

# ***Escherichia coli* pyruvate:flavodoxin oxidoreductase, YdbK - regulation of expression and biological roles in protection against oxidative stress**

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*E. coli* YdbK is predicted to be a pyruvate:flavodoxin oxidoreductase (PFOR). However, enzymatic activity and the regulation of gene expression of it are not well understood. In this study, we found that *E. coli* cells overexpressing the *ydbK* gene had enhanced PFOR activity, indicating the product of *ydbK* to be a PFOR. The PFOR was labile to oxygen. The expression of *ydbK* was induced by superoxide generators such as methyl viologen (MV) in a SoxS-dependent manner after a lag period. We identified a critical element upstream of *ydbK* gene required for the induction by MV and proved direct binding of SoxS to the element. *E. coli ydbK* mutant was highly sensitive to MV, which was enhanced by additional inactivation of *fpr* gene encoding ferredoxin (flavodoxin):NADP(H) reductase (FPR). Aconitase activity, a superoxide sensor, was more extensively decreased by MV in the *E. coli ydbK* mutant than in wild-type strain. The induction level of *soxS* gene was higher in *E. coli ydbK fpr* double mutant than in wild-type strain. These results indicate that YdbK helps to protect cells from oxidative stress. It is possible that YdbK maintains the cellular redox state together with FPR and is involved in the reduction of oxidized proteins including SoxR in the late stages of the oxidative stress response in *E. coli*.

**Key words:** Oxidative stress, *ydbK*, pyruvate:flavodoxin oxidoreductase, superoxide inducible genes, SoxRS regulon

## INTRODUCTION

Reactive oxygen species (ROS) are generated in living cells as inescapable by-products of incomplete reduction of molecular oxygen during normal cellular metabolism (Cui et al., 2012). The generation of ROS is enhanced by the exposure of cells to exogenous stimuli such as ionizing radiation and various chemical oxidants (Cui et al., 2012). Cellular components such as DNA, proteins and lipids are easily oxidized and inactivated through oxidation by ROS (Cui et al., 2012). DNA damage generated by ROS has been implicated in mutagenesis, carcinogen-

esis and aging (Cui et al., 2012; Klaunig et al., 2011). To overcome these deleterious effects, bacteria and eukaryotic cells have evolved defense mechanisms against oxidative stress caused by ROS (Imlay, 2008; Lushchak, 2011).

When exposed to excess ROS, cells respond by expressing a specific set of genes. A bacterial superoxide stress sensor, SoxR, is constitutively expressed but inactive in its reduced form (Li and Demple, 1994; Pomposiello and Demple, 2001). When its [2Fe-2S] cluster is oxidized, SoxR transcriptionally activates the *soxS* gene (Wu and Weiss, 1991; Gaudu and Weiss, 1996). SoxS is a transcriptional activator of the AraC/XylS family and induces the expression of a large number of regulon genes that play critical roles in protecting cells from oxidative stress (Wu and Weiss, 1991; Pomposiello et al., 2001). SoxS-activated genes include superoxide scavenging enzyme (*sodA*), DNA repair enzyme (*nfo*), ferredoxin (flavodoxin)-NADP(H) reductase (*fpr*), transcriptional repressor for iron uptake (*fur*), oxidative stress-resistant isozymes of the TCA cycle (*fumC*, *acnA*) and efflux pump (*acrAB*) (Pomposiello et al., 2001; Martin and Rosner, 2002). As a negative-feedback mechanism, SoxS interferes its own

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Abbreviations: ROS, reactive oxygen species; PFOR, pyruvate:flavodoxin oxidoreductase; MV, methyl viologen; FPR, ferredoxin:NADP(H) reductase; *soi*, superoxide-inducible; Ap, ampicillin; Km, kanamycin; IPTG, isopropyl-β-D-thiogalactopyranoside; CoA, coenzyme A; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; TPP, thiamine diphosphate.

expression by binding of excess SoxS proteins to the promoter region of the *soxS* gene (Nunoshiba et al., 1993). When superoxide stress diminishes, SoxR is reduced by NAD(P)H-dependent reductases (Kobayashi and Tagawa, 1999; Koo et al., 2003), and SoxS is degraded by proteases such as Lon (Griffith et al., 2004). The SoxRS regulon is known to be activated by the superoxide radical, but recent studies show that SoxR is also activated in the absence of superoxide when the cellular redox balance is disrupted (Krapp et al., 2002; Gu and Imlay, 2011). So, it is important to examine whether the SoxRS regulon includes certain reductases to restore and maintain the cellular redox state.

We previously isolated several novel superoxide-inducible (*soi*) genes in *E. coli* (Mito et al., 1993). Among these *soi* genes, *soi-9* was identified to be the *ydbK* gene. A homology search predicted that YdbK is a putative pyruvate:flavodoxin oxidoreductase (PFOR) which catalyzes the oxidation of pyruvate to acetyl-CoA and CO<sub>2</sub> and delivers electrons to flavodoxin (Serres et al., 2001). Flavodoxin is an electron transfer protein with FMN prosthetic groups and often substitutes for ferredoxin, another electron transfer protein with an iron-sulfur center, indicating that flavodoxin can also act as a substrate for the NADPH:ferredoxin oxidoreductases (Osborne et al., 1991). The *ydbK* gene is constitutively expressed in SoxS-overexpressing *E. coli* strains (Mito et al., 1993; Fabrega et al., 2012). *E. coli ydbK* mutants are highly sensitive to superoxide-generating agents (Mito et al., 1993; Eremina et al., 2010), indicating that YdbK plays a critical role in the defense against oxidative stress in *E. coli*. Furthermore, most PFORs are oxygen-labile (Williams et al., 1987; Wahl and Orme-Johnson, 1987; Kunow et al., 1995). Previously, it had been shown that *ydbK* gene and downstream *ompN* gene are co-expressed in the same mRNA transcript and activated by SoxS, and the authors suggested that this SoxS-dependency could be indirect via an unknown factor (Fabrega et al., 2012).

In this study, we found that *ydbK* was superoxide-inducible and obtained evidence for direct regulation of the gene expression by SoxS. We also found a critical role of YdbK in defense against oxidative stress. It is possible that YdbK and FPR function in restoring reducing equivalents in the late stages of the superoxide-induced response.

## MATERIALS AND METHODS

**Bacterial strains and plasmids** *E. coli* cells were grown in Luria-Bertani (LB) medium at 37°C with vigorous shaking. When necessary, ampicillin (Ap), kanamycin (Km) and chloramphenicol was added to the medium at 100, 50 and 30 µg/ml, respectively. *E. coli* BW25113, its derivatives and plasmid clones pNT3-*ydbK*, pNTR-SD and ASKA *soxS* were supplied by National BioResource

Project. A plasmid pMal-stop was constructed from pMal-c2 by introducing a stop codon in front of the *lacZ* coding region using oligonucleotides listed in Table 1. Other *E. coli* K-12 strains and plasmids used in this study are listed in Table 2 and Table 3, respectively (Carlioz and Touati, 1986; Nunoshiba and Demple, 1993; Datsenko and Wanner, 2000; Baba et al., 2006; Casadaban et al., 1980).

### Construction of the *E. coli ydbK fpr* double mutant

To remove the Km-resistance gene from the genome, JW3895 (*fpr::Km*) was transformed with pCP20, which carried FLP recombinase (Cherepanov and Wackernagel, 1995), and clones sensitive to both Km and Ap were selected (*fpr* mutants without a kanamycin cassette and pCP20). The *ydbK::Km* construct was then transduced from JW1372 to its *fpr* mutant strain using the P1 phage (Saka et al., 2005; Kitagawa et al., 2005).

### Construction and mutagenesis of gene fusion constructs with *lacZ*

The upstream sequences of *ydbK* (−562 to +17, −119 to +17 and −102 to +17) and *soxS* (−156 to +17) were amplified by PCR using primers for introducing *EcoRI* and *BamHI* restriction sites at the end of the product (Table 1). PCR products were purified using a High Pure PCR Cleanup Micro Kit (Roche). pMC1403 and purified PCR products were digested with *EcoRI* and *BamHI*, purified by agarose gel electrophoresis and recovered using a UltraClean 15 DNA Purification Kit (MO BIO). The ligation reaction was performed using a DNA Ligation Kit Ver. 2.1 (Takara), and the products were used to transform competent cells of the *E. coli* strain XL10-GOLD (Table 2). Transformants were selected on LB plates supplemented with Ap. Plasmid DNA was purified from Ap-resistant colonies using a Wizard minicolumn (Promega) and sequenced by a fluorescent dideoxy method (Smith et al., 1986).

Mutagenesis in the *ydbK* promoter sequence was performed by using primers that contain mutations (Table 1).

**Purification of Male-SoxS protein** The *soxS* coding sequence was amplified by PCR using primers to introduce *EcoRI* and *HindIII* restriction sites at the ends of the product (Table 1). pMal-c2 (New England BioLabs) and purified PCR products were digested with *EcoRI* and *HindIII*.

Purification of the Male-SoxS protein was conducted according to the method of Fawcett and Wolf (1994) with a slight modification. Overnight cultures were diluted 100-fold in LB medium supplemented with Ap (50 µg/ml) and glucose (2 mg/ml), and grown at 37°C until the OD<sub>600</sub> reached 0.4–0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM and incubation was continued for 4 h. Cells were pelleted by centrifugation at 7,000 rpm at 4°C, washed with PBS and

Table 1. Oligonucleotides used in this study

Sequence	Used for
CCGAATTCATAAGCTGGCGGATTTAATG	pMC1403- <i>ydbK'</i> :: <i>lacZ</i> , substrate for gel shift assay
CCGGATCCCCGTCAATAGTAATCATATG	Common reverse primer for <i>ydbK'</i> :: <i>lacZ</i> reporters substrate for gel shift assay (-562)
CACCTGACGTCTAAGAAAC	Sequencing for pMC1403
GGCGAAAGGGGATGTGCT	Sequencing for pMC1403
CCGAATTCCTTCTTCTCTGATCTTCAAG	for gel shift assay (-270)
CCGAATTCCTGATGTGGGGGACACAAAAG	pMC1403- <i>ydbK'</i> :: <i>lacZ</i> -119, substrate for gel shift assay (-119)
CCGAATTCAGCGAAAATGCAGAAGAAAG	pMC1403- <i>ydbK'</i> :: <i>lacZ</i> -101, substrate for gel shift assay (-102)
CCGAATTCGCGCGCAGCAATTTTCGTG	for gel shift assay (-50)
CCGAATTCGCGCAATGTAAGGGTGTGCAT	for gel shift assay (-20)
CCGGATCCGCGTAATGAATGTTTTGATC	Substrate for gel shift assay (-562 to -120)
GCGCGAATTCATGTCCCATCAGAAAATTATTCAG	pMal- <i>soxS</i>
TAATAAGCTTTTACAGGCGGTGGCGATAATC	pMal- <i>soxS</i>
GGTCGTCAGACTGTGCGATGAAGCC	Sequencing primer for pMal-c2
AATTCTGAGGATCCTCTAGAGTCGACCTGCAGGCA	pMal-stop
AGCTTGCCTGCAGGTCGACTCTAGAGGATCCTCAG	pMal-stop
ATGAATTCGCCACACCGCTGCGTTTCGC	pMC1403- <i>soxS'</i> :: <i>lacZ</i>
ATGGATCCATTTTCTGATGGGACATAAA	pMC1403- <i>soxS'</i> :: <i>lacZ</i>
ATGAATTCGGGGACACAAAAGCGAAAATGCAGAAGAAAGCC	pMC1403- <i>ydbK'</i> :: <i>lacZ</i> -112
ATGAATTCGGGGACACAAAAGCGAAAATATAGAAGAAAGCC	pMC1403- <i>ydbK'</i> :: <i>lacZ</i> -#1-1
ATGAATTCGGGGACACCCCAGCGAAAATGCAGAAGAAAGCC	pMC1403- <i>ydbK'</i> :: <i>lacZ</i> -#1-2 (#2-2)
ATGAATTCGGGGACATTTGAGCGAAAATGCAGAAGAAAGCC	pMC1403- <i>ydbK'</i> :: <i>lacZ</i> -#1-3
ATGAATTCGGGGATACAAAAGCGAAAATGCAGAAGAAAGCC	pMC1403- <i>ydbK'</i> :: <i>lacZ</i> -#2-1

Table 2. Bacterial strains used in this study

Strain	Genotype	Reference
GC4468	as BW581 but $\Delta(LAC4169)$ <i>rpsL sup (Am)</i>	Carlioz and Touati (1986)
BW831	as GC4468 but <i>soxS::Tn10</i>	Tsaneva and Weiss (1990)
TN521	as GC4468 but $\Delta$ <i>soxRS</i> $\lambda$ ( <i>soxR<sup>+</sup> soxS'::lacZ</i> ) <i>Km<sup>R</sup></i>	Nunoshiba and Demple (1993)
XL10-Gold	<i>Tet<sup>R</sup> <math>\Delta</math>(mcrA)183 <math>\Delta</math>(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F<sup>+</sup>proAB lacIqZAM15 Tn10 (Tet<sup>R</sup>) Amy Cam<sup>R</sup></i>	Obtained from Stratagene
BL21 (DE3)	<i>F- ompT hsdSB (rB- mB-) gal dcm (DE3)</i>	Obtained from Invitrogen
BW25113	<i>lacI<sup>q</sup> rrrnB<sub>T14</sub> DlacZ<sub>WJ16</sub> hsdR514 DaraBAD<sub>AH33</sub> DrhaBAD<sub>LD78</sub></i>	Datsenko and Wanner (2000)
JW4023	as BW25113 but <i>soxS::Km</i>	Baba et al. (2006)
JW1372	as BW25113 but <i>ydbK::Km</i>	Baba et al. (2006)
JW3895	as BW25113 but <i>fpr::Km</i>	Baba et al. (2006)
BW25113 <i>ydbK fpr</i>	as JW1372 but $\Delta$ <i>fpr</i>	This study

frozen at  $-20^{\circ}\text{C}$  before use. Cells were resuspended in 10 volumes of 1 x buffer A (20 mM HEPES (pH 7.4) containing 1 mM EDTA, 200 mM NaCl, 1 mM dithiothreitol (DTT)) and disrupted by sonication at  $4^{\circ}\text{C}$ . The lysates were centrifuged at 14,000 rpm for 10 min at  $4^{\circ}\text{C}$  and the supernatant was kept in fresh tubes. The cell pellets were resuspended in 5 volumes of buffer A, disrupted and centrifuged at 14,000 rpm for 10 min at  $4^{\circ}\text{C}$ . The super-

natants were mixed and centrifuged at 14,000 rpm at  $4^{\circ}\text{C}$  for 30 min. The supernatant was subsequently loaded on a 10-ml column packed with amylose resin (New England BioLabs). The column was washed twice with buffer A, and eluted with elution buffer (buffer A containing 20 mM maltose). Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). FactorXa (1% w/w, New England BioLabs) was added to the frac-

Table 3. Plasmids used in this study

Plasmid name	Features	Reference
pMC1403	Amp <sup>R</sup> <i>lacZ'</i>	Casadaban et al. (1980)
pMC1403- <i>ydbK'::lacZ</i>	Amp <sup>R</sup> <i>ydbK'::lacZ'</i>	This study
pMC1403- <i>ydbK'::lacZ</i> -119	Amp <sup>R</sup> <i>ydbK'::lacZ'</i>	This study
pMC1403- <i>ydbK'::lacZ</i> -101	Amp <sup>R</sup> <i>ydbK'::lacZ'</i>	This study
pMC1403- <i>ydbK'::lacZ</i> -#1-1	Amp <sup>R</sup> <i>ydbK'::lacZ'</i>	This study
pMC1403- <i>ydbK'::lacZ</i> -#1-2 (#2-2)	Amp <sup>R</sup> <i>ydbK'::lacZ'</i>	This study
pMC1403- <i>ydbK'::lacZ</i> -#1-3	Amp <sup>R</sup> <i>ydbK'::lacZ'</i>	This study
pMC1403- <i>ydbK'::lacZ</i> -#2-1	Amp <sup>R</sup> <i>ydbK'::lacZ'</i>	This study
pMC1403- <i>soxS'::lacZ</i>	Amp <sup>R</sup> <i>soxS'::lacZ'</i>	This study
pCP20	Amp <sup>R</sup> Cm <sup>R</sup> <i>flp</i>	Cherepanov and Wackernagel (1995)
pNT3- <i>ydbK</i>	Amp <sup>R</sup> <i>ydbK</i>	Saka et al. (2005)
pNTR-SD	Amp <sup>R</sup>	Saka et al. (2005)
ASKA <i>soxS</i>	Cm <sup>R</sup> <i>soxS</i>	Kitagawa et al. (2005)
pMal-c2	Amp <sup>R</sup> <i>malE-lacZα</i>	NEB
pMal-c2- <i>soxS</i>	Amp <sup>R</sup> <i>malE-soxS</i>	This study
pMal-c2-stop	Amp <sup>R</sup> <i>malE</i>	This study

tions exhibiting peak protein content and incubated for 20 h on ice. Then, an equal volume of 2 x buffer A containing 80% glycerol and 2 mM phenylmethylsulfonyl fluoride (PMSF) was added. Samples were stored at -20°C prior to use. Protein concentrations of undigested samples were determined using a Bradford assay with BSA as the standard.

MalE protein was purified from *E. coli* cells harboring pMal-stop with same procedures.

**Assay for pyruvate oxidoreductase activity** All buffers were freed from oxygen by flushing with nitrogen for at least 2 min immediately before use. *E. coli* cells were cultured aerobically in LB medium and suspended in extraction buffer (50 mM Tris-HCl (pH 7.5) containing 20% ethylene glycol, 1 mM MgCl<sub>2</sub>, 0.1 mM thiamine diphosphate (TPP), 10 mM thiosulfate, 50 mM β-mercaptoethanol and 1 mM DTT). Cells were disrupted by sonication at 4°C. The lysates were centrifuged at 14,000 rpm for 30 min at 4°C and the supernatants (crude extracts) were kept on ice. The reaction mixture (5 ml) contained 100 mM Tris-HCl (pH 7.5), 5 mM MgSO<sub>4</sub>, 6 mM DTT, 0.1 mM TPP, 0.2 mM CoA, 10 mM pyruvate and 1 mM MV. After oxygen was removed by aspiration for 10 min, the crude extract was added to the reaction mixture. PFOR activity was assayed at room temperature by monitoring the reduction of MV. The increase in absorbance at 578 nm was measured in anaerobic Thunberg tubes (φ18 mm).

**Assay for sensitivity to MV** *E. coli* cells were grown in LB medium at 37°C to a stationary phase, washed and diluted 10-fold with PBS. Sterilized paper strips (0.5 ×

12 cm) were put in the cell suspensions, kept at room temperature for 5 min and then placed on LB plates (10.4 × 14.4 cm) containing MV with a linear gradient of concentrations (0–0.8 mM or 0–0.2 mM for the *ydbK fpr* double mutant). After the paper strips were removed, the plates were incubated at 37°C for about 18 h, and the zone of growth inhibition was determined to estimate survival.

**Assay for aconitase activity** Aconitase activity was measured according to the method described by Skovran and Downs (2000) with a slight modification. Cells were grown in LB medium overnight and diluted 100-fold in 50 ml of fresh LB medium. The cultures were incubated until the OD<sub>600</sub> reached 0.5. After treatment with 100 μg/ml of MV for 60 min, the cells were harvested, washed and resuspended in 4 ml of buffer B (150 mM Tris-HCl (pH 8.0) containing 12.5 mM citrate and 1.5 mM NaCl). The cells were lysed by sonication at 4°C and the lysates were centrifuged at 14,000 rpm for 5 min at 4°C. The extracts were mixed with 20 mM isocitrate and aconitase activity was assayed at room temperature by monitoring the increase in absorbance at 240 nm after addition of the crude extract. Protein concentrations were determined using the Bradford assay with BSA as the standard, and specific activity was calculated as the change in absorbance/min/mg of protein.

**β-galactosidase assay** The overnight cultures were diluted 100-fold into fresh LB medium unless otherwise stated. The β-galactosidase activity of whole cell extracts was determined as described by Miller (Miller, 1972). The activity was estimated as units/OD<sub>600</sub>.

**Gel shift assay** DNA fragments were amplified by PCR using the primers listed in Table 1. Reverse primers were end-labeled with [ $\gamma$ - $^{32}$ P]-ATP using T4 polynucleotide kinase. The DNA-binding reaction for the gel shift assay was performed in 30  $\mu$ l of reaction mixture (24 mM HEPES (pH 7.9), 5 mM Tris, 20 mM NaCl, 50 mM KCl, 1.4 mM EDTA, 0.4 mg/ml BSA, 18% glycerol, 1 mM DTT, 500  $\mu$ g of calf thymus DNA, 5–10 fmol of substrate duplex oligonucleotide and 0–5 pmol of purified SoxS or 5 pmol purified Male). The reaction mixture was incubated on ice for 15 min and loaded on prerun native acrylamide gels. Gels were run against TGE buffer (25 mM Tris-HCl (pH 8.3), 190 mM glycine and 100 mM EDTA) at 300 V for 1 min, and then at 125 V for 3 h at 4°C.

## RESULTS

### The product of the *ydbK* gene has PFOR activity

Computational analysis has predicted that *E. coli* YdbK is PFOR (Serres et al., 2001). Because the PFOR was extremely labile to oxygen, its enzymatic activity and the regulation of its gene expression are not well understood. In this study, we attempted to characterize the enzymatic activity of YdbK *in vivo*. We measured the PFOR activity in crude extracts prepared from various *E. coli* cells by monitoring the reduction of MV. MV effectively substitutes for flavodoxin as an electron acceptor in pyruvate oxidation (Kaihoavaara et al., 1998). In the extract from the *E. coli ydbK* mutant, the oxidoreductase activity decreased to about 40% (Fig. 1). On the other hand, a

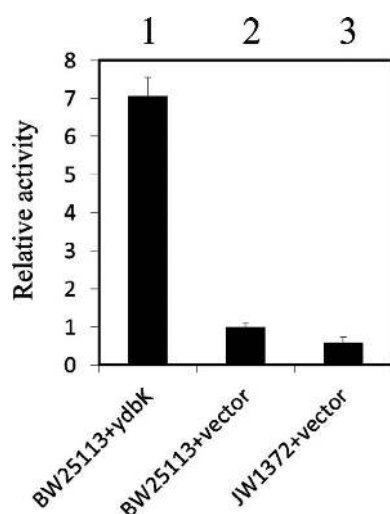


Fig. 1. Pyruvate:flavodoxin oxidoreductase (PFOR) activity of the *ydbK* gene product. PFOR activity in extracts from various *E. coli* cells. The activity was measured by monitoring the reduction of MV and normalized to that measured in extract from wild-type cells harboring the supporting vector. Bars indicate standard deviations based on the results of three independent experiments. Lane 1, BW25113/pNTR-*ydbK*; lane 2, BW25113/pNTR-SD (supporting vector); lane 3, JW1372 (*ydbK::Km*)/pNTR-SD.

highly elevated level of pyruvate oxidoreductase activity was detected in the crude extract from *E. coli* cells harboring a YdbK-expressing plasmid (Fig. 1). The PFOR activity increased about 7-fold. These results indicate that the *E. coli ydbK* gene product is PFOR.

### The *fpr ydbK* double mutant exhibits hypersensitivity to MV

*E. coli* with mutations in both *ydbK* and *fpr* genes displays abnormal sensitivity to MV (Eremina et al., 2010; Bianchi et al., 1995). The *fpr* gene is also regulated by SoxS and encodes a ferredoxin (flavodoxin)-NADP(H) reductase (FPR) (Krapp et al., 2002). It has been predicted that YdbK also delivers reducing equivalents to flavodoxin (Serres et al., 2001). Hence we constructed a *fpr ydbK* double mutant and characterized its phenotypes. The double mutant exhibited slow growth in LB medium under aerobic conditions compared with wild-type and single mutant strains, and sometimes aggregated in overnight cultures (data not shown). To assess MV sensitivity, overnight cultures were plated on agar plates containing a linear gradient of MV, and subjected to survival assays. The double mutant was more sensitive to MV than the *fpr* or *ydbK* single mutant (Fig. 2, Table 4).

### Decreased aconitase activity in *E. coli ydbK* mutant treated with MV

*E. coli* has two aconitases, AcnA and AcnB (Gruer and Guest, 1994). AcnB is the major aconitase and *acnB* gene was activated early in the exponential phase (Gruer et al., 1997). The active center of aconitase B, which contains an 4Fe-4S cluster, is labile to superoxide, therefore, aconitase activity is considered to be an indicator of oxidative stress in *E. coli*. To elucidate the *in vivo* functions of YdbK under oxidative stress, we measured the aconitase activity in wild-type and *ydbK* mutant strains incubated with or without 100  $\mu$ g/ml MV for 1 h. The aconitase activity was decreased by MV in both strains, and the decrease was greater in MV-treated

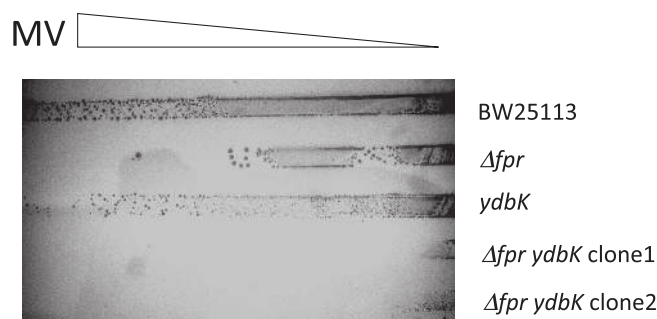


Fig. 2. Sensitivity of various strains of *E. coli* to MV. Sensitivity of *E. coli* BW25113 and its derivatives was determined by incubating the cells on LB plates containing MV with a gradient of concentration (0–0.2 mM) at 37°C for about 18 h. Three separate experiments were done and a representative result are shown.

Table 4. Zone of growth inhibition (mm)

Genotype	Zone of growth inhibition (mm)				
	Expt. 1	Expt. 2	Expt. 3	Mean	S.D.
wild-type	33	20	23	25.3	6.8
<i>ydbK</i>	60	55	50	55.0	5.0
<i>fpr</i>	100	94	94	96.0	3.5
<i>ydbK fpr</i> (clone 1)	123	103	120	115.3	10.8
<i>ydbK fpr</i> (clone 2)	117	126	107	116.7	9.5

*E. coli* cells were grown at 37°C to a stationary phase, washed and diluted 10-fold with PBS. Sterilized paper strips (0.5 × 12 cm) were put in the cell suspensions and then placed on LB plates (10.4 × 14.4 cm) containing MV with a linear gradient of concentrations, followed by incubation at 37°C for about 18 h. The zone of growth inhibition was determined to estimate survival.

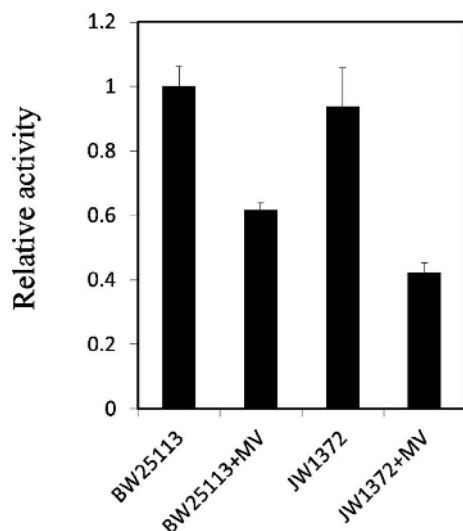


Fig. 3. Aconitase activity in wild-type and *ydbK* mutant strains. *E. coli* cells were grown in LB medium and treated as described in Experimental Procedures. Bars indicate standard deviations based on the results of three independent experiments. The activity level were 0.227 and 0.213 (decrease in absorbance)/min/mg protein for wild-type and *ydbK* mutant strains without MV treatment, respectively.

*ydbK* mutant (54.9%) than MV-treated wild-type strain (38.3%) (Fig. 3).

AcnA is an oxygen-resistant isozyme and induced by superoxide in a SoxS-dependent manner (Gardner and Fridovich, 1991; Varghese et al., 2003). However, the induction of *acnA* gene is slower and the induction level is lower than other superoxide-inducible genes (Lu et al., 2005). These facts suggest that MV-induced *acnA* expression did not completely rescue the loss of AcnB activity caused by MV and that the decrease in the AcnB activity was greater than the induction of AcnA activity after treatment with MV.

### SoxS-dependent induction of *ydbK::lacZ* fusion gene

To investigate the regulatory mechanisms of

expression of *ydbK* gene, we used a gene fusion construct with *lacZ* gene as a reporter. The resultant pMC1403/*ydbK::lacZ* plasmid contained the 562-bp upstream region and the first several N-terminal amino acids of the YdbK protein, fused to the *lacZ* gene of the plasmid vector in frame. We introduced the plasmid into *E. coli* GC4468 and GC4468*soxS* and assayed the  $\beta$ -galactosidase activity in these cells 1 h after treatment with various concentrations of MV. The expression of the *ydbK::lacZ* fusion gene was induced by MV, which was eliminated by the *soxS* gene disruption (Fig. 4A). Furthermore, SoxS overexpression by incubating *E. coli* cells with 0.4 mM IPTG for up to 90 min enhanced the expression of *ydbK::lacZ* without MV (Fig. 4B). Recent studies showed that SoxRS regulon genes are also induced in response to hydrogen peroxide ( $H_2O_2$ ) (Semchyshyn et al., 2005). In this study, however, we observed that  $H_2O_2$  did not induce the expression of *ydbK::lacZ* fusion gene (data not shown). Furthermore, we also obtained the results that the response of *ydbK::lacZ* to MV was significantly eliminated in *E. coli* by disruption of *soxR* gene (data not shown). These results indicate that transcriptional activation of the *ydbK* gene is markedly dependent on the function of SoxS. It was also found that the induction of *ydbK::lacZ* occurred after a lag time (Fig. 4B).

Next, to identify the region critical for the MV response, we generated a series of 5' deletions of the upstream region of the *ydbK* gene fused to *lacZ* gene. Cultures were treated with 10  $\mu$ g/ml (32  $\mu$ M) of MV for up to 1 h. The *lacZ* reporter could respond to MV treatment when fused to the 562-bp and 119-bp upstream region of the *ydbK* gene, but not when fused to the 101-bp region (Fig. 4, C and D), indicating that the sequence between -119 and -102 is critical for the MV response. The expression of *ydbK::lacZ* increased after a lag time of more than 20 min of exposure to MV (Fig. 4C).

### Binding of SoxS to the *ydbK* promoter region *in vitro*

Members of the AraC family share low solubility (Egan and Schleif, 1994; Rodgers and Schleif, 2009). Male is a very effective solubilizing fusion partner (Kapust and Waugh, 1999). Male-SoxS was previously shown to be able to recognize the SoxS-binding sequence (Fawcett and Wolf, 1994). Hence, we purified the Male-SoxS protein (Fig. 5A) and confirmed by SDS-PAGE. Fig. 5A indicates that we have purified Male-SoxS protein successfully, and the results of *in vitro* assay were not due to other contaminated proteins.

Prior to the *in vitro* analysis, we investigated whether overexpression of Male-SoxS by incubation with IPTG induces expression of the *ydbK* gene *in vivo*. We introduced pMal-c2-*soxS* or pMal-c2-stop into the *E. coli soxS* mutant and measured YdbK activity in the cell extract. As shown in Fig. 5B, we could detect approximately 10-fold higher activity of PFOR in *E. coli* overexpressing

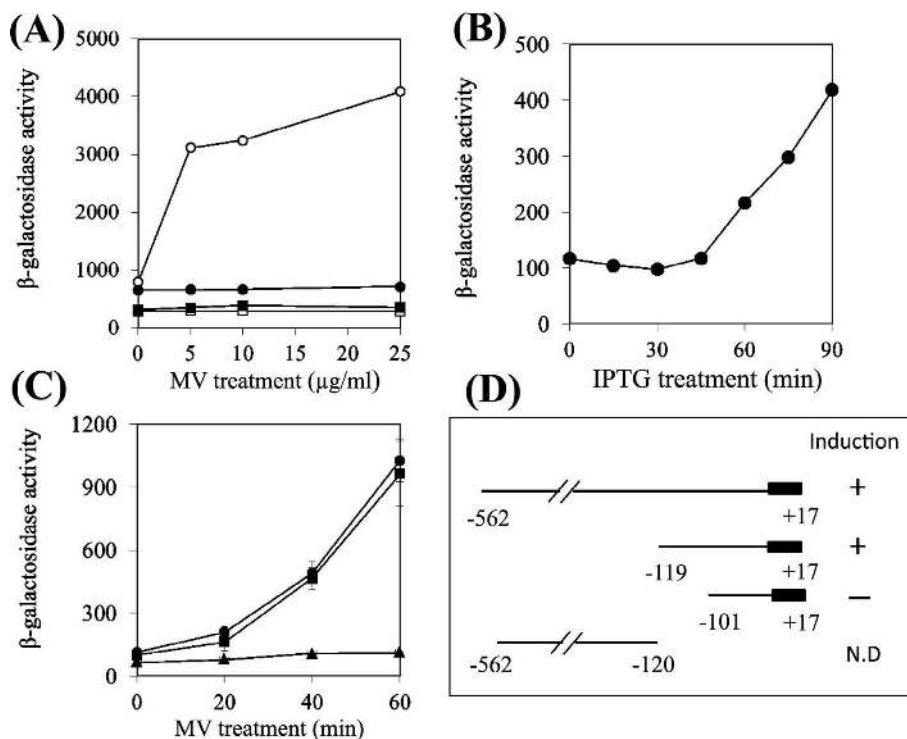


Fig. 4. SoxS-dependent expression of the *ydbK*::*lacZ* fusion gene. (A) SoxS-dependent expression of the *ydbK*::*lacZ* fusion gene. Exponentially growing *E. coli* cells containing *ydbK*::*lacZ* were treated with MV for 1 h, then assayed for  $\beta$ -galactosidase activity. -○-, GC4468/pMC1403-*ydbK*::*lacZ*; -●-, GC4468*soxS*/pMC1403-*ydbK*::*lacZ*; -■-, GC4468*soxS*/pMC1403. (B) The induction of *ydbK*::*lacZ* by overexpression of SoxS. SoxS was artificially induced by IPTG in *E. coli* GC4468*soxS* harboring ASKA *soxS*, then assayed for  $\beta$ -galactosidase activity. (C) Effect of the deletion of the upstream sequences on the induction of *ydbK* expression by MV. Exponentially growing *E. coli* cells containing *ydbK*::*lacZ* were treated with 10  $\mu\text{g/ml}$  MV for up to 1 h. *E. coli* GC4468 strains that possess pMC1403-derived plasmids; -●-, -562 to +17; -■-, -119 to +17; -▲-, -101 to +17. (D) Schematics of the series of 5' and 3' deletions of the upstream region of the *ydbK* gene.

Male-SoxS than in control *E. coli* cells. Furthermore, a high level of the 128-kDa protein was also observed in Male-SoxS-overexpressing *E. coli* cells by SDS-PAGE (data not shown). These results indicate that Male-SoxS can also induce the expression of *ydbK* *in vivo*.

However, the Male-SoxS showed a weak binding activity to the promoter of *ydbK* gene *in vitro*. So, we digested purified Male-SoxS with Factor Xa (1% w/w) and used the mixture in the presence of PMSF. Purified Male was used as a negative control.

A gel shift assay was conducted with  $^{32}\text{P}$ -labeled duplex oligonucleotides prepared by PCR using primers listed in Table 1. As shown in Fig. 6A, SoxS formed a complex with DNA fragments that contain the sequence from -562 to +17 (fragment I), but such binding was not observed with Male. With fragment I, two complexes with different sizes were observed as the amount of SoxS increased. Similar binding properties of SoxS have been also reported in *micF*, *zwf*, *nfo* and *sodA* (Li and Demple, 1994). Oligomerization of SoxS is unlikely because it binds as a monomer (Jair et al., 1996), suggesting that there are multiple SoxS-binding sites in the *ydbK* promoter. This is consistent with the fact that SoxS

could also form a complex with a DNA fragment containing the sequence between -562 and -120 (data not shown). However, because the deletion of this secondary binding site did not influence the induction of *ydbK*::*lacZ* fusion by MV (Fig. 4C), this site is not involved in the MV response.

When we tested DNA fragments that contained the sequence from -119 to +17 (fragment II), SoxS formed two complexes with different size, while only single complex was observed with DNA fragments that contained the sequence from -101 to +17 (fragment III) (Fig. 6A). Importantly, SoxS also formed complex with DNA fragments that contained the sequence from -50 to +17 (Fig. 6B, fragment IV). Since -35 hexamer that is recognized by RNAP is known to overlap or lie the downstream of soxbox, deletion of sequence from -119 to -102 did not remove -35 hexamer of the downstream binding site.

Since SoxS did not form complex with DNA fragments that contained the sequence from -20 to +17 (Fig. 6B, fragment V), indicating that at least part of downstream binding site is overlapped at the region from -49 to -21.

To examine a possibility of contamination of other proteins that bind *ydbK* promoter sequence, we performed a

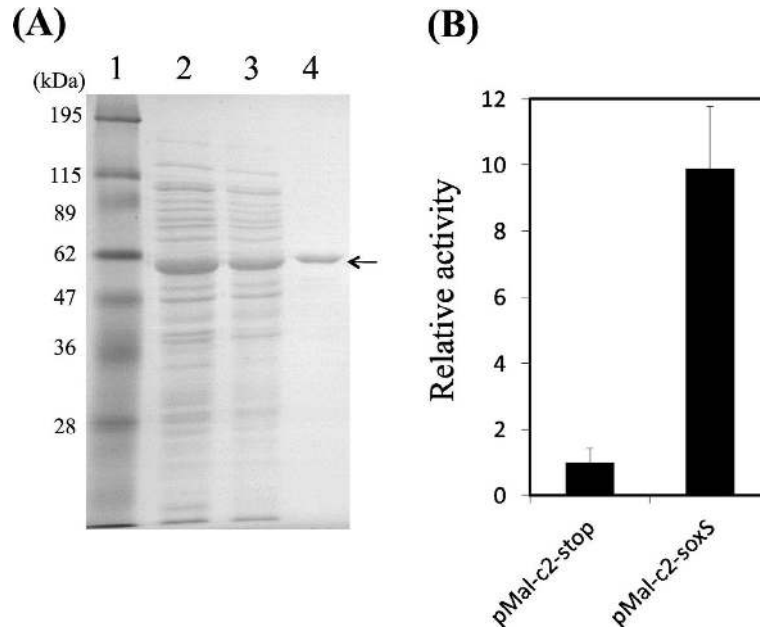


Fig. 5. Purification and functional analysis of MalE-SoxS. (A) SDS-PAGE analysis of purified proteins. Overexpression of MalE-SoxS was induced in strain BL21 (DE3) with 0.1 mM IPTG and the protein was purified on an amylose resin column. Lane 1, Pre-stained protein markers; lane 2, whole cell extract; lane 3, crude extract; lane 4, purified MalE-SoxS (55 kDa). (B) The pyruvate oxidoreductase activity in extract from MalE-SoxS-expressing *E. coli* strains. Bars indicate standard deviations based on the results of three independent experiments. A plasmid pMal-c2-soxS or pMal-c2-stop was introduced into *E. coli* JW4023 (*soxS::Km*). Pyruvate oxidoreductase activity was measured as described in MATERIALS AND METHODS.

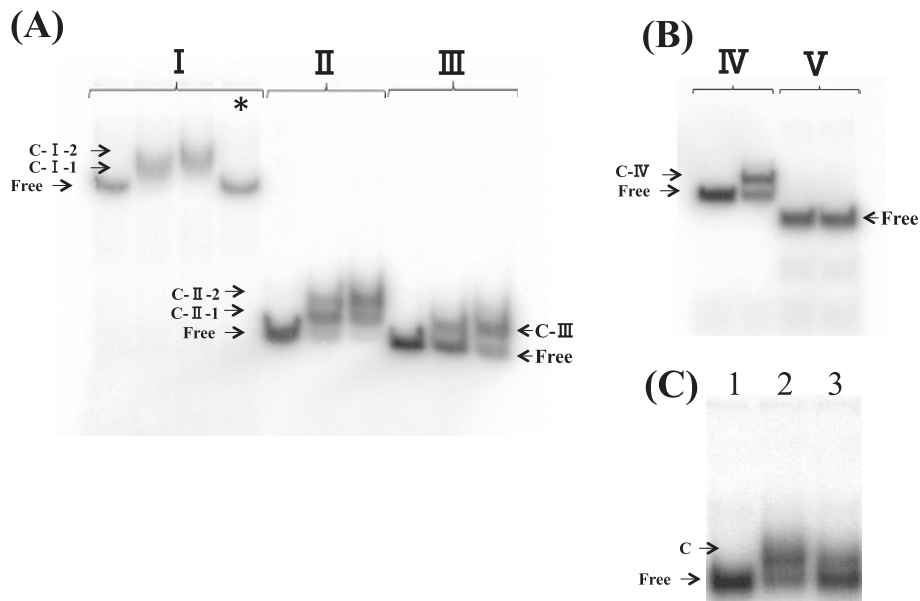


Fig. 6. Binding of SoxS to the DNA fragments *in vitro*. (A) Gel shift assay of SoxS-binding to the *ydbK* promoter. DNA fragments were incubated with increasing amounts of purified SoxS (0, 2.5 and 5 pmol) or MalE (5 pmol, indicated by \*) and the binding was analyzed by electrophoresis.; (I), -562 to +17; (II), -119 to +17; (III), -101 to +17. (B) DNA fragments containing the *ydbK* promoter and upstream regions were incubated with purified SoxS (0 or 5 pmol) and the binding was analyzed by electrophoresis. ; (IV), -50 to +17; . (V), -20 to +17. (C) DNA fragments containing the *zwf* promoter and upstream regions were incubated with purified SoxS (0 or 5 pmol) and the binding was analyzed by electrophoresis.; lane 1, no enzyme; lane 2, only non-specific competitor; lane 3, additional 60 ng *ydbK* fragment. The positions of the free probe and complexes are indicated.



competition assay. We used  $^{32}\text{P}$ -labeled *zwf* upstream sequence (-300 to +17) as a probe that contains a SoxS-binding site (Fawcett and Wolf, 1994), and added unlabeled *ydbK* upstream sequence with similar length (-270 to +17) as a competitor. When 60 ng of *ydbK* fragment was added, significant inhibitory effect was observed (Fig. 6C). All samples used contained 500 ng of calf thymus DNA to stabilize SoxS protein. More than 1,200 ng of calf thymus DNA was required to obtain the same extent of inhibitory effect. These results indicate that SoxS specifically binds to *ydbK* promoter sequence.

**Mutation analysis of the *ydbK* promoter** Previously reported consensus sequences (“Soxbox”) for SoxS-binding have three characteristics (Griffith and Wolf, 2001); (1) an invariant A at position 1, (2) two recognition elements (RE1 and RE2), to which two HTH motifs of SoxS bind, and (3) an AT-rich spacer between the two REs (Fig. 7). Promoters can be divided in two classes based on the position and orientation of the soxbox in relation to the -35 hexamer.

In the region of the upstream sequence of *ydbK*, there were two putative SoxS-binding sites (Fig. 7). In the non-template strand, which is in the reverse orientation, there is an element (soxbox 1) that is similar to the Soxbox of the *rob* promoter (Schneiders and Levy, 2006). This element shares a 7nt stretch that includes the RE1 element (GCAT). The same 7nt stretch is also found in the Soxbox of the *nfnB* promoter (Barbosa and Levy, 2002). This putative element did not have the invariant A of *rob* and *nfnB*. There is no similarity with the consensus sequence (CAAA) in the RE2 element. Instead of a string of A’s, this putative binding site has a T-rich

sequence on its 3’ side. In the template strand (in the forward orientation), we could detect a 20-bp element (soxbox 2) that shared 12/17 specified nucleotides with the consensus sequence. This element also lacked the invariant A and the first nucleotide of the RE1 element was A instead of G. This stretch (ACAC) was also found in the soxbox of the *inaA* promoter (Martin and Rosner, 2011).

To clarify which element is the genuine binding site, we introduced several mutations and investigated the effect on the expression of *ydbK*::*lacZ* fusion and on the binding of MalE-SoxS. The results are shown in Table 5. The oligonucleotides #1-1 and #2-1 contained a mutation in the RE1 element of soxbox 1 and soxbox 2, respectively. #1-2 carried a mutation in the RE2 element of soxbox 1, and #2-2, which had the same sequence as #1-2, carried a mutation in the AT-rich spacer of soxbox 2. In #1-3, the RE2 element of soxbox 1 was modified to generate the consensus sequence CAAA. In the case of soxbox 2, mutation of RE2 was constrained because it probably overlaps with the -35 hexamer.

In the case of #1-2 and #1-3, *ydbK*::*lacZ* fusions were not induced by MV, and MalE-SoxS did not bind to the DNA fragments. On the other hand, the expression of *lacZ* and the binding of MalE-SoxS were not affected by the mutations introduced in #1-1. These results indicate that at least 3 T’s located in the RE2 of soxbox 1 are important but soxbox 1 was not a genuine binding site.

Because #2-2 had the same sequence as #1-2 but was located in the opposite strand, the 3 A’s located in the spacer region must be critical. Moreover, a single mutation in the RE1 of soxbox 2 (ACAC to ATAC) was deleterious, indicating that soxbox 2 was the genuine binding site.

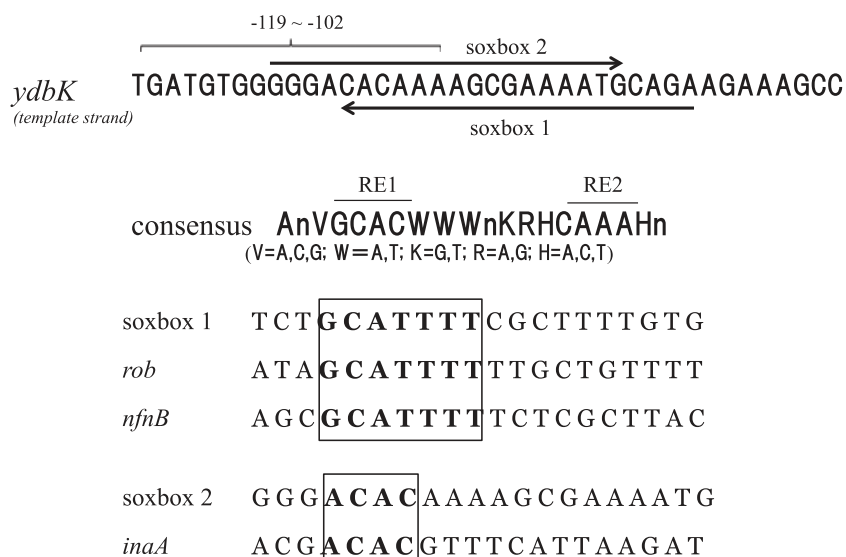


Fig. 7. Sequence analysis of the upstream sequence of *ydbK*. The region includes two putative “soxboxes”. The orientation of the “soxboxes” is depicted by arrows. The previously reported consensus sequence and soxboxes of SoxS binding sites with similarities with the putative binding sites are also shown.

Table 5. Mutagenesis of soxboxes

	Feature	Sequence	Induction (%)	Binding of MalE-SoxS
soxbox 1	non-template strand, -107 to -88	TCTGCATTTTCGCTTTTGTG	100.0	+
#1-1	mutation in RE1	TCTATATTTTCGCTTTTGTG	93.6	+
#1-2	#1-2 mutation in RE2	TCTGCATTTTCGCTGGGGTG	11.0	—
#1-3	RE2→consensus	TCTGCATTTTCGCTCAAATG	15.3	—
soxbox 2	template strand, -111 to -92	GGGACACAAAAGCGAAAATG	100.0	+
#2-1	mutation in RE1	GGGATACAAAAGCGAAAATG	12.7	—
#2-2	mutation in spacer	GGGACACCCCAGCGAAAATG	15.3	—

Various *E. coli* cells were treated with MV and the  $\beta$ -galactosidase activity of whole cell extract was determined as described by Miller (1972). The DNA-binding reaction for the gel shift assay was performed as described in EXPERIMENTAL PROCEDURES. Samples were loaded on prerun 6% native acrylamide gels. Gels were run against TGE buffer (25 mM Tris-HCl (pH 8.3), 190 mM glycine and 100 mM EDTA) at 300 V for 1 min, and then at 125 V for 3 h at 4°C.

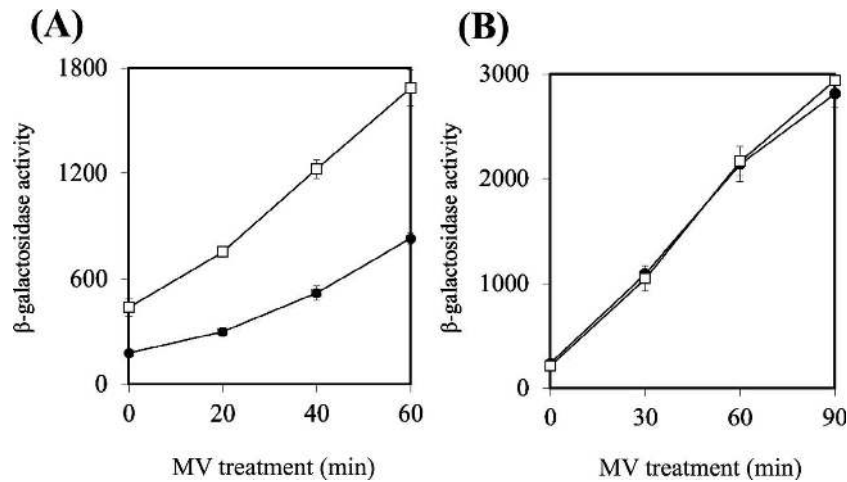


Fig. 8. Effects of YdbK levels on the expression of *soxS':lacZ* fusion gene induced by MV. (A) The expression of *soxS':lacZ* was assayed in the *E. coli ydbK fpr* mutant. Exponentially growing cells of *E. coli* were treated with 2  $\mu$ g/ml of MV for up to 1 h, then assayed for  $\beta$ -galactosidase activity. -●-, BW25113/pMC1403 *soxS':lacZ*; -□-,  $\Delta ydbK fpr$ /pMC1403-*soxS':lacZ*. (B) The expression of *soxS':lacZ* in YdbK-overexpressing *E. coli* cells. Exponentially growing cells of *E. coli* were treated with 10  $\mu$ g/ml of MV for up to 90 min. -●-, TN521/pNT3-*ydbK*; -□-, TN521/pNTR-SD.

We could not find the downstream soxbox, and introduction of mutations was constrained because of its influence on other important elements such as Shine-Dalgarno sequence.

#### Effects of YdbK levels on the induction of SoxS

The results described above suggest that the cellular redox state is disturbed in the *E. coli ydbK* mutant. Hence, we investigated whether the induction of SoxS was influenced by the level of YdbK. A *soxS':lacZ* fusion gene was constructed on a plasmid and introduced into a *ydbK*-proficient or deficient *E. coli* strain. In the presence of 10  $\mu$ g/ml MV, the expression level of *soxS':lacZ* was not significantly affected by a single mutation in the *ydbK* gene (data not shown). Furthermore, previous

studies showed that a single mutation of *fpr* did not affect the induction of the SoxRS regulon (Krapp et al., 2002). The *E. coli ydbK fpr* double mutant was highly sensitive to MV (Table 4), indicating their mutual backup interactions. Therefore, we measured the expression of *soxS':lacZ* in the double mutant. Overnight culture was diluted appropriately and grown until the OD<sub>600</sub> reached 0.2, followed by treatment with 2  $\mu$ g/ml (6.4  $\mu$ M) of MV for up to 1 h. Compared with the wild-type strain, the expression of *soxS':lacZ* was remarkably higher in the mutant strain (Fig. 8A). Even in the absence of MV, significantly higher expression was observed in the mutant strain, indicating that disruption of both *ydbK* and *fpr* was enough to disturb the cellular redox state in *E. coli* under aerobic conditions.

We next investigated the effect of YdbK overexpression. pNT3-*ydbK* or the supporting vector was introduced into *E. coli* TN521 carrying *soxS::lacZ* and an intact *soxR* gene in  $\lambda$  lysogen. The overnight culture was diluted appropriately and grown until the OD<sub>600</sub> reached 0.4–0.5. IPTG was then added at a final concentration of 0.5 mM and further incubated for 3 h. The cells were treated with 10  $\mu$ g/ml (32  $\mu$ M) of MV for up to 90 min. Although a high level of production of the 128-kDa protein was detected by SDS-PAGE (data not shown), the expression of *soxS::lacZ* was not changed by overexpression of YdbK (Fig. 8B).

## DISCUSSION

**YdbK has a PFOR activity** Serres et al. (2001) has predicted that the *E. coli* YdbK protein is PFOR. However, its enzymatic activity is still unclear. In this study, we characterized the enzymatic activity of YdbK *in vitro* and *in vivo*. Purification of YdbK was so difficult because of its extreme sensitivity to oxygen. Furthermore, in the extract from the *E. coli ydbK* mutant, the oxidoreductase activity decreased to only 40% compared with wild-type cells (Fig. 1). The residual activity is probably due to other pyruvate-oxidizing enzymes such as pyruvate formate-lyase and pyruvate dehydrogenase complex (Blaschkowski et al., 1982; Knappe et al., 1984). Hence, we attempted to characterize the enzymatic activity in crude extract prepared from *E. coli* harboring a YdbK-overexpressing plasmid. We detected a highly elevated level of PFOR activity in crude extract from YdbK-overexpressing *E. coli* (Fig. 1). It is therefore concluded that the *E. coli ydbK* gene encodes PFOR.

***ydbK* is induced by superoxide and directly regulated by SoxS** The *ydbK* gene was induced by MV in a SoxS-dependent manner under aerobic conditions (Fig. 4). The expression of *ydbK* (previously named *soi-9*) was induced by MV, menadione bisulfite and exposure to high pressure oxygen (Mito et al., 1993), but not by H<sub>2</sub>O<sub>2</sub>, indicating that it is a cellular response to superoxide radical. In *Salmonella enterica*, a *ydbK*-like gene was also shown to be induced by MV in a SoxS-dependent manner and the mutant of this gene was sensitive to MV (Pomposiello and Demple, 2000). In addition, flavodoxin A and flavodoxin B, the redox partners of YdbK, are members of the regulon (Gaudu and Weiss, 2000).

MarA and Rob also belong to the AraC/XylS family and are known to activate transcription of a common set of genes (Martin and Rosner, 2011). Salicylate and 2,2'-dipyridyl specifically activate the Mar- and Rob-regulon, respectively (Pomposiello and Demple, 2001; Martin and Rosner, 2004; Rosner et al., 2002). However, in the *E. coli soxS* mutant strain, salicylate (5 mM) and 2,2'-dipyridyl (5 mM) did not induce *ydbK::lacZ* expression significantly (data not shown), indicating that MarA and Rob are not involved in *ydbK* expression. YdbK's induction seems to be specific to oxidative stress.

In this study, it is concluded that the sequence between –119 and –102 upstream of the *ydbK* promoter is critical for the MV response. The argument was derived from the following results; (1) The *lacZ* reporter could respond to MV treatment when fused to the 562-bp and 119-bp upstream region of the *ydbK* gene, but not when fused to the 101-bp region (Fig. 4C). (2) SoxS formed two complexes with DNA fragment that contain the sequence between –119 to +17, but only one complex was formed when the fragment containing the sequence from –101 and +17 was used.

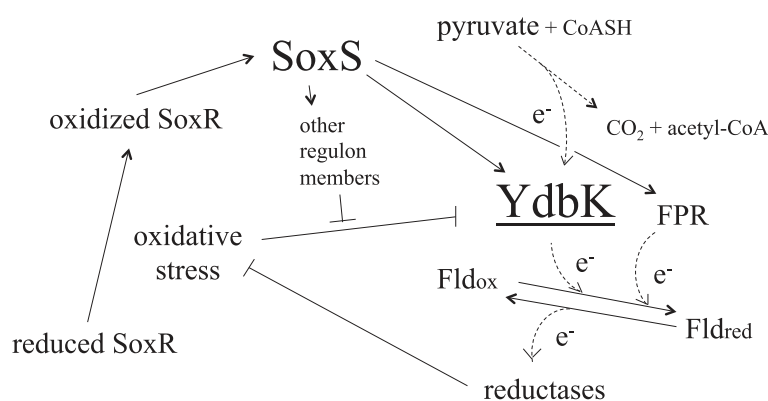


Fig. 9. A model for the role of YdbK in prevention of oxidative stress. Under oxidative stress, SoxR is activated and induces the expression of SoxS, which in turn activates the expression of *ydbK*. YdbK oxidizes pyruvate, and liberated electrons ( $e^-$ ) are transferred via flavodoxin to various reductases, which overcome oxidative stress and restore a reducing environment in cells. Other components of the SoxRS regulon possibly protect YdbK from oxidative inactivation. Fld, flavodoxin. “ $\uparrow$ ” indicates activation of induction, and “ $\downarrow$ ” indicates inhibition of induction. Dashed arrows indicate the current of electrons.

### The expression of *ydbK* was induced after a lag period

It has been shown that the extent of SoxR activation is greater in FPR-overexpressing cells, because FPR consumes NADPH, an important reducing source, and SoxS induces FPR only after a lag period of several minutes to avoid this problem (Krapp et al., 2002). *ydbK::lacZ* was also expressed after a lag period (Fig. 4). Both YdbK and FPR transfer an electron to flavodoxin. These results indicate that, in addition to loss of NADPH and oxidative deactivation of YdbK, excess reduction of flavodoxin in the presence of redox-cycling agents may need to be avoided.

**Protection against oxidative stress** The *fpr ydbK* double mutant was more sensitive to MV than the *fpr* or *ydbK* single mutant (Table 4). These results indicate that FPR and YdbK play antioxidative roles in independent but related pathways. FPR functions to restore reducing equivalents and/or reactivates damaged enzymes (Fig. 9). Giró et al. (2006) reported that FPR-deficient mutants were still able to reactivate Fe-S clusters and suggested the existence of back-up routes. Because aconitase activity was reduced in YdbK-deficient cells (Fig. 3), YdbK may function in this back-up route (Fig. 9).

From the view point of iron metabolism, it seems reasonable that oxidative stress induces YdbK expression. It has been shown that superoxide liberated iron from storage proteins or iron-sulfur clusters (Keyer and Imlay, 1996; McCormick et al., 1998). Free iron easily reacts with H<sub>2</sub>O<sub>2</sub> and generates hydroxyl radicals that damage cellular components such as DNA. SoxRS regulon also includes Fur, the global repressor of iron uptake (Zheng et al., 1999). It is known that, under iron-deficient conditions, ferredoxin-NADP<sup>+</sup> oxidoreductase is reduced by flavodoxin instead of by ferredoxin, an iron-containing electron donor (Osborne et al., 1991). So, YdbK, a redox partner of flavodoxin, may be necessary to reserve reducing equivalents under such conditions.

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