genomic DNA isolation from *E. coli* wild type strain, MG1655. Antibacterial compounds were used at the following concentrations: ampicillin, 50 µl/ml; chloramphenicol, 20 µl/ml. In order to determine the effect of paraquat in the exponential and stationary phases, cells were grown in LB medium up to an optical density of 0.2 at 600 nm or overnight and treated with 0.1 mM paraquat for 1 hour, followed by β-galactosidase assay.

### DNA manipulation

General procedures described by Sambrook *et al.* (24) were followed to purify, digest, and ligate DNAs. Preparation of chromosomal DNA was performed as described by Koh *et al.* (15). DNA sequencing was done using oligonucleotide primers flanking the cloning site in pRS415.

### Construction and screening of promoter library

Chromosomal DNA from *E. coli* MG1655 strain was partially digested with *Alu*I or *Hae*III to the extent where the fragments abound in the range of 200~1000 bp. The promoter-probing pRS415 DNA was completely digested with *Sma*I, treated with calf intestine alkaline phosphatase, and ligated with the chromosomal fragments at a molar ratio of 1:3. The library was transformed into *E. coli* DH 5α strain, and the 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 (about 5 units), the concentration of X-Gal was lowered to 20 µl/ml in order to discriminate against vectors without promoter inserts. For screening paraquat-inducible promoters, the 96-well microtiter plate method as described by Koh *et al.* (15) was employed.

**Table 1.** Bacterial strains and plasmids used in this study

Strains and plasmid	Description	Source or reference
Strains		
MG1655	Wild type <i>E. coli</i>	5
GC4468	( <i>argF-lac</i> ) 169 <i>rpsL</i> <i>sup</i> (Am)	26
BW829	GC4468 <i>sox-8::cat</i> *	27
Plasmid		
pRS415	<i>lacZYA</i> operon fusion vector, Amp <sup>r</sup>	25

## Results

### Screening of paraquat-inducible promoters

A random promoter library was made using pRS 415 plasmid which harbors 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 can be selected on the basis of  $\beta$ -galactosidase expression by screening for blue colonies on X-Gal selective plates. Among 30,000 transformants, we selected 2,700 blue transformants as promoter-containing candidates. In order to isolate promoters specifically induced by the superoxide radical, 2,700 transformants were grown in microtiter plate and screened for paraquat-inducible clones as described in Materials and Methods. 21 clones which are induced by treatment with 0.1 mM paraquat were finally obtained. The induction of  $\beta$ -galactosidase by paraquat was measured separately for each of these promoters. As shown in Table 2, all of these promoters were induced more than 1.5 fold on multi-copy plasmid (about 50–100 copies/cell).

### Sequence analysis of paraquat-inducible promoter fragments

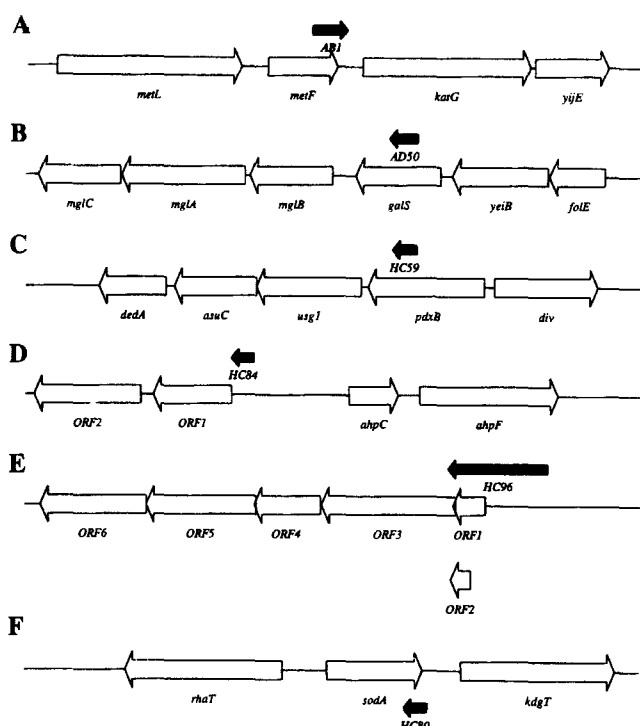
We determined the nucleotide sequences of 13 promoter fragments less than 1 kb of length and searched for homologous sequences in the DNA database (GenBank/EMBL/DDBJ) using the BLAST program (1). Since the sequencing of the full *E. coli* genome had been completed, the chromosomal loci of all 13 promoter clones could be determined. Table 2 shows the loci of these 13 promoter clones as well as some flanking genes of known functions and ORFs of unidentified function. Some promoter

clones were mapped directly upstream of the coding region, suggesting that they are likely to function as promoters of the genes or the ORFs. For example, AB1 was mapped to the upstream region of *katG*, overlapping with the C-terminal region of the *metF* gene (Fig. 1A). *katG* encodes bifunctional catalase-peroxidase hydroperoxidase I (catalase HPI) and *metF* encodes 5, 10-methylenetetrahydrofolate reductase in *E. coli*. *katG* is a member of the *oxyR* regulon and has been intensively investigated in relation with oxidative stress. Its known promoter is induced by H<sub>2</sub>O<sub>2</sub>, but not by paraquat, and is located about 130 bp downstream from the end of the AB1 promoter fragment. It will be interesting to examine whether HPI (*katG*) expression is also regulated by superoxide anion. The AD50 promoter was mapped to the upstream region of the *mglB* gene, overlapping with the central region of the *galS* gene (Fig. 1B). *mglB* encodes D-galactose-binding periplasmic protein (periplasmic galactose receptor), which constitutes high-affinity galactose permease with MglC, which is the membrane translocator, and MglA, which is the membrane-associated ATPase (23). *mglABC* has been shown to be coexpressed as an operon (13). The upstream gene *galS* encodes *mgl* repressor or galactose ultra-induction factor. These genes have not been previously reported to be related with the oxidative stress and require further investigation in this respect. Another promoter clone,

**Table 2.** Properties of paraquat-inducible promoters

Promoter clones	Locus	Genes in the locus	$\beta$ -galactosidase activity (Miller units)		Induction fold	Regulation by SoxS	Stationary phase induction
			Paraquat (-)	Paraquat (+)			
AB1	89.0 min	<i>metF</i> , <i>katG</i>	151.6	420.4	2.8	+	-
AB45	68.7 min	<i>mtr</i>	326.5	2060.6	6.3	+	-
HA84	41.0 min	<i>sdaA</i>	368.8	1564.3	4.2	+	-
HA25	N.D.		103.7	284.6	2.7	+	-
HA64	N.D.		234.6	299.9	1.3	N.D.	N.D.
HE43	87.3 min	<i>dsbA</i> , <i>ppfA</i>	747.6	2440.7	3.3	+	+
AD50	46.5 min	<i>galS</i>	267.7	713.5	2.7	+	-
HB13	20.1 min	<i>dmsA</i> , <i>dmsB</i> , <i>dmsC</i>	68.8	211.4	3.1	+	-
HB43	N.D.		150.0	529.1	3.5	+	-
HC1	44.8 min	unknown ORFs	37.3	151.2	4.0	+	-
HC41	N.D.		47.8	124.3	2.6	+	-
HC59	50.2 min	<i>pdxB</i> , <i>usg1</i>	37.2	408.2	11.0	+	-
HC65	90 or 5 min	<i>rrnB</i> or <i>rrnH</i>	45.8	1872.2	40.9	+	-
HC84	13.7 min	<i>ahpC</i> , <i>ahpF</i>	3170.1	6558.8	2.1	+	-
HC48	N.D.		478.9	727.8	1.5	N.D.	-
HC70	N.D.		308.4	634.2	2.1	+	-
HC71	N.D.		342.9	502.0	1.5	N.D.	+
HC80	88.5 min	<i>sodA</i>	26.6	737.2	27.7	+	-
HC96	38.1 min	unknown ORFs	2840.5	4640.2	1.6	-	-
HD7	N.D.		2221.3	3942.2	1.8	+	-
HD94	54.9 min	<i>cysP</i>	126.4	536.5	4.2	+	+

\* N.D. : not determined.



**Fig. 1.** Genetic loci of six paraquat-inducible promoters. The location of cloned promoter fragments (closed arrow) and the neighboring genes or ORFs (open arrows) were demonstrated. A, *metL* encodes aspartokinase II/homoserine dehydrogenase II. *metF* encodes 5, 10-methylenetetrahydrofolate reductase. *katG* encodes bifunctional catalase-peroxidase (hydroperoxidase I). B, *mglB* encodes D-galactose-binding periplasmic protein. *galS* encodes *mgl* repressor and galactose ultrainduction factor. *folE* encodes GTP-cyclohydrolase I. C, *dedA* encodes DedA protein. *asuC* encodes pseudouridylylase I. *usg1* encodes unknown protein. *pdxB* encodes erythronate-4-phosphate dehydrogenase. *div* encodes Div protein. D, *ahpC* encodes alkylhydroperoxide reductase C22 protein. *ahpF* encodes alkylhydroperoxide reductase F52A protein. F, *rhaT* encodes rhamnose permease. *sodA* encodes manganese superoxide dismutase. *kdgT* is the locus associated with the function of 2-keto-3-deoxygluconate transport. *yjiE*, *yeiB*, and all other ORFs are of unknown function deduced from sequence analysis.

HC59, was mapped to the upstream of the *usg1* gene, overlapping with the C-terminal region of the *pdxB* gene (Fig. 1C). These genes also have not been reported to be related with the oxidative response. *usg1* was originally reported as one of the upstream genes of *asuC* (*hisT*), which encodes pseudouridylylase I, and was temporally named as "*usg1*" (upstream gene) because of its unknown function (3, 21). *usg1* and *asuC* had been reported to be cotranscribed from the *usg1*-upstream promoter, whose -35 element is located 23 bp-downstream from the 3'-end of our HC59 promoter fragment. However, it was clear from S1

mapping that the known promoter acts only in part, and the possible existence of a further upstream promoter had been strongly proposed (3). Later, another upstream gene turn out to be the *pdxB* gene, which encodes erythronate-4-phosphate dehydrogenase, and *pdxB* has been suggested to be cotranscribed with *usg1*, *asuC*, and *dsg1* (this was temporally named as "downstream gene" but now is called *dedA*), as an operon (4). However, the possibility still remains that HC59 promoter may act as an upstream promoter of *usg1*, *asuC*, and *dsg1* operon, especially in response to the oxidative stress. This can be tested in further study.

The HC84 and HC96 promoters were mapped to the upstream regions of unknown ORFs (Fig. 1D, E). The downstream ORF of HC84, ORF1 in Fig. 1D, contains the specific DNA binding motif significantly homologous with that of the LysR family members of *E. coli* transcriptional regulator; the characteristic helix-turn-helix DNA binding motif. The calculated molecular mass of the predicted ORF1 protein is 34.4 kDa, consisting of 300 amino acids. ORF2 (Fig. 1D), whose deduced molecular weight is 47.8 kDa, does not have any significant homology with any known genes. It is intriguing to note that the genes for thiol peroxidase system (*ahpCF*) regulated by OxyR are located upstream of the HC84 promoter in diverging orientation. Considering the functions of AhpC and AhpF, it will be interesting to investigate whether the LysR-type regulator transcribed from the HC84 promoter in response to superoxide regulates the expression of the *ahpCF* genes. ORFs 3-6 are located in close proximity downstream of the HC96 promoter (Fig. 1E). ORF1 [encodings 122 amino acids (13.3 kDa)] overlaps with HC96 and is highly homologous with the N-terminal domain of the putative heat shock protein *hscA* (about 47% identity and 71% similarity over the entire region; 105 amino acids of all 122 amino acids). ORF2 is overlapped with ORF1 in -1 frame and is predicted to encode a small protein of about 8.5 kDa, containing only 74 amino acids. We could not find any ORF2-homologous genes which have ever been reported. ORF3, whose deduced molecular mass is 56.3 kDa, consists of 508 amino acids with high homology to the subunit of ABC type transporter from *Synechocystis* sp. (67% identity and 83% similarity). ORF4, whose deduced molecular mass is 27.6 kDa, consists of 248 amino acids with high homology to another subunit of the same ABC transporter and contains ATP-binding motif (58% identity and 77% similarity). The probable ABC-type transporters from other organisms also show high homology with ORF4, including those from *Cyanophora para-*

*doxa* (56% identity and 73% similarity), chloroplast of *Porphyra purpurea* (56% identity and 74% similarity), *Odontella sinensis* (51% identity and 73% similarity), chloroplast of *Odontella sinensis* (51% identity and 72% similarity), and chloroplast of *Anthamion sp.* (51% identity and 72% similarity). Consistently, ORF3 also shows high homology with corresponding proteins from the above organisms. Therefore it is very likely that ORF3 and 4 downstream of the HC96 promoter may function as an ABC-transporter in *E. coli*. ORF5 also shows high homology with yet another subunit constituting an ABC transporter complex. In comparison, homologues of ORF3 and ORF4 all appear in the same orientation whereas ORF5 does not exist commonly in other organisms. ORF6 is NifS homologue of *E. coli*. Further investigation is necessary to elucidate their function as well as their operon structure and regulation.

We found that some of our promoter clones lay in a nonconventional orientation. For example, the HC80 promoter is located at the 3' end of the *sodA* gene (encoding superoxide dismutase), in the reverse orientation (Fig. 1F). No other ORF is found downstream of the HC80 promoter. We can postulate that the HC80 promoter plays a role in feed back regulation of *sodA* gene expression. Likewise, the HD94 promoter lies in the central region of the *cysP* gene (encoding thiosulfate binding protein precursor) in the reverse orientation (data not shown). No ORF is found in opposite direction, either. The function of these promoters is hard to predict, but they may promote the transcription of regulatory antisense RNA. Of other promoters, AB45, HB13, and HC1 promoters were mapped to the downstream region of *mtr*, *dmsC*, and unknown ORF, respectively (data not shown). *mtr* and *dmsC* are genes for tryptophan-specific permease and anaerobic dimethyl sulfoxide reductase, respectively. These promoters are also in the reverse orientation, like the HC80 and HD94 promoters, suggesting that they may play similar roles in feed-back regulation.

#### Regulation of paraquat-inducible promoters by *soxRS* and *rpoS*

Since our 21 promoter clones were induced by superoxide-generating agent paraquat, we examined whether the induction was regulated by *soxRS*, the responsible regulator for superoxide stimuli. For this purpose, we introduced our promoter-*lacZ* fusion in pRS415 into the  $\Delta$ *soxRS* strain, BW829 (Table 1). The effect of the *soxRS* mutation on the paraquat-inducibility of the promoters was examined in the multicopy state (Table 2). 17 out of 18 promoters tested were not induced by paraquat

in the *soxRS* mutant, suggesting that these promoters are positively regulated by SoxR and SoxS (Table 2). Their specific roles in response to superoxide stress remain to be further studied.

One exception is the HC96 promoter. Contrary to our expectation, the HC96 promoter was substantially induced by paraquat in the *soxRS* deletion mutant (Fig. 2). Therefore, induction of the HC96 promoter is independent of *soxRS* locus. So far, no genes have been reported to be activated by paraquat, independent of SoxRS regulator in *E. coli*. How the HC96 promoter is regulated will be a very interesting subject for further investigation.

Previously, we isolated paraquat-inducible genes using the same strategy. One of those genes, *pqi5*, is under dual regulation by SoxRS and RpoS (19). RpoS is a regulatory protein which controls genes induced at the onset of stationary phase, nutrient starvation, and high osmolarity. We tested whether our promoters were under the control of RpoS. Three promoters, HE43, HC71, and HD94 were significantly induced at the onset of stationary phase (Fig. 3, Table 2). The expression of these clones increased at least three fold in the stationary phase compared with exponential phase. It is likely that these promoters may be also under the dual regulation of SoxRS and RpoS.

Our approach to isolating paraquat-inducible promoters using promoter-probing plasmids has proven to be quite fruitful. By analyzing the functions of each responsive genes, we expect to elucidate

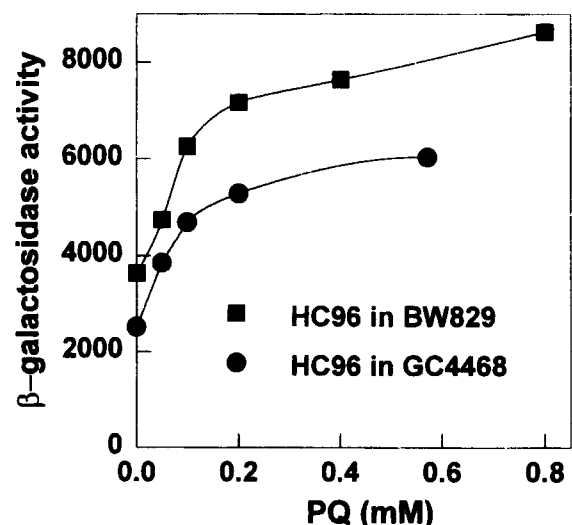
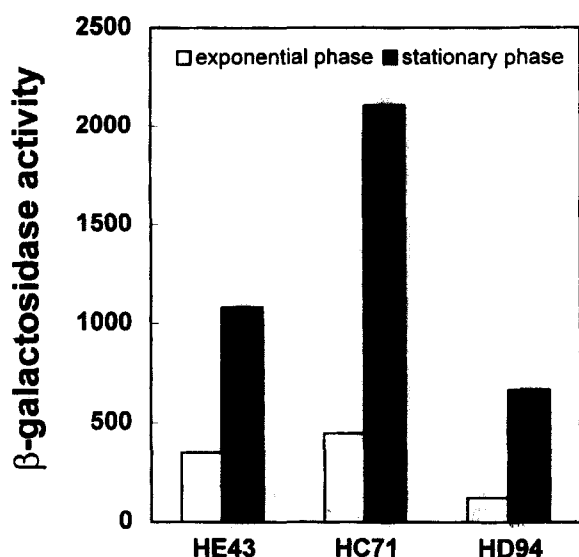


Fig. 2. Effect of *soxRS* deletion mutation on paraquat-inducibility of HC96 promoter.  $\beta$ -galactosidase activity derived from HC96 promoter on pRS415 plasmid was measured and presented in Miller units. GC4468 (wild type) and BW829 (*soxRS* deletion mutant) were treated with various concentration of paraquat (PQ).



**Fig. 3.** Induction of HE43, HC71, and HD94 promoters in the stationary phase.  $\beta$ -galactosidase activity (in Miller units) of cells containing HE43, HC71, or HD94 promoters on pRS415 was measured at the exponential ( $OD_{600}=0.2-0.5$ ) and stationary phase (more than 12 h culture).

the response mechanism of a bacterial cell toward superoxide stress. It will also be intriguing to investigate whether and how the induction is mediated by superoxide anion. The presence of SoxRS-independent activation is another interesting subject to be studied.

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