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Regulation of *fpr* Gene Encoding NADPH:Ferredoxin Oxidoreductase by the *soxRS* Locus in *Escherichia coli*

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We isolated a promoter inducible by paraquat, a superoxide-generating agent, from *Escherichia coli* using a promoter-probing plasmid pRS415. From sequence analysis we found out that the promoter is for *fpr* encoding NADPH:ferredoxin oxidoreductase. We constructed an operon fusion of *lacZ* gene with *fpr* promoter to monitor the expression of the gene in the single-copy state. LacZ expression by *fpr* promoter was induced about 20-fold by 0.8 mM 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 by treatment with hydrogen peroxide, ethanol, and heat shock. Induction of β-galactosidase was significantly reduced by introducing a △*sox-8*::cat or *soxS3*::Tn*10* mutation into the fusion strain, indicating that *fpr* gene is a member of the *soxRS* regulon. The transcriptional start site was determined by primer extension analysis. Possible roles of *fpr* induction in superoxide stress were discussed.

Key words: *Escherichia coli*, *fpr*, NADPH:ferredoxin oxidoreductase, oxidative stress, *soxRS* regulon

All aerobic organisms 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 (12, 14, 19, 30, 34). *Escherichia coli* possesses two separate oxidative stress regulons, one for hydrogen peroxide (*oxyR* regulon) (10, 11) and the other for superoxide stress (*soxRS* regulon) (16, 17, 39).

Redox-cycling compounds such as paraquat, plumbagin, or menadione serve as a continuing source of superoxide as they undergo repeated cycles of oxidation and reduction (20). Upon exposure to redox-cycling compounds which are thought to generate superoxide in a cell, *E. coli* induces the synthesis of about 40 proteins (16, 40). A subset of these proteins are produced by a regulon controlled by two regulatory genes, *soxR* and *soxS*, constituting a *soxRS* regulon (3, 29, 41, 42). The products of the 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*), GTP cyclohydrolase II (encoded by *ribA*), *inaA* and *pqi*-5 with unknown functions (8, 9, 18, 24, 26, 31).

A complex protection mechanism that cells employ upon exposure to highly oxidative conditions could be fully revealed when the gene products induced or repressed during this process are fully characterized. We have previously used a multi-copy promoter-probing plasmid, pJAC4, to isolate DNA fragments containing promoters inducible by paraquat (23, 24). In this report, we demonstrate the isolation of promoter for *fpr* gene using another promoter-probing plasmid, pRS415, and evidence for the regulation of this gene by the *soxRS* locus.

Materials and Methods

Strains, phages, and plasmids

The strains used in this study were listed in Table 1. *E. coli* MG1655, a wild-type K-12 strain, was used as the source 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

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

Strain, plasmid or phage	Description	Source or reference
Strain		
MG1655	Wild type *E. coli*	(4)
GC4468	△(*argF-lac*)*169 rpsL* sup(Am)	(37)
BW829	GC4468 △*sox-8*::cat[a]	(39)
BW831	GC4468 *soxS3*::Tn*10*[b]	(39)
BW847	GC4468 *soxR4*::cat[c]	(39)
GSO18	*recD1014 lac gal rpsL* △*oxyR*::kan[d]	(2)
JC102	GC4468 (λJC102)	This work
JC202	JC102 △*sox-8*::cat; P1(BW829)×JC102	This work
JC302	JC102 *soxS3*::Tn*10*; P1(BW831)×JC102	This work
JC402	JC102 *soxR4*::cat; P1(BW847)×JC102	This work
JC502	JC102 △*oxyR*::kan; P1(GSO18)×JC102	This work
Plasmid		
pRS415	*lacZYA* operon fusion vector, Amp[r]	(36)
pJC102	*fpr* promoter cloned upstream of *lacZ* in pRS415	This work
Phage		
λRZ5	Φ(*bla*'-'*lacZ*) *lacY*[+]	R. Zagursky
λJC102	Φ(*fpr-lacZ*) *lacY*[+] *bla*[+]	This work
λ*cI*	wild type λ phage	(35)
λ*vir*	virulent derivative of λ phage	(35)
P1*vir*	virulent derivative of P1 phage	(35)

[a] △*sox-8*::cat is a deletion mutation of *soxR* and *soxS*.
[b] *soxS3*::Tn*10* is an insertion mutation of *soxS*.
[c] *soxR4*::cat is a *soxR* constitutive mutation.
[d] △*oxyR*::kan is an insertion mutation of *oxyR*.

were introduced by P1 transduction to *fpr-lacZ* fusion strain (JC102), selecting for antibiotics resistance associated with the 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 (35). 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 up to an optical density of 0.2 at 600 nm and were treated with those agents at various concentrations for 1 hr.

Enzymes and chemicals

Restriction enzymes, Klenow fragment of DNA polymerase I, and DNA ligas ewere obtained from New England Biolabs, Boehringer Mannheim Biochemicals, or Promega Corporation. 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.* (32). DNA sequencing was done by Sanger's dideoxy chain termination method using Sequenase from United States Biochemicals (33).

Construction of random promoter library

Chromosomal DNA of *E. coli* MG1655 strain was partially digested with *Alu*I under the conditions where the fragments abound in the range of 500~1000 bp. The promoter-probing vector, pRS415 was completely digested with *Sma*I and treated with calf intestine alkaline phosphatase. The prepared plasmid was ligated with the chromosomal fragments at a molar ratio of 1 : 5 and was transformed into *E. coli* DH5α strain. The inserted fragment lies upstream of the *lacZ* reporter gene, and if it contains an intact promoter region 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 the 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 with the addition of o-nitrophenyl-β-D-galactopyranoside after permeabilization of the bacteria with sodium dodecyl sulfate (SDS)-chloroform (28). Cells were grown in LB medium up to an optical density of 0.2 at 600 nm and were aliquoted into two neighboring wells, one of which was treated with 0.1 mM paraquat for 1 hr at 37°C. β-Galactosidase activity was measured using microplate reader (Molecular Devices). Cells expressing more β-galactosidase in the presence of paraquat were again grown in 3 ml of LB medium and confirmed for paraquat-inducibility.

Construction of *lacZ* operon fusions

In order to construct a strain containing *fpr*-driven *lacZ* gene on the chromosome, we followed the procedure developed by Simons *et al.* (36). The promoter-*lacZ* fusion of pJC102 plasmid, in which relevant promoter region was cloned in front of *lacZ* gene, was then transferred onto λRZ5 by homologous recombination *in vivo*. The recombinant phage was used to lysogenize GC 4468 strain. Amp[r] Lac[+] colonies were selected and confirmed to be λ lysogens by cross-streaking against λ*cI*

and λ*vir* (35). Single lysogens were furthers elected by measuring the level of β-galactosidase activities.

RNA isolation

Cellular RNA was extracted using RNAzol B (Biotecx Laboratories, Inc.) according to manufacturer's recommendation except that the cells were previously 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'-AACTGCGCGTCGCCGCTTTCA-TCG-3') complementary to sequences of *trp-lac* junction of pRS415 plasmid was synthesized and labeled at 5' end with [γ-^{32}P]ATP. The labeled probe was extended by avian myeloblastosis virus (AMV) reverse transcriptase (Promega) as described by Sambrook *et al.* (32). The resulting cDNAs were analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea.

Results

Isolation of promoter clones inducible by paraquat, a superoxide generator

In order to isolate promoters specifically induced by paraquat, we constructed and screened a random promoter library made with a multicopy promoter-probing plasmid, pRS415, for inducibility by paraquat. The pRS 415 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 detected to be induced more than 2.5-fold by treatment with 0.1 mM paraquat. We determined the nucleotide sequences of these promoter fragments and searched for homologous sequences in the DNA database (GenBank, EMBL) (1). Sequence analysis revealed that one promoter clone was identical to the upstream sequence of *fpr* gene encoding NADPH:ferredoxin (flavodoxin) oxidoreductase (Fig. 1).

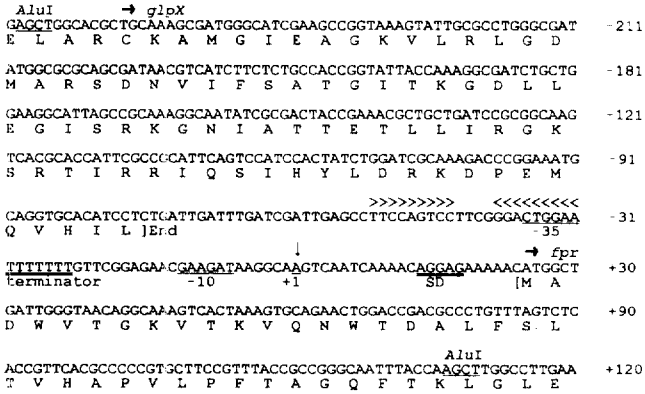

Fig. 1. Nucleotide sequence of promoter fragment of *fpr*. The nucleotide sequences as well as the deduced amino acid sequences of *Alu*I fragments cloned into pRS415 plasmid are shown. The location of the transcription start site determined by primer extension analysis (see Fig. 4) is indicated by an arrow. The putative Shine-Dalgarno sequence (SD) as well as -35 and -10 promoter elements recognized by Eσ^{70} RNA polymerase are underlined. Convergent arrowheads indicate regions of dyad symmetry. Numbering is based on the reference of transcription start site (+1).

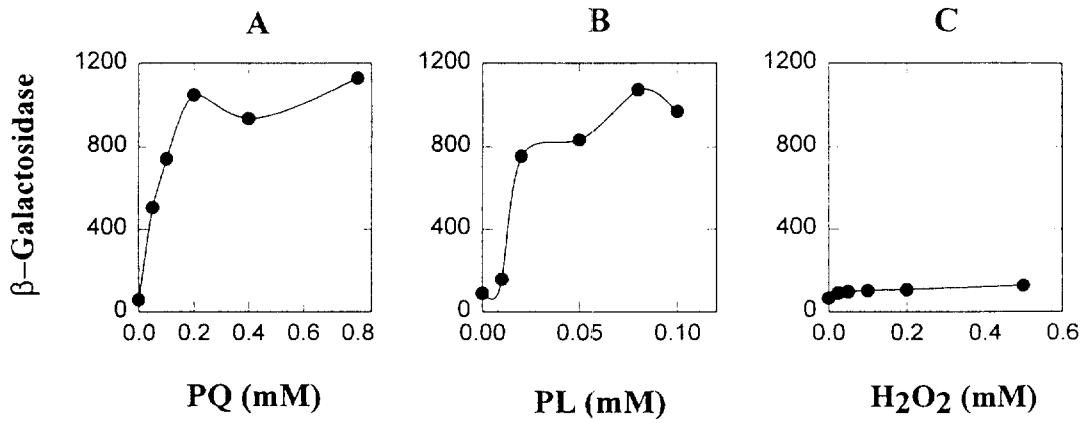

Fig. 2. Induction of β-galactosidase by various treatments in the *fpr-lacZ* fusion stain. Exponentially growing *E. coli* cells containing the *fpr-lacZ* fusion (JC102) were treated with various concentrations of paraquat (PQ) (A), plumbagin (PL) (B), or hydrogen peroxide (C) for 1 hr. β-Galactosidase, expressed in Miller units, was assayed as described in Materials and Methods.

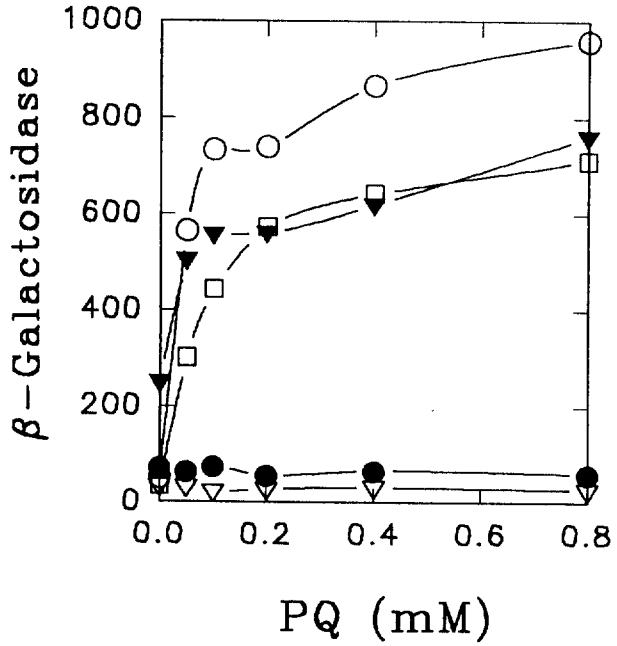

Fig. 3. *soxRS*-dependent induction of β-galactosidase in the *fpr-lacZ* fusion strain by paraquat (PQ). Various regulatory mutant alleles were transduced to the *fpr-laZ* fusion strain (JC102; ○) by phage P1 to generate strain JC202 (△*sox-8*::cat; ●), JC302 (*soxS3*::Tn*10*; ▽), JC402 (*soxR4*::cat; ▼), and JC502 (△*oxyR*::kan; □), respectively. Following treatment for 1 hr with paraquat at various concentrations, β-galactosidase activity (in Miller units) was measured.

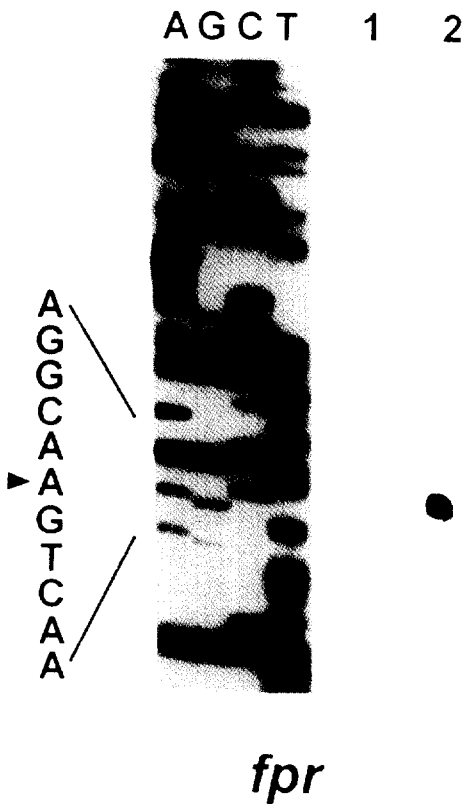

Fig. 4. Mapping 5' ends of *fpr* transcript by primer extension analysis. Radiolabelled primer was extended on the RNA samples (50 μg) isolated from the cells with (lane 2) or without (lane 1) paraquat treatment. DNA sequencing ladders were generated from the purified recombinant pRS415 plasmid (pJC102) with the same primer (lanes A, G, C, and T). The nucleotide sequence of the coding strand is shown on the left, with the specific transcription initiation site indicated by an arrowhead.

Induction of *fpr* gene by redox-cycling agents

The inducibility of *fpr* gene by redox-cycling compounds and other agents was investigated using single-copy *lacZ* fusions (Fig. 2). For this purpose, promoter-*lacZ* operon fusion was constructed as described in Materials and Methods, and the lysogen (JC102) was treated with various redox-cycling agents, known to produce superoxide within the cell, as well as with other agents. *fpr* was induced up to 20-fold at 0.8 mM paraquat (Fig. 2A). This level of induction was comparable to the approximately 10-fold induction of *nfo, zwf, sodA*, and *micF* upon paraquat treatment in the single-copy state (9, 39). Treatment with plumbagin displayed a similar response except that induction occurs at much lower concentrations than paraquat and decreases at higher concentrations, which is 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℃ to 42℃ did not cause a significant induction of the promoter (Fig. 2C; data on ethanol and heat treatment not shown). From these results we were able to confirm that *fpr* is specifically induced by superoxide-generating agents in general.

Effect of *soxRS* or *oxyR* mutation on the paraquat-inducibility of *fpr* gene

Since *fpr* gene was induced by various redox-cycling agents, we examined whether the induction is regulated by *soxRS*. The effect of mutations in *soxR* and *soxS* on the induction of *fpr-lacZ* fusion by paraquat was examined (Fig. 3). △*sox-8*::cat, which is a deletion mutation of *soxR* and *soxS*, and *soxS3*::Tn*10*, which is a null mutation in *soxS*, were transduced into *fpr-lacZ* fusion lysogen. Induction by paraquat at various concentrations was then measured. It was clearly demonstrated in Fig. 3 that *lacZ* expression was not induced by paraquat in *soxR* and *soxS* mutant strains, suggesting that *fpr* is positively regulated by *soxR* and *soxS*. When a *soxR* constitutive mutation (*soxR4*::cat) was transduced into *lacZ* fusion strains, the basal level of β-galactosidase was 5-fold higher than in wild type, again confirming the role of *soxR* as a positive regulator of *fpr*. The mutations in *oxyR* had no significant effect on the inducibility by para-

quat. These genetic data are consistent with the observation that *fpr* was not induced by hydrogen peroxide. Therefore, we conclude that *fpr* gene is a member of the *soxRS* regulon.

Analysis of *fpr* transcript

To precisely locate the 5' end of the *fpr* transcript, primer extension was performed as described in Materials and methods. RNA was isolated from cells harboring pJC102 plasmid with or without paraquat treatment. Only one major primer-extended product was observed (Fig. 4). The induction fold was estimated to be about 20-fold similar to the result by β-galactosidase assay(see Fig. 2). We were able to locate the 5' end of the *fpr* transcript on G precisely 24 nucleotide upstream of the start codon of *fpr* coding region (see Fig. 1). From the location of transcription start site, we were able to tentatively locate -35 and -10 elements of *fpr* promoter, which is overlapped with ρ-independent transcription terminator of the preceding *glpX* gene (see Fig. 1) (38).

Discussion

Ferredoxin (flavodoxin) $NADP^+$ reductase (FNR protein, NADPH : ferredoxin oxidoreductase) was first characterized as a component required for the activation of methionine synthase (15), pyruvate : formate lyase (22), andribonucleotide reductase (6). The gene encoding this enzyme (*fpr*) was clonedrecently in *E. coli* (6). Liochev *et al.* have identified one of their NADPH : PQ^{2+} diaphorases as NADPH : ferredoxin reductase (27). They have shown that thisdiaphorase is a member of the *soxRS* regulon and that upon paraquat treatment, the level of enzyme activity increases. Our genetic data on *fpr* transcription (see Fig. 2 and 3) confirm their results. NADPH : ferredoxin(flavodoxin) reductase has been shown to be one enzymatic route for ferredoxin/flavodoxin reduction in *E. coli*, the other being pyruvate : ferredoxin reductase (7). Reduced ferredoxin/flavodoxin subsequently participate in a variety of metabolic reactions, many of which involve reduction of an iron center; for example, the reductive activation of a number of enzymes includinig methionine synthase, ribonucleotide reductase, and pyruvate : formate lyase (6, 15, 22). Liochev *et al.* have proposed that the ratio of reduced/oxidized doxins affects the redox state of the cell, which could be the signal that SoxR responds to and therefore *soxRS* regulon system is self-regulating (27). Induction of *fpr* as part of the *soxRS* regulon may be useful to the cell because it can be viewed as replacement of oxidation-sensitive pyruvate : ferredoxin reductase by oxidation-stable NADPH : ferredoxin reductase, as an analogy to stable fumarase C replacing sensitive fumarases A and B (26). Very recently Bianchi *et al.* reported that *E. coli* with disrupted *fpr* gene shows increased sensitivity to paraquat, whereas the overproducer is considerably more resistant than the wild-type strain (5). This suggests that NADPH : ferredoxin reductase seems to function in protecting cells from oxidative stress, but the mechanism for the protective effect remains to be elucidated.

We analyzed the *fpr* promoter region in search for putative SoxS binding sequences upstream of transcription start sites. Sequences from -179 to -150 is similar to the proposed SoxS binding sequence (13, 25). However, very recently Jair *et al.* reported that regions from -73 to -46 were protected by MalE-SoxS fusion protein from DNase I cleavage (21). However, these sequences have no obvious homology to the proposed SoxS binding sequence. Moreover, there is no report to genetically define the sequences required for SoxS-mediated transcriptional activation *in vivo*. Further investigation is necessary to identify the target sites for SoxS and to reveal the activation mechanism of SoxS.

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