JOURNAL OF BACTERIOLOGY, May 1995, p. 2673–2678 Vol. 177, No. 10
0021-9193/95/$04.00+0

Isolation of a Novel Paraquat-Inducible (*pqi*) Gene Regulated by the *soxRS* Locus in *Escherichia coli*

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Received 1 November 1994/Accepted 13 March 1995

We have isolated promoters inducible by paraquat, a superoxide radical-generating agent, from *Escherichia coli*, using promoter-probing plasmid pJAC4 (Y. S. Koh and J. H. Roe, Korean J. Microbiol. 31:267–273, 1993). One promoter clone *pqi-5* (*pqi* denotes paraquat-inducible gene) was mapped at 21.8 min on the *E. coli* chromosome by using the Kohara phage library. We constructed an operon fusion of the *lacZ* gene with the *pqi-5* promoter to monitor the expression of the gene in the single-copy state. LacZ expression was induced about 7- to 13-fold by 77 to 780 μM paraquat. Other known superoxide generators such as menadione, phenazine methosulfate, and plumbagin also induced the expression of β-galactosidase in this fusion strain. On the other hand, no significant induction was observed with treatment with hydrogen peroxide, ethanol, and heat shock. Induction of β-galactosidase was significantly reduced by introducing a Δ*sox-8*::*cat* or *soxS3*::Tn*10* mutation into the fusion strain, indicating that *pqi-5* is a member of the *soxRS* regulon. A DNA fragment containing the *pqi-5* promoter was cloned and sequenced from the Kohara phage E2E5. We identified two *pqi-5* open reading frames (ORFs); ORF-A encodes a predicted protein of 342 amino acids, and ORF-B is truncated at the cloning site. The transcription start site from the *pqi-5* promoter was determined by primer extension and S1 nuclease protection analyses. Northern (RNA) and S1 analyses indicated that there are two kinds of *pqi-5* transcript; one covers ORF-A only and the other covers ORF-A and possibly also ORF-B. Levels of both transcripts increased about 20-fold by paraquat treatment. The protein products of ORF-A were detected as 38- and 35-kDa proteins in the membrane fraction.

Reactive oxygen species such as superoxide radical, hydrogen peroxide, and hydroxyl radical naturally arise during normal metabolism in aerobically growing cells as a result of the incomplete reduction of molecular oxygen and also arise from a variety of environmental sources such as ionizing radiation and redox-cycling agents (17, 33). Enteric bacteria have evolved adaptive responses that adjust the expression of numerous genes to the stresses exerted by reactive oxygen (12, 14, 36). In *Escherichia coli*, two separate oxidative stress regulons are known to be triggered by either hydrogen peroxide (the *oxyR* regulon) (9, 10) or redox-cycling agents (the *soxRS* regulon) (14, 15, 39, 40). Both regulons achieve coordinate transcriptional induction of disparate genes and have exhibited no known regulatory overlap with each other so far.

Upon exposure to redox-cycling compounds, which are thought to generate superoxide radicals in a cell, *E. coli* induces the synthesis of about 40 proteins (14, 41). At least nine of these proteins are produced by a regulon controlled by two regulatory genes, *soxR* and *soxS*, constituting a *soxRS* regulon (3, 28, 40, 42, 43). 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*); and NADPH:ferredoxin oxidoreductase (encoded by *fpr*) (8, 15, 16, 23, 24, 40).

Efforts have been made to discover more genes induced by paraquat, since the majority of the paraquat-inducible proteins in *E. coli* have not been identified. One convenient approach is to screen random operon fusions to a reporter gene (e.g., *lacZ*) for inducibility by superoxide stress, using the Mud (Ap *lac*) phage. Using this approach, Kogoma et al. (19) and Mito et al. (26) have isolated several new superoxide-inducible gene fusions regulated by the *soxS* locus. Since the fusion can often lead to inactivation of the fused genes, this approach cannot isolate essential genes in the stressed condition. In order to circumvent this problem, we previously screened a random promoter library with a multicopy promoter-probing plasmid, pJAC4, for inducibility by paraquat and isolated three clones (*pqi-5*, *pqi-15*, and *pqi-34*) (20). Here, we report that *pqi-5* is a novel gene mapped at 21.8 min on the *E. coli* chromosome and is a member of the *soxRS* regulon.

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 as the source for isolating chromosomal DNA and RNA. Mutations in the *soxRS* and *oxyR* genes were introduced by P1 transduction to a *pqi-5*—*lacZ* fusion strain (YS101), selecting for antibiotic resistance associated with the mutation. The *pqi-5* promoter fragment was originally obtained from recombinant pJAC4 plasmid (pJAC4-*pqi5*) which exhibited induction of the reporter gene, β-lactamase, upon paraquat treatment (18, 20).

Media and cell growth. In order to determine the effect of various redox-cycling agents or oxidants, cells were grown in Luria-Bertani medium up to an optical density of 0.2 at 600 nm and were treated with those 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 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. (31). DNA sequencing was done by Sanger's dideoxy chain termination method with Sequenase from United States Biochemicals (32).

Gene mapping. An ordered array of λ-phage clones (21) immobilized onto a sheet of positively charged nylon membrane (Gene Mapping Membrane; Takara Biochemical Inc.) was kindly provided by A. Ishihama, National Institute of Genetics, Mishima, Japan. This membrane was hybridized with a *Sau*3AI frag-

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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*	5
Q358	Kohara library host strain	21
GC4468	Δ(*argF-lac*)*169 rpsL sup*(Am)	38
BW829	GC4468 Δ*sox-8*::*cat*[a]	40
BW831	GC4468 *soxS3*::Tn*10*[b]	40
BW847	GC4468 *soxR4*::*cat*[c]	40
AQ4591	*oxyΔ3*[*oxyΔ3*(*oxyR-butB*)*3*] *argE*::Tn*10*[d]	19
GSO18	*recD1014 lac gal rpsL* Δ*oxyR*::*kan*[e]	2
YS101	GC4468 (λYS101)	This work
YS104	GC4468 (λYS104)	This work
YS201	YS101 Δ*sox-8*::*cat*; P1(BW829) × YS101	This work
YS301	YS101 *soxS3*::Tn*10*; P1(BW831) × YS101	This work
YS401	YS101 *soxR4*::*cat*; P1(BW847) × YS101	This work
YS501	YS101 *oxyΔ3 argE*::Tn*10*; P1(AQ4591) × YS101	This work
YS601	YS101 Δ*oxyR*::*kan*; P1(GSO18) × YS101	This work
BL21(DE3)	Strain expressing T7 RNA polymerase	37
CP367	*polA*(Ts)	C. K. Park
Plasmids		
pRS415	*lacZYA* operon fusion vector, Amp[r]	36
pBR322	Amp[r] Tet[r]	31
pJAC4-*pqi5*	*pqi-5* promoter cloned upstream of *ampC* in pJAC4	20
pBE	2.4-kbp *Bam*HI-*Eco*RI fragment of λE2E5 subcloned in pBR322	This work
pYS101	*pqi-5* promoter cloned upstream of *lacZ* in pRS415	This work
pUC4-KIXX	Plasmid containing kanamycin resistance gene block	Pharmacia Inc.
pBSORF-A	*Pvu*II-*Eco*RI fragment containing ORF-A cloned in pBluescript (KS)	This work
pORFA-kan	1.2-kbp *Sma*I fragment from pUC4-KIXX cloned into the *Pst*I site of pBSORF-A	This work
Phages		
λRZ5	Φ(*bla'-'lacZ*) *lacY*$^+$	R. Zagursky
λYS101	Φ(*pqi-5-lacZ*) *lacY*$^+$ *bla*$^+$	This work
λYS104	Φ(pRS415-*lacZ*) *lacY*$^+$ *bla*$^{+f}$	This work
λ*c*I	Wild-type λ phage	34
λ*vir*	Virulent derivative of λ phage	34
P1*vir*	Virulent derivative of P1 phage	34
λE2E5	Kohara phage clone hybridizing with *pqi-5*	21

[a] Δ*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] *oxyΔ3* is a deletion mutation of *oxyR*.
[e] Δ*oxyR*::*kan* is an insertion mutation of *oxyR*.
[f] This phage was constructed by crossing pRS415 with λRZ5 as a control for basal level expression of β-galactosidase directed by promoterless vector sequences.

ment recovered from plasmid pJAC4-*pqi5* containing the *pqi-5* promoter which was labelled with [α-^{32}P]dATP by using random primers.

Construction of *lacZ* operon fusions. In order to construct a strain containing a *pqi-5*-driven *lacZ* gene on the chromosome, we followed the procedure developed by Simons et al. (35). The *Sau3*AI fragment containing the *pqi-5* promoter region in pJAC4-*pqi5* was cloned into the *Bam*HI site of pRS415 in front of the *lacZ* gene. The fusion was then transferred onto λRZ5 by homologous recombination in vivo. The resultant phage was used to lysogenize the GC4468 strain. Amp[r] Lac$^+$ colonies were selected and confirmed to be λ lysogens by cross-streaking against λ*c*I and λ*vir* (34). Single lysogens were further selected by measuring the level of β-galactosidase activities. β-Galactosidase activity was assayed as described by Miller (25).

RNA isolation. Cellular RNA was extracted with RNAzol B (Biotecx Laboratories, Inc.) according to the manufacturer's recommendations, 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.

Northern (RNA) hybridization analysis. RNA samples were denatured with formamide and formaldehyde, electrophoresed on a 1% agarose gel containing formaldehyde, and transferred to a Hybond-N$^+$ membrane (Amersham Co.) as described by Sambrook et al. (31). The blot was then hybridized with three kinds of *pqi-5* gene fragments (see Fig. 5A) that had been radiolabelled with random primers.

S1 nuclease protection analysis. S1 analysis was performed essentially as described by Berk and Sharp (6). The probes used are shown in Fig. 5A. They were labelled with either [γ-^{32}P]ATP or [α-^{32}P]dATP by standard procedures (31). The S1-protected DNAs were analyzed by electrophoresis on a 5% polyacrylamide gel containing 7 M urea. The amount of DNA in each band was quantitated by measuring radioactivity with a Molecular Imager (Bio-Rad).

Primer extension analysis. A synthetic oligonucleotide (5′-CACTTGCGTCT GTAGGGCTTCCAG-3′) complementary to the sequence between 263 and 286 nucleotides upstream of the translational start site of the *pqi-5* open reading frame A (ORF-A) was synthesized and labelled at the 5′ end with [γ-^{32}P]ATP. The labelled probe was extended by avian myeloblastosis virus reverse transcriptase (Promega) as described by Sambrook et al. (31). The resulting cDNAs were analyzed by electrophoresis on a 6% polyacrylamide gel containing 7 M urea.

Gene disruption. *pqi-5* disruption mutants were obtained by replacing the chromosomal *pqi-5* gene with the in vitro-disrupted one. The kanamycin resistance cassette of pUC4-KIXX (1.2 kbp) (Pharmacia Inc.) was inserted into the *Pst*I site of the *pqi-5* ORF-A gene of pBSORF-A to produce pORFA-kan. This recombinant plasmid was introduced into a temperature-sensitive *polA* mutant (CP367, a kind gift from C. K. Park, Korea Advanced Institute of Science and Technology) and allowed to form cointegrates by selecting ampicillin-resistant colonies at 42°C. The cointegrates were isolated as single colonies and cultured to stationary phase at 30°C for five consecutive times to allow resolution by a second recombination event. The desired mutants, in which the chromosomal *pqi-5* gene had been replaced by the *pqi-5*::*kan* construction, were obtained by selecting Kan[r] Amp[s] colonies. Gene replacement was confirmed by Southern hybridization. The mutated *pqi-5* allele was transduced into MG1655 and GC4468.

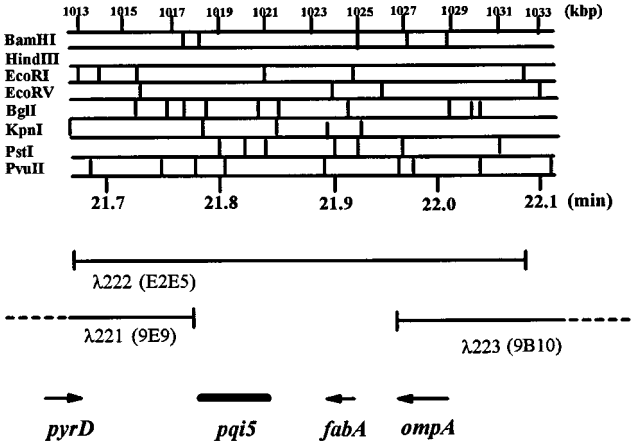


FIG. 1. Map position of *pqi-5* on the *E. coli* chromosome. A cloned *pqi-5* promoter fragment was hybridized to the *E. coli* Kohara library on a gene mapping membrane. The hybridizing region in the positive phage clone (E2E5) was determined as described in the text. The 2.4-kbp *Bam*HI-*Eco*RI fragment which hybridized to the *pqi-5* promoter fragment probe is indicated (*pqi-5*) along with adjacent known genes *pyrD* for dihydroorotate oxidase, *fabA* for β-hydroxydecanoyl thioester dehydrase, and *ompA* for outer membrane protein 3a.

Nucleotide sequence accession number. The DNA sequence of the *pqi-5* region (see Fig. 4) has been registered in the EMBL library under accession number X81561.

RESULTS

Mapping the *pqi-5* gene on the *E. coli* chromosome. In order to identify the *pqi-5* gene, we first tried to locate the position of the cloned *pqi-5* promoter on the *E. coli* chromosome by hybridizing the labelled promoter fragment with the Kohara λ library of the *E. coli* genome (21). The *pqi-5* probe hybridized to a single lambda clone, E2E5, containing *E. coli* DNA located at about 21 min on the chromosome. Further refinement of mapping was done by using various restriction enzymes, and we were able to map *pqi-5* to a precise locus between *pyrD* and *fabA* (Fig. 1). This locus is different from any previously identified loci or genes in the *soxRS* or *oxyR* regulon.

Induction of the *pqi-5* gene by redox-cycling agents. The inducibility of the *pqi-5* gene on the chromosome by redox-cycling compounds and other treatments was investigated. For this purpose, a *pqi-5-lacZ* operon fusion was constructed as described above, and the lysogen (YS101) was treated with various redox-cycling agents, known to produce superoxide

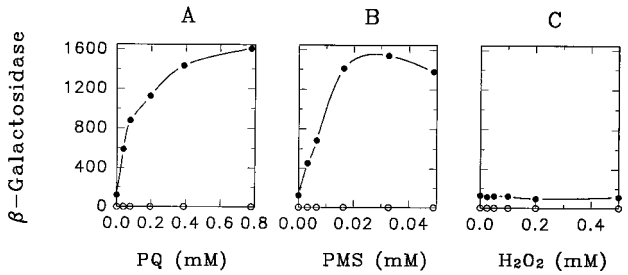


FIG. 2. Induction of β-galactosidase in the *pqi-5-lacZ* fusion strain by various treatments. Exponentially growing *E. coli* cells containing the *pqi-5-lacZ* fusion on the chromosome (YS101; ●) were treated with various concentrations of paraquat (PQ) (A), phenazine methosulfate (PMS) (B), or hydrogen peroxide (C) for 1 h. β-Galactosidase activity, expressed in Miller units, was assayed as described in Materials and Methods. YS104 (○), a promoterless construct, acting as a control, was treated in the same way.

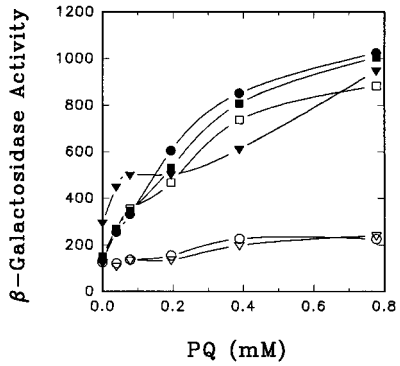


FIG. 3. *soxRS*-dependent induction of *pqi-5-lacZ* by paraquat (PQ). Various regulatory mutant alleles were transduced to the *pqi-5-lacZ* fusion strain (YS101; ●) by phage P1 to generate strains YS201 (Δ*sox-8*::*cat*; ○), YS301 (*soxS3*::Tn*10*; ▽), YS401 (*soxR4*::*cat*; ▼), YS501 (*oxyΔ3 argE*::Tn*10*; □), and YS601 (Δ*oxyR*::*kan*; ■). Following treatment for 1 h with paraquat at various concentrations, β-galactosidase activity (in Miller units) was measured.

radicals within the cell. As shown in Fig. 2, the level of β-galactosidase increased by ninefold at 0.2 mM paraquat and increased further at higher concentrations. Phenazine methosulfate induced *pqi-5* at much lower concentrations than paraquat. At 0.02 mM it increased the level of β-galactosidase by 12-fold. Plumbagin and menadione were effective also (data not shown). Treatment with hydrogen peroxide, ethanol, and heat did not cause any changes (Fig. 2C; data on ethanol and heat treatment not shown). From these results we were able to confirm that *pqi-5* is induced by redox-cycling agents in general.

Effect of *soxRS* or *oxyR* mutation on the paraquat inducibility of *pqi-5*. Since *pqi-5* 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 *pqi-5* by paraquat was examined (Fig. 3). Δ*sox-8*::*cat*, which is a deletion mutation of *soxR* and *soxS*, and *soxS*3::Tn*10*, which is a null mutation of *soxS*, were transduced into the *pqi-5-lacZ* fusion lysogen (YS101). Induction of β-galactosidase by paraquat at various concentrations was then measured. It was clearly demonstrated that *lacZ* expression was not induced by paraquat in these mutant strains, suggesting that *pqi-5* is positively regulated by *soxR* and *soxS*. When a *soxR* constitutive mutation (*soxR4*::*cat*) was transduced into strain YS101, the basal level of β-galactosidase was twofold higher than in the wild type, again confirming the role of *soxR* as a positive regulator for *pqi-5*. The mutations in *oxyR* had no effect on the inducibility by paraquat. These genetic data are consistent with the above-mentioned observation that *pqi-5* was not induced by hydrogen peroxide. Therefore, we conclude that the *pqi-5* gene is a new member of the *soxRS* regulon.

The cloning and nucleotide sequence determination of the *pqi-5* gene. To investigate further the function and regulation of transcription of the *pqi-5* gene, we cloned the DNA fragment containing the *pqi-5* promoter. A 2.4-kbp *Bam*HI-*Eco*RI fragment of lambda E2E5 was subcloned into pBR322 (to produce pBE) and sequenced. The whole nucleotide sequence revealed two ORFs (Fig. 4). The first ORF (ORF-A) is from nucleotides 852 to 1877 and encodes a predicted protein of 342 amino acids and 37,950 Da. The second ORF (ORF-B) is truncated at the *Eco*RI site. Since the N-terminal amino acid sequence of the *pqi-5* gene product is unknown, the beginning of the ORF-A coding sequence was assigned next to a putative Shine-Dalgarno sequence, AAGGAG. The BLAST program (1) was used to search for homology between the predicted

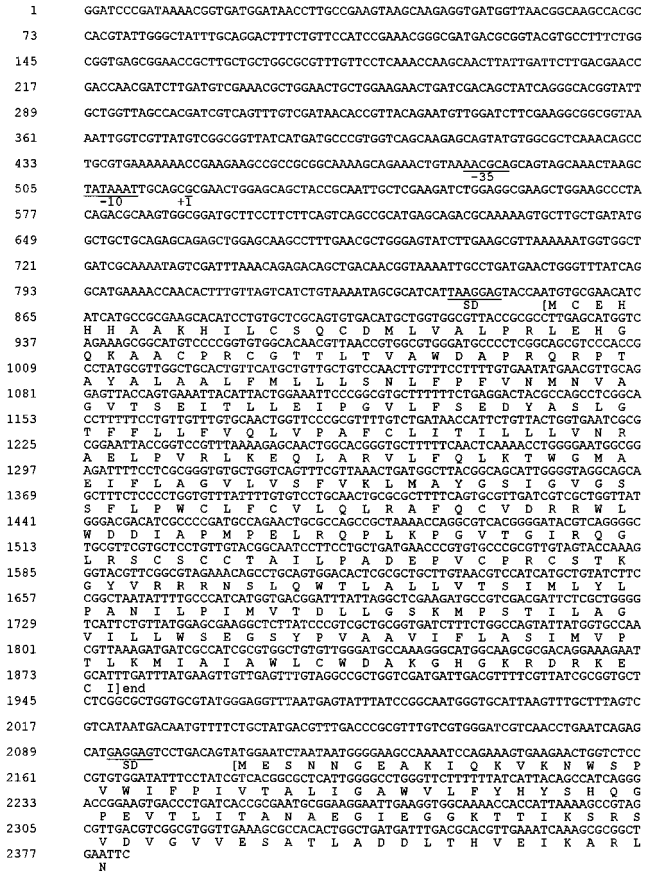


FIG. 4. Nucleotide sequence of 2.4-kbp *Bam*HI-*Eco*RI fragment containing the *pqi-5* gene. The nucleotide sequences as well as the deduced amino acid sequences of the putative ORFs are shown. The first ORF (ORF-A) consists of 342 amino acids, and the second one (ORF-B) is truncated at the *Eco*RI site. The location of the transcription start site determined by S1 and primer extension analyses (Fig. 6) is indicated (+1). The putative Shine-Dalgarno sequence (SD) as well as the −35 and −10 promoter elements recognized by $E\sigma^{70}$ RNA polymerase is underlined.

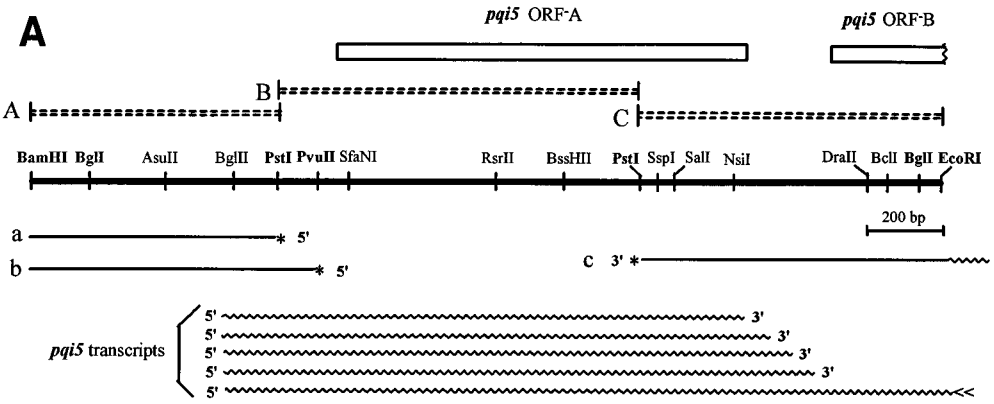
ORF-A protein and proteins of the various protein and translated DNA databases (GenBank, EMBL, PIR, Brookhaven Protein Data Bank, and SWISS-PROT). ORF-A contains 16 cysteine residues and shared some marginal homology with proteins such as alcohol dehydrogenase or metallothioneins that have many cysteines involved in metal binding. The cysteines may play a role in polypeptide folding, multimer formation, or even redox sensing. Some homology was also found with two separate domains of NADH-ubiquinone oxidoreductase from a *Synechococcus* sp. Hydrophobicity analysis revealed that this protein is most likely an integral membrane protein, containing seven putative transmembrane domains (22).

Detection of *pqi-5* transcripts. In order to identify the in vivo transcripts from the *pqi-5* region, Northern hybridization analysis was performed with three separate DNA probes (Fig. 5A, probes A to C) which spanned the entire 2.4-kbp DNA containing the *pqi-5* ORFs. RNAs were prepared from wild-type MG1655 cells with or without paraquat treatment (780 μM) for 1 h. As shown in Fig. 5B, probes A and B detected three transcripts with sizes of about 2.4, 1.3, and 0.6 kb. Probe C detected 2.4- and 1.3-kb transcripts. The small size of the 0.6-kb transcript as well as its lack of hybridization with probe C excludes this transcript from being a candidate for the *pqi-5* ORF-A or ORF-B transcript. The ratio of the quantities of the 2.4- and 1.3-kb RNAs was about 1:1.2. The induction of the 2.4- and 1.3-kb transcripts by paraquat was estimated to be about 13- to 30-fold when probes B and C were used. The 0.6-kb RNA from upstream of ORF-A was also induced significantly by paraquat. In order to determine precisely the location of the 5′ and 3′ ends of the *pqi-5* transcripts, we performed S1 nuclease mapping as well as primer extension.

Endpoint mapping of *pqi-5* transcripts. Two separate DNA probes (Fig. 5A, probes a and b) were used to determine the 5′ end of the *pqi-5* transcripts with S1 nuclease. RNA was isolated from cells harboring the pBE plasmid with or without paraquat treatment. Each probe yielded a single S1-protected fragment when RNA was isolated from cells treated with paraquat (data not shown). The 5′ endpoint of the *pqi-5* transcripts was calculated to be about 333 nucleotides upstream of the start codon of the *pqi-5* ORF-A. This indicated that there is only one major transcription initiation site for both the 1.3- and 2.4-kb *pqi-5* transcripts. The extent of induction of the *pqi-5* transcripts by paraquat was estimated to be about 20-fold when the radioactivity was quantitated with a Molecular Imager. To precisely locate the 5′ end of the *pqi-5* transcripts, primer extension was performed as described above (Fig. 6A). Only one major primer-extended product was observed when the total RNA was prepared from cells treated with paraquat. The level of induction was estimated to be about 20-fold, similar to the result obtained by S1 analysis. Several minor bands were detected when the film was overexposed. We were able to locate the 5′ end of the *pqi-5* transcripts on G precisely 334 nucleotides upstream of the start codon of *pqi-5* ORF-A.

For 3′ end mapping, DNA probe c (Fig. 5A), which was labeled at the 3′ end of the *Pst*I restriction site, was used. This labeled site is located 268 nucleotides upstream from the end of the stop codon for ORF-A. This probe yielded heterogeneous S1-protected fragments which were induced markedly by paraquat treatment (Fig. 6B). The sizes of the protected fragments were about 268, 363, 397, 449, and 770 nucleotides. The shortest fragment indicated the presence of the 3′ end very near the translational stop codon. The midsized transcripts have their 3′ ends at around 95, 129, and 181 nucleotides downstream from the stop codon of ORF-A. The longest transcript, which generated a 770-nucleotide band, encompassed the whole length of the probe up to the *Eco*RI site and therefore must have its 3′ end downstream of the *Eco*RI site. These results enable us to propose that the 1.3-kb transcript detected by Northern analysis corresponds to the transcripts whose 3′ ends are located adjacent to or 95 to 181 nucleotides downstream of the ORF-A stop codon, whereas the 2.4-kb transcript corresponds to the longer one whose 3′ end extends beyond the *Eco*RI cloning site. The relative abundance of the four shorter transcripts (~1.3 kb) and the longest one (~2.4 kb) was estimated to be 1.3:1. This is comparable to the ratio determined by Northern blot analysis. Therefore, we conclude that the *pqi-5* locus in *E. coli* produces two types of transcripts, one encoding ORF-A only and the other encoding ORF-A and possibly ORF-B also.

Gene disruption and expression of the *pqi-5* ORF-A gene. We obtained several disruption mutants of *pqi-5* ORF-A by replacing the wild-type gene with truncated ORF-A containing a kanamycin resistance gene block. The mutants displayed no obvious changes in growth rates in Luria-Bertani medium or in resistance against oxidants such as paraquat, plumbagin, menadione, phenazine methosulfate, and hydrogen peroxide or antibiotics such as tetracycline and chloramphenicol. Expression of the wild-type ORF-A gene on the multicopy plasmid



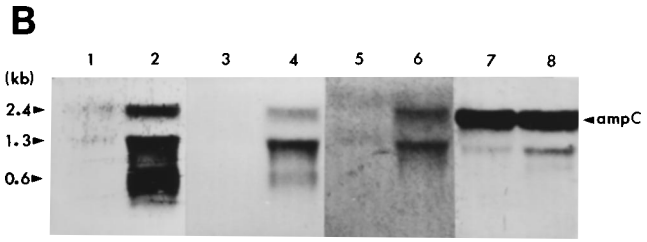


FIG. 5. Detection of *pqi-5* transcripts. (A) DNA probes for Northern and S1 protection analyses. The 2.4-kbp *Bam*HI-*Eco*RI DNA fragment is shown in the middle, with the restriction enzyme sites indicated. The locations of the enzyme sites in boldface type were previously determined by Kohara et al. (21) and were confirmed by us. The probes for Northern analysis (A, B, and C) and S1 protection analysis (a, b, and c) are shown, and the positions of the radioactive labels for the S1 probes are indicated by asterisks. Probe c contained extra DNA derived from the cloning vector pGEM3zf(+) (wavy line). The boundaries of the *pqi-5* transcripts determined by the results shown in Fig. 6 are indicated at the bottom. (B) Northern blot analysis of *pqi-5* transcripts. RNA (100 μg) isolated from exponentially growing *E. coli* MG1655 cells treated with 780 μM paraquat (lanes 2, 4, 6, and 8) or plain buffer (lanes 1, 3, 5, and 7) for 1 h was hybridized with labelled probes (probe A for lanes 1 and 2, probe B for lanes 3 and 4, and probe C for lanes 5 and 6). Transcripts from the *ampC* gene of MG1655 were hybridized with the β-lactamase gene fragment from plasmid pJAC4 as a loading control (lanes 7 and 8). The positions of three transcripts detected by the probes are indicated on the left (approximate sizes are shown).

(pBE) did not cause any changes in resistance against such oxidants or antibiotics either. More systematic examination of the phenotype of the mutants under various culture conditions is necessary in order to define the role of the *pqi-5* gene product under oxidative conditions.

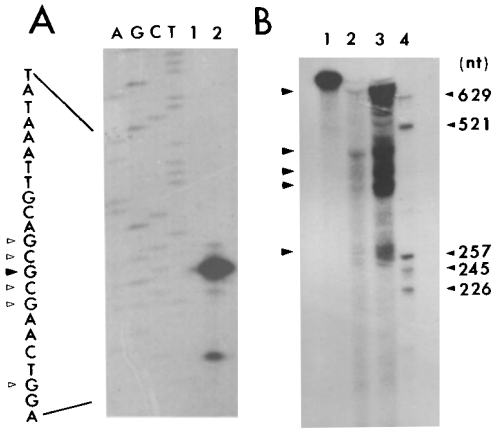


FIG. 6. Mapping 5′ and 3′ ends of *pqi-5* transcripts. (A) Fine mapping of 5′ end of *pqi-5* transcripts by primer extension analysis. Radiolabelled primer (24-mer) was extended on the RNA samples (100 μg) isolated from cells with (lane 2) or without (lane 1) paraquat treatment. DNA sequencing ladders were generated from the purified recombinant pBE plasmid with the same primer (lanes A, G, C, and T). The nucleotide sequence of the coding strand is shown on the left, with the *pqi-5*-specific transcription initiation sites indicated by arrowheads (filled and open arrowheads, major and minor sites, respectively). (B) Mapping 3′ ends of *pqi-5* transcripts by S1 analysis. RNAs isolated from cells with (lane 3) or without (lane 2) paraquat treatment were hybridized with probe c (Fig. 5A) labelled at its 3′ end. One-tenth of the amount of probe used for S1 analysis was loaded on lane 1. The prominent S1-protected bands are indicated by arrowheads. nt, nucleotides.

We detected the protein product of *pqi-5* ORF-A by expressing the gene on vector pET15b with the exclusive T7 promoter expression system. The gene products were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as doublet bands with sizes of 38 and 35 kDa (data not shown). Since the predicted molecular mass of the ORF-A protein is 37,950 Da, the upper band might be the full-sized gene product whereas the lower one might be the processed product. There are two predicted processing sites on the ORF-A polypeptide; one is behind alanine at residue 37, counting from the C terminus, and the other is behind alanine at residue 112, counting from the N terminus. Judging from the small size difference between the doublet bands, it is more likely that the former cleavage site was used. The gene products were detected only in the membrane fraction, confirming the prediction that ORF-A protein is an integral membrane protein.

DISCUSSION

Several genes of the *soxRS* regulon have been mapped on the *E. coli* chromosome. These are *sodA* (88 min), *nfo* (47 min), *zwf* (41 min), *micF* (48 min), *fumC* (35.5 min), *acnA* (28 min), *fpr* (88 min), and *inaA* (48 min) (4, 7, 11, 16, 23, 24, 29, 30, 38). The *soxS* gene, which is regulated by *soxR*, is located at 92 min (3, 15, 40, 42). In addition, two superoxide-inducible (*soi*) genes isolated by Kogoma et al., *soi-28* and *soi-17*/*soi-19*, are located at 47 and 45 to 61 min, respectively (19). The *pqi-5* gene is located at 21.8 min, according to the results of this study, and is a novel operon containing more than one ORF. Recently, Mito et al. isolated eight *soi*::*lacZ* fusions inducible by paraquat under the control of the *soxS* gene (26). Five of

these fusions are located at 6 to 26 min. At present, we cannot tell whether the *pqi-5* gene was represented by any one of these fusions.

A putative promoter was found upstream of the transcriptional start site (Fig. 4). It has the −10 hexamer sequence, which is in good agreement with the consensus sequence recognized by $E\sigma^{70}$. However, the −35 region was very poorly matched with the consensus sequence. Overall, the homology score of this putative *pqi-5* promoter is about 34%, which leads to a prediction that it is a weak promoter (27). This prediction is consistent with the observation that only a very low level of transcripts was present in the cell without paraquat treatment (Fig. 5 and 6). Sequences just upstream of −35 reveal significant homology with known SoxS binding sequences (13).

The lack of any prominent phenotypes in disruption mutants as well as predictable functional domains makes it hard to suggest a role for *pqi-5* in *E. coli* under oxidative conditions. Preliminary results indicate that this gene is substantially induced in the stationary phase (20a), suggesting its role under stressful conditions. Changes in the activity of oxidoreductases, transport functions, survival under conditions of prolonged starvation, and starvation-induced cross protection in disruption mutants are under examination in more systematic ways.

ACKNOWLEDGMENTS

We express our gratitude to D. Touati, B. Weiss, R. W. Simons, G. Storz, and C. K. Park for providing various *E. coli* mutant strains, phages, and plasmids and to A. Ishihama and Y. Kohara for the gene mapping membrane and Kohara phages, respectively.

This work was supported equally by a Genetic Engineering Research Grant from the Ministry of Education and a KOSEF Research Grant to the Research Center for Molecular Microbiology in Korea.

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