The EMBO Journal Vol. 22 No. 11 pp. 2614–2622, 2003

A reducing system of the superoxide sensor SoxR in *Escherichia coli*

Mi-Sun Koo, Joon-Hee Lee, So-Yeon Rah, Won-Sik Yeo, Jin-Won Lee[1], Kang-Lok Lee, Young-Sang Koh[2], Sa-Ouk Kang[1] and Jung-Hye Roe[3]

Laboratory of Molecular Microbiology and [1]Laboratory of Biophysics, School of Biological Sciences, and Institute of Microbiology, Seoul National University, Seoul 151-742, Korea

[2]Present address: Department of Microbiology, Cheju National University Medical School, Cheju 690-756, Korea

[3]Corresponding author
e-mail: jhroe@plaza.snu.ac.kr

M.-S.Koo and J.-H.Lee contributed equally to this work

The *soxRS* regulon functions in protecting *Escherichia coli* cells against superoxide and nitric oxide. When SoxR is activated by oxidation of its [2Fe–2S] cluster, it increases the synthesis of SoxS, which then activates its target gene expression. How the oxidized SoxR returns to and is maintained in its reduced state has been under question. To identity genes that constitute the SoxR-reducing system, we screened an *E.coli* mutant library carrying a chromosomal *soxSp::lacZ* fusion, for constitutive mutants. Mutations mapped to two loci: the *rsxABCDGE* operon (named for reducer of SoxR) that is highly homologous to the *rnfABCDGE* operon in *Rhodobacter capsulatus* involved in transferring electrons to nitrogenase, and the *rseC* gene in the *rpoE–rseABC* operon. In-frame deletion of each open reading frame in the *rsxABCDGE* operon produced a similar constitutive phenotype. The double mutation of *rsx* and *rseC* suggested that *rsxABCDGE* and *rseC* gene products act together in the same pathway in reducing SoxR. Electron paramagnetic resonance analysis of SoxR and measurement of re-reduction kinetics support the proposal that *rsx* and *rseC* gene products constitute a reducing system for SoxR.

Keywords: ferredoxin motif/iron–sulfur cluster/redox regulation/reduction of SoxR/superoxide stress

Introduction

Cells are equipped with a defense system to cope with the harmful effect of reactive oxygen species encountered during aerobic growth or environmental stress conditions. Bacteria have evolved sophisticated molecular mechanisms to sense the oxidant levels and to activate protective systems. Much progress has been made in understanding these mechanisms, especially in *Escherichia coli*. *Escherichia coli* cells utilize two transcription factors OxyR and SoxR to sense the oxidants and then induce various genes against oxidative stress that are involved in removing oxidants, repairing damaged cell components and maintaining reducing conditions in the cell (Bauer *et al*., 1999; Storz and Imlay, 1999; Pomposiello and Demple, 2001). Whereas OxyR responds primarily to H_2O_2 and nitrosylating agents, SoxR is known to respond primarily to superoxide and nitric oxide (Nunoshiba *et al*., 1993; Hausladen *et al*., 1996; Storz and Imlay, 1999).

SoxR is a 17 kDa transcriptional regulator of the MerR family (Amabile-Cuevas and Demple, 1991; Wu and Weiss, 1991). It forms a dimer in solution, with each monomer containing a [2Fe–2S] cluster (Hidalgo *et al*., 1995; Wu *et al*., 1995). For SoxR, the [2Fe–2S] cluster is not required for initial folding, or for maintaining its structure or DNA-binding activity. Instead, the [2Fe–2S] cluster undergoes reversible one-electron oxidation and reduction and thereby modulates its activity (Hidalgo and Demple, 1994; Wu *et al*., 1995). When the [2Fe–2S] center of SoxR is in the fully oxidized state (Fe^{3+}–Fe^{3+}), SoxR can activate the transcription of its only known target gene *soxS*. When it is reduced by one electron (Fe^{2+}–Fe^{3+}), the ability to activate *soxS* transcription is lost (Ding *et al*., 1996; Gaudu and Weiss, 1996). Thus, the Fe–S cluster serves as an elaborate redox-sensitive switch for SoxR activation to modulate *soxS* gene transcription. The mechanism of target gene activation by oxidized SoxR involves promoter distortion, being similar to that by MerR in response to Hg^{2+} (Ansari *et al*., 1995; Hidalgo and Demple, 1997) and to that by another MerR family member ZntR in response to Zn^{2+} (Outten *et al*., 1999). The oxidative activation of SoxR is distinguished from another well-known Fe–S-containing transcription factor Fnr, which contains a [4Fe–4S] cluster. Fnr loses its DNA-binding activity upon oxidation, due to disassembly of its Fe–S cluster (Lazazzera *et al*., 1996; Popescu *et al*., 1998).

It has been estimated that the [2Fe–2S] clusters in SoxR are >90% reduced during aerobic growth, as monitored by electron paramagnetic resonance (EPR) analysis of *E.coli* cells overproducing SoxR protein (Ding and Demple, 1997; Gaudu *et al*., 1997). Upon exposure of *E.coli* cells to paraquat, the EPR signal from the reduced [2Fe–2S] cluster in SoxR disappears rapidly, but returns within a few minutes after the withdrawal of the oxidative stress (Ding and Demple, 1997). The *in vivo* kinetics of the activation and inactivation of SoxR monitored by the increase and decrease in *soxS* mRNA level parallel the change in the oxidation and reduction state of SoxR monitored by EPR (Ding and Demple, 1997).

A question to be resolved is how the oxidized SoxR is reduced rapidly upon removal of oxidative stress condition and how the reduced state of SoxR is maintained against auto-oxidation during aerobic growth. It has been hypothesized that activation of the SoxR system by redox cycling agents might be mediated via limiting reductase activity through depletion of NADPH, a possible

Table I. Bacterial strains used in this study

Strains	Relevant genotype	Reference
GC4468	(*argF-lac*) *169 rpsL sup*(Am)	Laboratory collection
MS1343	GC4468, *soxSp::lacZ*, Amp^r	This study
MC1306	MS1343, *rseB::*Tn*10*	This study
MC1392	MS1343, *rsxB::*Tn*10*	This study
MC1323	MS1343, *rsxB::*Tn*10*	This study
MC1353	MS1343, *rsxB::*Tn*10*	This study
MS11	GC4468, Δ*rsxC::kan*	Koo (2001)
MC1393	MS1343, Δ*rsxC::kan*	This study
MC1394	MS1343, Δ*rsxBC::kan*	This study
BW900	GC4468, *soxR9::cat*	Wu and Weiss (1991)
BW829	GC4468, Δ*sox-8::cat*	Tsaneva and Weiss (1990)
BW847	GC4468, *soxR4::cat*	Tsaneva and Weiss (1990)
BM900	MS1343, *soxR9::cat*	This study
BMC9-23	MS1343, *soxR9::cat*, *rsxB::*Tn*10*	This study
BMC9-06	MS1343, *soxR9::cat*, *rseB::*Tn*10*	This study
BM847	MS1343, *soxR4::cat* (*soxR4^c*)	This study
BMC847-93	MS1343, *soxR4^c*, Δ*rsxC::kan*	This study
CP367	Temperature-sensitive *polA* mutant	Laboratory collection
DY330	W3110 *lacU169 gal490* cI857 (*cro-bioA*)	Yu *et al.* (2000)
SYPT1	MS1343, Δ*rsxP::tet*	This study
SYAK1	MS1343, Δ*rsxA::kan*	This study
SYBK1	MS1343, Δ*rsxB::kan*	This study
SYCK1	MS1343, Δ*rsxC::kan*	This study
SYDK1	MS1343, Δ*rsxD::kan*	This study
SYGK1	MS1343, Δ*rsxG::kan*	This study
SYET1	MS1343, Δ*rsxE::tet*	This study
SYNT1	MS1343, Δ*nth::tet*	This study
JHRA1	MS1343, Δ*rseA nadB3140::*Tn*10*, Tet^r	This study
JHRB1	MS1343, Δ*rseB nadB3140::*Tn*10*, Kan^r	This study
JHRC1	MS1343, *rseC::*Ω *nadB3140::*Tn*10*, Kan^r, Chl^r	This study
JHRC2	GC4468, *rseC::*Ω *nadB3140::*Tn*10*, Kan^r, Chl^r	This study
JHRBC1	MS1343, Δ*rseBC::*Ω *nadB3140::*Tn*10*, Kan^r, Chl^r	This study
JHRMC1	MC1392, *rseC::*Ω *nadB3140::*Tn*10*, Kan^r, Chl^r, Tet^r	This study

electron donor for the reductase (Liochev and Fridovich, 1992). Recently, an NADPH-dependent SoxR-reducing activity was isolated in *E.coli*, but the protein has not been characterized further (Kobayashi and Tagawa, 1999). Flavodoxins (FldAB) and ferredoxin-NADPH-oxidoreductase (Fpr), both being the components of the *soxRS* regulon, have been examined for SoxR-reducing activity, and turned out not to affect the level of *soxS* expression (Gaudu and Weiss, 1996, 2000). In this study, we explored factors involved in reducing SoxR, taking advantage of random Tn*10* insertional mutagenesis, and selected mutants that constitutively express the *soxS* gene, excluding mutations in the *soxR* gene itself. We report on finding two genetic loci (*rsx* and *rseC*) whose products are necessary for maintaining the reduced state of SoxR.

Results

Tn10 insertion in rsx and rseC loci caused constitutive expression of soxSp::lacZ

To screen for genes involved in reducing SoxR, random insertional mutations were created in the chromosome of the Δ*soxRS* strain of *E.coli* (BW829, Table I) using mini-Tn*10*. About 200 000 independent mutants were pooled and their genes were transduced by P1 phage into the reporter strain MS1343 containing a chromosomal copy of the *soxSp::lacZ* fusion gene. Constitutive mutants expressing a red color on MacConkey plates in the absence of added oxidants were selected. To exclude the possibility of second site mutations, we confirmed the mutant phenotype by re-transducing the Tn*10* marker into fresh MS1343 host. From the transductants, we isolated four constitutive mutants. While *soxS* promoter-driven β-galactosidase activity in the wild-type is 80–100 Miller units at exponential phase (A_{600} = 0.5), the isolated mutants exhibited an ~6- to 8-fold elevated basal level of *soxS* expression in the absence of any oxidants (Figure 1).

To identify the mutated genes, the regions of Tn*10* insertion were cloned and sequenced. Three mutations (MC1323, MC1353 and MC1392) were all mapped at the same region, called the *ydg* locus, showing high homology in sequence and gene (operon) structure to the *rnf* (Rhodobacter nitrogen fixation) genes of *Rhodobacter capsulatus* (Figure 2A). We named this locus *rsx* (reducer of SoxR) and used the same alphabetical numbering in accordance with the *rnf* genes in *R.capsulatus*. In all three mutations, Tn*10* was found inserted at the C-terminal part of the *rsxB* gene. One remaining mutation (MC1306) was mapped in the *rseB* gene of the *rpoE-rseABC* operon encoding a sigma factor (σ^E) and its regulators (Figure 2A).

Both RsxB and RsxC contain two ferredoxin-like motifs of C-X_2-C-X_2-C-X_3-CP, a putative binding site of the [4Fe–4S] cluster (Figure 2B). The homologous genes in *Hemophilus influenzae* and *R.capsulatus* also contain these ferredoxin-like motifs (Kumagai *et al.*, 1997). RsxC, which is longer by 220 amino acids than *R.capsulatus* RnfC, contains an NADH-binding motif that is also conserved in other RnfC homologs. The RnfABCDGE

proteins are known to be required for nitrogen fixation in *R.capsulatus*, most probably in transferring electrons to nitrogenase as a membrane-bound complex (Schmehl *et al*., 1993; Jouanneau *et al*., 1998). The gene arrangement in the *E.coli rsx* locus matches well with that in *R.capsulatus rnf* except that *E.coli* lacks an *rnfH* homolog and instead contains a non-homologous gene *rsxP* preceding *rsxA* and *nth* downstream of *rsxE*. It has been reported previously that the *nth* gene encoding endonuclease III is co-transcribed with the upstream *rnf* homolog genes (Gifford and Wallace, 2000).

The hydrophobicity prediction suggests that RsxA, RsxD and RsxE contain >6 transmembrane domains, whereas RsxB and RsxG contain one hydrophobic domain at each N-terminus. Only RsxC lacks any hydrophobic patch. The topology of RsxA (YdgL) and RsxE (YdgQ) has been examined previously to reveal that they contain six transmembrane domains with both ends of RsxA protruding into the periplasm and both ends of RsxE protruding into the cytoplasm (Sääf *et al*., 1999). In *R.capsulatus*, RnfA has also been demonstrated to be a membrane protein with six transmembrane domains. RnfB and RnfC have been shown to locate at the periphery of the membrane, stabilizing each other (Kumagai *et al*., 1997). Therefore, it is most likely that Rsx proteins in *E.coli* form a multisubunit complex in the membrane.

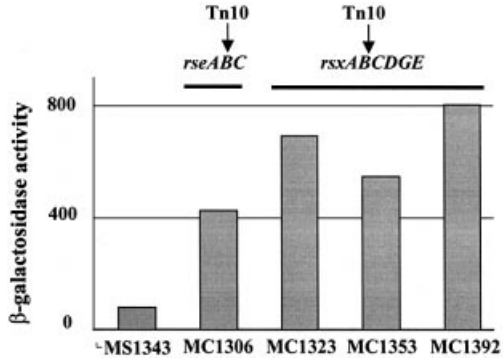

Fig. 1. Constitutive mutants with elevated *soxS* expression. Deep red colonies that express the *soxS* gene on McConkey plates in the absence of oxidants were selected from the Tn*10*-insertional mutant pool in the MS1343 (*soxSp::lacZ*) background. The β-galactosidase activity was determined at early exponential phase and is presented in Miller units, as described in the text.

The *rseABC* genes constitutes an operon with *rpoE* encoding σ^E, which transcribes its target genes in response to cell envelope stress. RseA, an anti-σ^E factor located in the inner membrane, binds and negatively regulates σ^E. RseB is an accessory factor that binds to RseA in the periplasmic face and negatively regulates σ^E. No particular role for RseC has been assigned (Missiakas and Raina, 1997; De Las Peñas *et al*., 1997; Ades *et al*., 1999). RseC is predicted to be a membrane protein with two transmembrane segments at the C-terminus. We found that *rseC* is homologous to the N-terminal half of the *rnfF* gene in the *fdxC–fdxN* gene cluster located upstream of the *rnfA* gene in divergent orientation in *R.capsulatus* (Figure 2A and C). They share many residues in common, including the cysteines that also appear in two hypothetical proteins (P44020 and P44060) from *H.influenzae*.

Effect of rsx and rse mutations is mediated via SoxR

To test whether the elevated *soxS* expression in *rsx::*Tn*10* and *rse::*Tn*10* mutants in the absence of oxidative stress is mediated via SoxR, a *soxR* deletion allele (*soxR9::cat* from BM900, Table I) was introduced into MC1392 (*rsx*::Tn*10*) and MC1306 (*rse*::Tn*10*) mutants by P1 transduction. In double mutants, the level of *soxS* expression decreased to the low level observed in the *soxR*

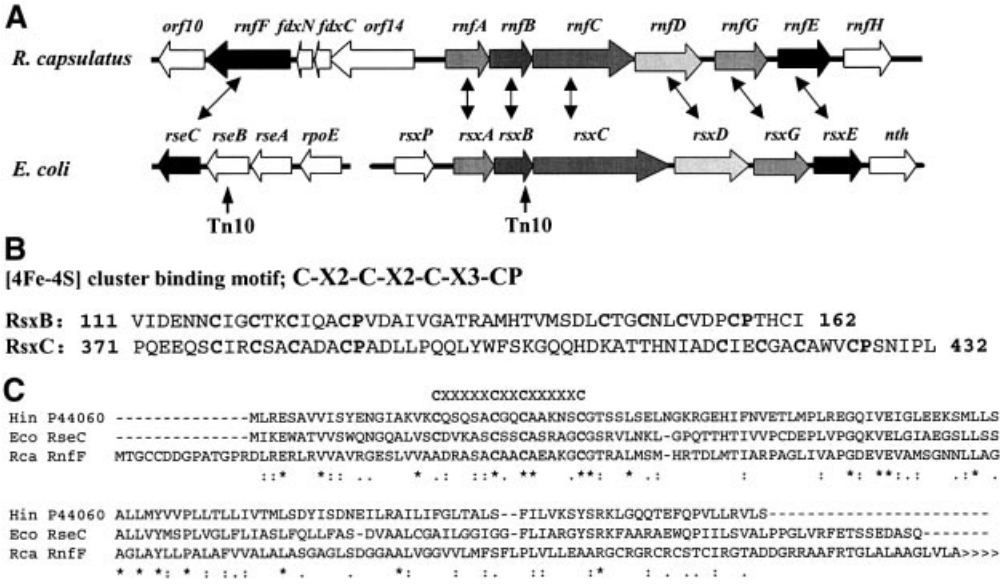

Fig. 2. The gene structure of the *rsx* and *rpoE-rse* operons. (**A**) The map of *rsxPABCDGE, nth* and *rpoE-rseABC* genes in comparison with the *rnf* genes of *R.capsulatus*. Homologous gene pairs are indicated with the same shadings and bi-directional arrows. The sites of Tn*10* insertions are indicated. (**B**) The [4Fe–4S] binding motifs in RsxB and RsxC. Two consecutive $CX_2CX_2CX_3CP$ motifs are highlighted. (**C**) Conserved regions among gene products of *E.coli rseC* (Eco RseC), *R.capsulatus rnfF* (Rca RnfF) and *H.influenzae* (Hin) P44060. The conserved cysteine residues are presented in bold.

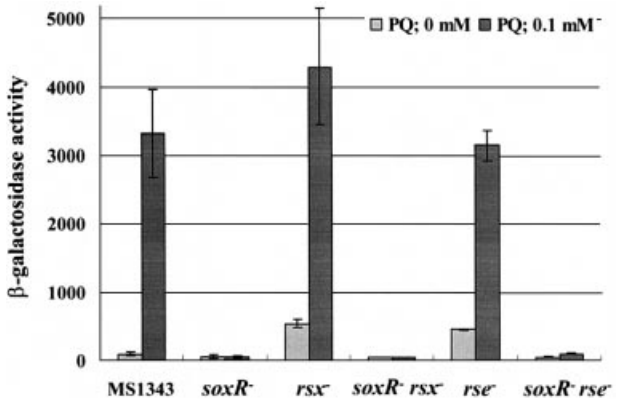

Fig. 3. SoxR-mediated *soxS* activation in *rsx* and *rse* mutants. Expression of *soxSp*-driven β-galactosidase was determined for wild-type (MS1343) and various mutants [BM900(*soxR*⁻), MC1392(*rsx*⁻), BMC9-23(*soxR*⁻ *rsx*⁻), MC1306(*rse*⁻) and BMC9-06(*soxR*⁻ *rse*⁻)]. Cells were grown in LB to early exponential phase (A_{600} = 0.2), and then either left untreated or treated with paraquat (PQ, 0.1 mM) for 60 min, followed by β-galactosidase activity assay. The mean value from four independent experiments is presented.

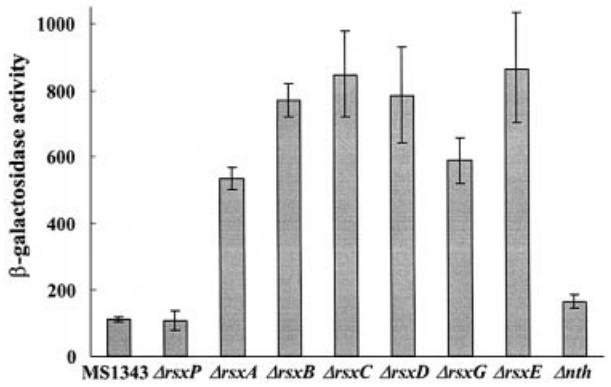

Fig. 4. Effect of each ORF in the *rsx* operon on *soxS* expression. An in-frame deletion of each gene in the *rsx* operon was constructed in the MS1343 background to create SYPT1 (Δ*rsxP::tet*), SYAK1 (Δ*rsxA::kan*), SYBK1 (Δ*rsxB::kan*), SYCK1 (Δ*rsxC::kan*), SYDK1 (Δ*rsxD::kan*), SYGK1 (Δ*rsxG::kan*), SYEK1 (Δ*rsxE::kan*) and SYNT1 (Δ*nth::tet*). All strains were grown to an OD_{600} of 0.5 and their basal β-galactosidase activities were measured. The mean value from three independent experiments is presented.

mutant (BM900) in the absence or presence of paraquat (Figure 3). This epigenetic effect of *soxR* mutation indicates that the *rsx* and *rse* mutations caused a constitutive phenotype through the action of SoxR. To investigate whether the effect of *rsx* and *rse* mutations is specific for the *soxRS* system and not for other oxidant-responsive system such as *oxyR*, we introduced *rsx::*Tn*10* and *rse::*Tn*10* alleles from MC1392 and MC1306 into the *oxyS::lacZ* fusion strain. The expression level of *oxyS* did not change by introducing these alleles, confirming that the effect of these mutations is specific for the *soxRS* system (data not shown).

Effect of each gene in rsx and rse operons on soxS expression

The *rsxPABCDGE* (*ydgKLMNOPQ*) and *nth* genes have been suggested to constitute an operon transcribed from two promoters immediately upstream of *rsxP* and *rsxA* (Gifford and Wallace, 2000). We confirmed the location of a promoter in front of the *rsxA* gene by S1 mapping. This promoter is stronger than the upstream one, whose start site we did not determine, by >5-fold as judged by S1 analysis (data not shown).

The original Tn*10* insertions at *rsx* (MC1323, 1353 and 1392) and at *rse* (MC1306) loci most probably have caused polar effects on the genes located downstream of the insertion site. Therefore, we constructed in-frame deletion mutants of each gene by swapping the entire coding region from the start to stop codons with an antibiotic resistance gene in-frame, avoiding the polar effect on downstream genes. When each in-frame deletion mutant of *rsxA*, *B*, *C*, *D*, *G* and *E* was constructed in the reporter strain MS1343, we found that the mutations all enhanced *soxS* expression to a level comparable with that observed in the original *rsx::*Tn*10* mutants (Figure 4). On the other hand, deletion of *rsxP* or *nth* did not cause any significant change in *soxS* expression. Introduction of each of the *rsxA*, *B*, *C*, *D*, *G* and *E* genes on a multicopy vector to each corresponding deletion mutant decreased *soxS* expression, confirming the effect of each gene (data not shown). Since six proteins are all required to maintain the low level of *soxS* expression, it is most likely that RsxA, B, C, D, G and E proteins might all act together in reducing SoxR, possibly as a complex, as their homologs may do in *R.capsulatus* in reducing nitrogenase.

We then examined the role of each gene in the *rseABC* locus. When we introduced individual mutant alleles of *rseA*, *rseB*, *rseC* and *rseBC* into MS1343, only the *rseC* mutation (JHRC1, Table I) enhanced *soxS* expression (Figure 5A, lanes 1–4). Deletion of both *rseB* and *rseC* gave results similar to *rseC* mutation (lane 5). Since Tn*10* was inserted at the *rseB* gene in the original mutant, the effect of mutation must be due to its polar effect downstream of the *rseC* gene. To confirm this, we performed a complementation experiment, introducing the wild-type *rseB* or *rseC* gene on a multicopy vector to the Δ*rseBC* mutant. As expected, only the wild-type *rseC* gene reduced *soxS* expression in the Δ*rseBC* mutant to the wild-type level. These results clearly demonstrated that RseC functions in keeping the level of *soxS* expression low, most probably via keeping SoxR in a reduced (inactive) state.

To test whether RseC protein works together with or independently of Rsx proteins in modulating SoxR activity, we constructed a double mutant (JHRMC1, Table I) containing *rsxB::*Tn*10* and *rseC*⁻ alleles in the MS1343 background. The double mutation did not cause any additive increase in *soxS* expression (Figure 5B), suggesting that *rseC* works together with *rsx* gene products in the same pathway in modulating SoxR activity.

Redox state of SoxR in rsx and rseC mutants

To assess the redox state of SoxR in *rsx* and *rseC* mutants, we performed EPR spectroscopic analysis using whole cells. EPR spectroscopy has been used to analyze the redox state of the overproduced proteins containing an iron–sulfur cluster in intact cells, since it can determine the unpaired electron of the Fe–S cluster (Johnson *et al.*, 1985), and has been applied successfully to monitor the redox state of SoxR *in vivo* (Ding and Demple, 1997; Gaudu *et al.*, 1997). The [2Fe–2S] cluster of SoxR

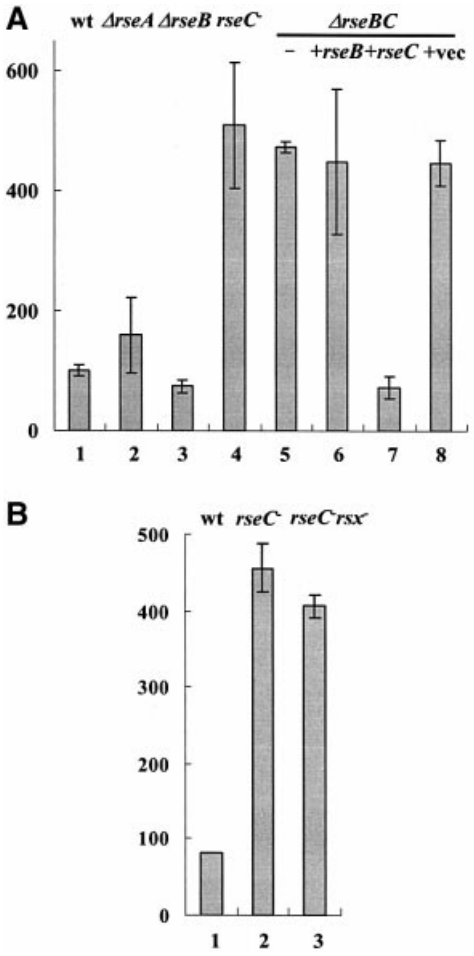

Fig. 5. Effect of each ORF in the *rse* operon and double mutations of *rsx* and *rseC* on *soxS* expression. (**A**) An in-frame deletion of each gene in the *rse* operon was constructed in the MS1343 (wt) background to create JHRA1 (Δ*rseA*, lane 2), JHRB1 (Δ*rseB*, lane 3), JHRC1 (Δ*rseC*⁻, lane 4) and JHRBC1 (Δ*rseBC*, lane 5) mutants. For complementation experiments, *rseB* and *rseC* genes were expressed in Δ*rseBC* cells on multicopy vector pTrc99A (Pharmacia) controlled by the fused *trc* promoter (lanes 6 and 7, respectively). Complementation with the parental pTrc99A vector was performed in parallel (lane 8). (**B**) The double mutant JHRMC1 (*rsx*⁻ *rseC*⁻) created by transducing the original *rsxB*::Tn*10* mutant allele of MC1392 to JHRC1 (*rseC*⁻) was measured for *soxS* expression along with MS1343 (wt) and JHRC1 (*rseC*⁻). The basal β-galactosidase activity was measured at OD_{600} = 0.5. The mean values from three independent experiments are presented.

produces a characteristic EPR spectrum in its reduced form ([2Fe–2S]$^{+}$), which disappears on oxidation to [2Fe–2S]$^{2+}$ (Hidalgo *et al*., 1995). Since only a small amount of SoxR exists in wild-type cells (<100 molecules per cell), spectroscopic observation *in vivo* requires the overproduction of SoxR. We overproduced SoxR protein in wild-type and *rsxC* or *rseC* mutants, and confirmed that similar amounts of SoxR were present in the soluble fraction, as judged by SDS–PAGE. The X-band EPR spectra from these cells were recorded at 96 K as described in Materials and methods.

SoxR in wild-type cells demonstrated its characteristic spectrum as a reduced form (Figure 6A). The intensity of the EPR signal was significantly decreased in *rseC* and more in *rsxC* mutants. Since only the reduced form of the [2Fe–2S] cluster produces the EPR signal, the data demonstrated clearly that the amount of reduced SoxR decreased significantly in *rsxC* and *rseC* mutants compared with the wild-type. When we used sodium dithionite, a strong reductant, to convert all the [2Fe–2S] clusters in SoxR to the reduced form, the EPR signal from the wild-type sample did not change significantly, whereas those from mutant samples increased significantly (Figure 6B). Assuming that the integrated value of the EPR signal from the dithionite-treated sample represents total [2Fe–2S] clusters of SoxR in each strain, we calculated the percentage of reduced SoxR in each cell type by taking the ratio of signal values from untreated versus dithionite-treated samples. The data indicate that only ~60 and 56% of the [2Fe–2S] clusters in overproduced SoxR exist as reduced forms in *rseC* and *rsxC* mutants, respectively, whereas nearly all SoxR are reduced in the wild-type. The signal intensity of the fully reduced (dithionite-treated) SoxR in mutant cells was lower than that of the wild type. Normalizing the integrated values to the same amount of SoxR polypeptides overproduced, the mutant signals corresponded to ~50 and 30% of the wild-type level in *rseC* and *rsxC* mutants, respectively. We interpret these data to suggest that the [2Fe–2S] cluster in SoxR might be unstable in the mutants, with a more drastic effect in the *rsxC* mutant. This is in accordance with a previous observation that some SoxR^{c} mutant proteins that exist in more oxidized forms tend to lose iron more easily (Gaudu *et al*., 1997). Overall, the EPR analysis demonstrated clearly that in *rsx* and *rseC* mutants, SoxR exists more in the oxidized form, agreeing with the prediction from the genetic data that the elevated *soxS* expression in the mutants is due to the activated (oxidized) SoxR. Thus we propose that the *rsxABCDGE* and *rseC* gene products are required in maintaining the reduced state of SoxR.

Effect of rsx and rse mutations on re-reduction of SoxR after oxidative stress

The [2Fe–2S] cluster of SoxR is oxidized rapidly on exposure to redox-cycling agents when the O_2 supply is sufficient. Limiting the O_2 supply by stopping aeration leads to rapid re-reduction of SoxR, allowing it to return to its inactive (reduced) state within 10 min as monitored by EPR spectroscopy (Ding and Demple, 1997). The rapid change in SoxR activity can be monitored by measuring the level of *soxS* mRNA due to its very short half-life *in vivo*. If RsxABCDGE and RseC function in reducing SoxR, mutations in *rsx* or *rseC* will retard the rate of re-reduction of SoxR, causing the elevated soxS mRNA level to persist for longer. We monitored the decay rate of *soxS* mRNA after stopping aeration in the presence of paraquat in wild-type, *rsxC* and *rseC* mutants. The *soxS* mRNA decreased to ~15% of the level within 10 min in the wild-type cell. In *rsxC* or *rseC* mutants, the *soxS* mRNA decreased more slowly than the wild-type (Figure 7), consistent with the proposal that Rsx and RseC proteins function in reducing SoxR.

Additive effect of rsx and soxR4^{c} constitutive mutations

Several constitutive mutants of SoxR, whose C-termini just downstream of the [2Fe–2S] site are truncated, have been reported (Nunoshiba and Demple, 1994; Gaudu and

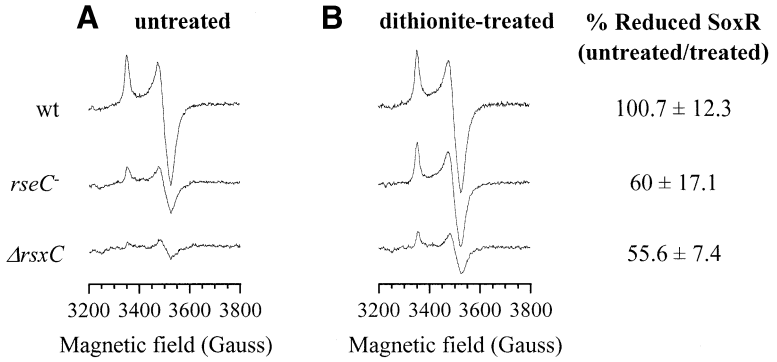

Fig. 6. EPR analysis of SoxR overproduced in *rsx* and *rseC* mutant cells. Wild-type (GC4468), MS11(Δ*rsxC::kan*) or JHRC2 (*rseC*–) cells harboring pTac1-SoxR were grown to OD_{600} = 0.5 and induced with 1 mM IPTG for 2 h at 25°C. Cells were harvested and resuspended to an equal cell density in 50 mM MOPS pH 7.6, 0.2 M KCl and 0.2 M $LiCl_2$. The concentrated cell suspension was then transferred directly to an EPR tube pre-cooled in liquid nitrogen. Sodium dithionite was mixed immediately before EPR measurement, as described in the text. The EPR signals were obtained at 96 K. The signal from cells containing control plasmid (pTac1) was subtracted from each measurement. The percentage of reduced SoxR in each cell type was determined by double integration of the signals, and taking the ratio of values from untreated versus dithionite-treated samples. Average values from three independent experiments are presented with standard deviations.

Weiss, 1996). One of those mutants, *soxR4*^{c} (*soxR4::cat*), whose C-terminal 19 amino acids were replaced with 49 residues from the *cat* cassette, was examined in conjunction with the *rsx* mutant. The level of *soxS* expression in the *soxR4*^{c} mutant was slightly higher than but comparable with the level observed in *rsxC* mutants (Figure 8). In the *soxR4*^{c}Δ*rsxC* double mutant, the basal level of *soxS* expression was elevated further to about half the level of full induction, exhibiting an additive effect of *rsx* and *soxR4*^{c} mutation. These results suggest that the mechanism for the increased oxidation of SoxR in the Δ*rsx* mutant is different from that in the *soxR4*^{c} mutant.

Discussion

The redox potential of SoxR at pH 7.6 has been estimated to be about –285 mV (Ding *et al*., 1996; Gaudu and Weiss, 1996). More than 40% and up to 95% of SoxR exists as a form containing reduced [2Fe–2S] during aerobic growth (Hidalgo *et al*., 1997). Since the cytoplasmic redox potential of *E.coli* has been estimated to be in the range of –260 to –280 mV (Gilbert, 1990; Hwang *et al*., 1995), it is very likely that SoxR *in vivo* is actively maintained in the reduced state, implying the presence of a specific reducing system for [2Fe–2S] of SoxR. In this work, we have presented evidence that *rsx* and *rseC* gene products constitute the components of a reducing system for SoxR.

The estimated redox potential of –340 mV for NAD(P)H/$NAD(P)^+$ suggests that electrons may be transferred from NAD(P)H to SoxR in the reducing system. The structural similarity between the *E.coli rsxABCDGE* and *R.capsulatus rnfABCDGE* gene products supports the prediction that the Rsx proteins are likely to convey electron transfer from NAD(P)H to SoxR as Rnf proteins might do in mediating electrons from NADH to nitrogenase in *R.capsulatus* (Schmehl *et al*., 1993; Kumagai *et al*., 1997). Unlike *E.coli*, *R.capsulatus* is able to fix dinitrogen, which is a process requiring high energy and high potential electrons. RnfB of *R.capsulatus* contains potential binding sites for a [2Fe–2S] and two [4Fe–4S] clusters, whereas RnfC has potential binding sites for two [4Fe–4S] clusters, NAD(H) and FMN (Kumagai *et al*., 1997). Considering the similarity of RsxB and RsxC to RnfB and C, it is very likely that RsxB and C may function as the core of the reductase system, taking part in transferring electrons to SoxR, instead of nitrogenase which is absent in *E.coli*. The purified RsxC exhibits NADPH-dependent cytochrome *c* reducing activity *in vitro* even in the absence of SoxR,

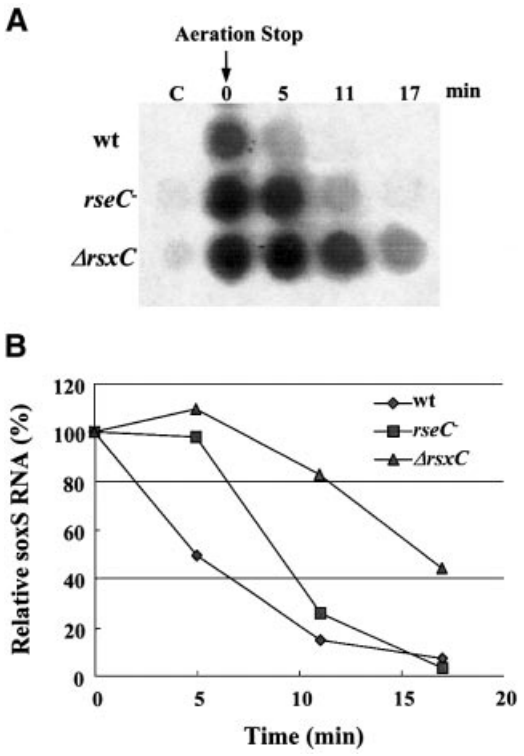

Fig. 7. Re-reduction kinetics of SoxR monitored by the change in *soxS* mRNA level. (**A**) Wild-type, JHRC1 (*rseC*–) and MC1393 (Δ*rsxC::kan*) cells were treated with 0.1 mM paraquat at OD_{600} = 0.2 for 30 min with vigorous shaking to activate SoxR to its full level. Activation was interrupted by limiting the O_2 supply by stopping shaking. Cells were removed at 0, 5, 11 and 17 min after the shaker stopped. Total RNA was prepared and analyzed by dot-blot hybridization using a specific probe for *soxS* mRNA. (**B**) The intensities of the hybridized dots were quantified by autoradiographic image analyzer (Fuji, FLA-2000) and plotted relative to the value (100%) at the 0 time point.

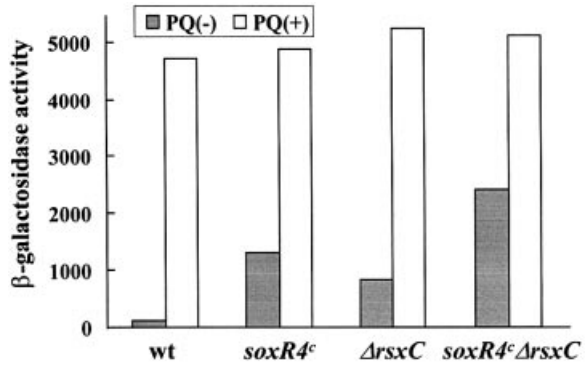

Fig. 8. Additive effect of *soxR^c^* and *rsx* constitutive mutations. The *soxR* constitutive mutant allele from BW847 was transduced into MS1343 (wt), resulting in BM847 (*soxR4^c^*). BM847 was transduced further with the Δ*rsxC* allele from MC1393, resulting in a *soxR4^c^*Δ*rsxC* double mutant (BMC847-93). MS1343 (wt), BM847, MC1393 and BMC847-93 cells were grown to an OD_{600} = 0.2, treated or not with paraquat (0.1 mM) for 1 h and assayed for β-galactosidase activity.

implying that it can transfer electrons from NADPH (M.-S.Koo and J.-H.Lee, unpublished results). Whether RsxB and RsxC function as the core components of the reductase for SoxR, forming a complex with other Rsx proteins, and whether it consumes NADPH or NADH need to be elucidated in the future.

Recently, Kobayashi and Tagawa (1999) reported that *E.coli* cells overproducing SoxR have SoxR-enhanced cytochrome *c* reductase activity in the presence of NADPH, and they isolated the responsible protein from the cytoplasm. Since the rate of cytochrome *c* reduction by the protein was enhanced ~7- to 10-fold when SoxR was added, the protein was inferred to be SoxR reductase. Interestingly, the molecular weight of the protein is similar to that of RsxC. However, the identity of the protein is uncertain since its coding gene has not been characterized. Our result does not exclude the possibility that there still remain reducing systems other than the Rsx–RseC system. In the absence of Rsx and RseC proteins, a back-up system may operate to reduce SoxR, as hinted at by the slow return of the elevated *soxS* mRNA to the uninduced level in the *rsx* and *rseC* mutants.

The level of constitutive *soxS* expression in *rsx* and *rseC* mutants was lower than the fully induced level, being ~15–20% of the full induction. The redox potential of SoxR is comparable with or slightly more negative than that of the cytoplasm. Thus in the absence of the reduction system, SoxR will exist as a partially but not fully oxidized form. In light of this, it is understandable that the basal constitutive level of SoxS expression in the mutants reflects this partially oxidized (activated) state of SoxR. Under condition of excess superoxide generation, all SoxR will be converted into the completely oxidized state, and thus it makes sense that paraquat treatment further increases the *soxS* expression in the mutant to the maximum level comparable with the full induction level in the wild-type (Figures 3 and 8). The observation that the double mutation of *rsx* and *soxR4^c^* increased *soxS* expression additively (to ~50% of full induction) leads us to exclude the possibility that the C-terminally modified *soxR4^c^* mutation caused hindrance of reduction, by limiting access to the reducing system composed of Rsx proteins (Nunoshiba and Demple, 1994; Gaudu and Weiss, 1996). The mechanism for SoxR activation by the *soxR4^c^* mutation could include a shift in redox equilibrium to a more oxidized form or a conformational change to an active form. If there exist other reducing path(s) for SoxR separate from the Rsx system, it is also possible that the *soxR4^c^* mutation could hinder the reduction mediated not by Rsx but by other system(s). Whether other *soxR^c^* mutants would behave similarly to *soxR4^c^* in terms of interaction with the Rsx system remains an intriguing question to be addressed. The *soxR^c^* mutations, whose effect is not additive with that of *rsx*, might reveal a possible mechanism for the Rsx system to convey electrons to the oxidized SoxR.

Although RsxB and RsxC may play a central role as the core electron mediators in reducing SoxR, we propose that they are part of the reductase complex in the cytoplasmic membrane composed of several Rsx proteins plus RseC. The membrane localization of RsxA, RsxE and RseC has been demonstrated previously (Missiakas *et al*., 1997; Sääf *et al*., 1999). We observed the distrubution of RsxB, C and G in the membrane fraction, as predicted (J.-H.Lee and S.-Y.Rah, in preparation). As an analogy to the observation that the membrane-bound RnfB and C proteins interact with each other (Kumagai *et al*., 1997; Jeong and Jouaneau, 2000), we hypothesize that Rsx proteins might also form a membrane-bound complex. The finding that *rseC* and *rsx* mutations are not additive, and that *rseC* is homologous to *rnfF* in *R.capsulatus* that is required for nitrogen fixation (Schmehl *et al*., 1993), led us to propose that they may act in the same electron transfer pathway and probably function within the same membrane-bound complex. Whether any of the components in the reducing complex interacts with SoxR and whether RseC interacts with any of the Rsx proteins need to be elucidated in the future.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table I. Cells were grown at 37°C in Luria–Bertani (LB) broth with vigorous aeration. Appropriate antibiotics were added at 100 μg/ml (ampicillin), 34 μg/ml (chloramphenicol), 50 μg/ml (kanamycin) and 20 μg/ml (tetracycline). Isopropyl-β-D-thiogalactopyranoside (IPTG) and paraquat were added at concentrations described in the text.

Construction of soxSp::lacZ reporter strain

A DNA fragment containing the *soxS* promoter (from nucleotide –135 to +65 relative to the transcription start site) was amplified by PCR using the primer pair SoxSF (TACTTTCATAGAATTCCAGCGCCGAT, *Eco*RI site underlined) and SoxSR (TATTCTAGGAGGATCCAAAAGACTAC, *Bam*HI site underlined). The fragment was cloned into the *Eco*RI–*Bam*HI site of pRS415 (Simons *et al*., 1987) to direct the expression of *lacZ* under the control of the *soxS* promoter (*soxSp*). The recombinant pRS415 containing the *soxSp::lacZ* fusion gene was transformed into the wild-type *E.coli* GC4468. It was then infected with phage λRZ5, allowing homologous recombination with the plasmid to occur *in vivo*. The recombinant phages were then allowed to lysogenize GC4468 cells via insertion through the *att* site in the chromosome. Single copy lysogens were screened by pale blue color of the colonies on X-Gal plates containing ampicillin, and selected on the basis of the lowest basal level of β-galactosidase activity. The resulting strain (MS1343) containing the *soxSp::lacZ* fusion gene on the chromosome was isolated and used as the wild-type reporter strain for the rest of this study.

β-galactosidase assay

Cells were grown in LB medium to an optical density of 0.2 at 600 nm and then either left untreated or treated with various concentrations of paraquat for 0.5–1 h at 37°C. β-galactosidase activity was assayed by adding *o*-nitrophenyl-β-D-galactopyranoside (ONPG) after permeabilization of the cell with SDS–chloroform (Miller, 1972).

Random insertional mutagenesis by mini-Tn10

A random mutation library was constructed with mini-Tn*10* as described by Kleckner *et al*. (1991). To avoid the mutation in the *soxRS* locus itself, we constructed the mutant library in the Δ*soxRS* strain (BW829, Δ*sox-8::cat*). BW829 cells were infected with λNK1323 carrying the mini-Tn*10*. Mutant colonies with Tn*10* insertion were selected on plates containing tetracycline and chloramphenicol. They were pooled and their genes were transduced into MS1343 using the $P1_{vir}$ transduction system (Sternberg and Mauer, 1991). Transductants that exhibit constitutive phenotype for *soxS* expression, forming blue or red colonies on X-Gal or MacConkey plates in the absence of any oxidants, were isolated. The mutated locus was cloned and identified by Southern hybridization using a Tn*10*-specific probe. The precise location of the Tn*10* insertion was determined by nucleotide sequencing.

Sequence analysis of the rsx locus

Nucleotide sequences encompassing the *rsxPABCDGE* and *nth* genes (DDBJ/EMBL/GenBank accession No. AE000258) were compared with the public database using BLAST and CLUSTAL W programs. The localization and topology of RsxABCDGE and RseC proteins were predicted using the SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html) and TMHMM programs (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

Construction of deletion mutants

Two methods were used to construct deletion mutants: plasmid integration/resolution (Koh and Roe, 1995) and open reading frame (ORF) replacement with linear DNA (Yu *et al*., 2000). MC1393 (Δ*rsxC::kan*), MC1394 (Δ*rsxBC::kan*) and SYBK1 (Δ*rsxB*) strains were constructed by plasmid integration and resolution. The *Nru*I–*Nhe*I internal fragment of *rsxC* and the *Nar*I–*Nhe*I internal fragment of *rsxBC* were replaced with a Kan^r cassette from pUC4-KIXX (Pharmacia) to generate MC1393 and MC1394, respectively. To generate SYBK1 (Δ*rsxB*), an internal 411 bp between two *Nru*I sites in *rsxB* was deleted in-frame, with a Kan^r block inserted at the *Ssp*I site upstream of the *rsxP* promoter to provide the selection marker (Koo, 2001; Rah, 2002). Co-integrates were generated in the CP367 (*polA^{ts}*) strain, and the desired mutants were selected as described in Koh and Roe (1995). The mutated gene was then transferred into the wild-type strain GC4468 or MS1343 by P1 transduction. The constructs were confirmed by Southern hybridization and PCR.

In-frame ORF replacement was performed as described by Yu *et al*. (2000) to create SYPT1 (Δ*rsxP::tet*), SYAK1 (Δ*rsxA::kan*), SYCK1 (Δ*rsxC::kan*), SYDK1 (Δ*rsxD::kan*), SYGK1 (Δ*rsxG::kan*), SYET1 (Δ*rsxE::tet*) and SYNT1 (Δ*nth::tet*) mutants (Rah, 2002). Either the Kan^r or the Tet^r cassette was amplified by PCR with a 50 nucleotide flanking sequence of the target gene. The start and stop codons of the target gene were replaced precisely with the start and stop codons of the Kan^r or Tet^r genes. Amplified linear DNA was introduced by electroporation into DY330, which contains the *exo*, *bet* and *gam* genes of λ phage regulated by the λcI repressor to enhance recombination frequency of the linear DNA. The gene replacement by homologous recombination was selected on antibiotic plates and confirmed by PCR and Southern hybridization. The mutated alleles were transferred to GC4468 or MS1343 strains by P1 transduction.

The *rse* mutants (JHRA1, JHRB1, JHRC1 and JHRBC1) were created by P1 transduction of mutant alleles from CAG22977(Δ*rseA*), CAG22974(Δ*rseB*), CAG22682 (*rseC*$^-$) and CAG22681 (Δ*rseBC*) into MS1343 (De Las Peñas *et al*., 1997).

Electron paramagnetic resonance (EPR) spectroscopy of SoxR

Wild-type (GC4468), MS11(Δ*rsxC::kan*) and JHRC2 (*rseC*$^-$) cells containing pTac1 vector or pTac1-SoxR, which overproduces SoxR under the control of the *tac* promoter (Koo, 2001), were grown to OD_{600} of 0.5 in LB containing ampicillin (100 μg/ml). SoxR expression was induced with 1.0 mM IPTG for 2 h at 25°C. Cells from 200 ml of culture were harvested at 4°C and resuspended in 7 ml of ice-cooled EPR buffer (50 mM MOPS pH 7.6, 0.2 M $LiCl_2$ and 0.2 M KCl). Following measurement of OD_{600} of the whole cell resuspension, cells were re-harvested at 4°C and suspended in 0.7–1 ml of EPR buffer, aiming to produce equal cell density among samples. Aliquots of 0.3 ml of cell suspension were then transferred to a pre-cooled EPR tube, and X-band EPR spectra were recorded at 96 K on a Bruker model EMX spectrometer (Bruker, Germany), equipped with a continuous flow N_2 temperature controller (Bruker, model BVT3000). To obtain EPR spectra of fully reduced SoxR in cells, the concentrated whole-cell suspensions were treated with 210 mM (final concentration) sodium dithionite. A 1 M solution of sodium dithionite was prepared anaerobically in 50 mM MOPS buffer pH 7.6, and aliquots of 80 μl were transferred to EPR tubes and mixed with cell suspension immediately before spectral measurements. The expression level of SoxR in the soluble fraction of cells subjected to EPR analysis was confirmed on SDS–PAGE in a parallel experiment. A nearly equal amount of SoxR polypeptide was over-produced reproducibly in the soluble fraction to ~5% of the total protein at maximum in wild-type and the mutants. The spectrum from cells containing control vector was subtracted from the data to obtain SoxR-specific signals. Typical EPR parameters used were as follows: temperature of 96 K, microwave frequency of 9.449 GHz, microwave power of 10 mW, modulation frequency of 100 kHz and modulation amplitude of 1 mT.

Isolation and dot-blot analysis of soxS mRNA

To induce *soxS* mRNA, GC4468 cells were treated with 0.1 mM paraquat for 30 min at an OD_{600} of 0.2, under vigorous shaking. Shaking was then stopped to limit aeration, and cells were taken at various time points after the shaker stopped. Total RNAs were prepared using the Ultraspec™-II total RNA isolation kit (Biotecx Laboratories, Inc.) as recommended by the manufacturer, except that cells were pre-treated with lysozyme (4 mg/ml) in 50 mM glucose, 25 mM Tris–HCl pH 8.0 and 10 mM EDTA for 2–5 min on ice. RNA (30 μg) was loaded on a Hybond-N$^+$ membrane (Amersham Pharmacia Biotech) equilibrated with 10× SSC by applying a vacuum in a dot-blotting apparatus (Hoefer) and immobilized by UV cross-linking. Hybridization was performed with a *soxS*-specific probe labeled by random priming with [α-^{32}P]dATP (Sambrook *et al*., 1989).

Acknowledgements

We are grateful to Drs B.Weiss, B.Demple and C.A.Gross for their kind donation of strains. This work was supported by a research grant (R03-2001-00047) from the Korea Science and Engineering Foundation to J.-H.R. J.-H.L. was the recipient of a BK21 fellowship for post-doctoral fellows from the Ministry of Education and Human Resources.

References

Ades,S.E., Connolly,L.E., Alba,B.M. and Gross,C.A. (1999) The *Escherichia coli* σ^E-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-α factor. *Genes Dev.*, **13**, 2449–2461.

Amabile-Cuevas,C.F. and Demple,B. (1991) Molecular characterization of the *soxRS* genes of *Escherichia coli*: two genes control a superoxide stress regulon. *Nucleic Acids Res.*, **19**, 4479–4484.

Ansari,A.Z., Bradner,J.E. and O'Halloran,T.V. (1995) DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature*, **374**, 371–375.

Bauer,C.E., Elsen,S. and Bird,T.H. (1999) Mechanisms for redox control of gene expression. *Annu. Rev. Microbiol.*, **53**, 495–523.

De Las Peñas,A., Connolly,L. and Gross,C.A. (1997) The σ^E-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of σ^E. *Mol. Microbiol.*, **24**, 373–385.

Ding,H. and Demple,B. (1997) *In vivo* kinetics of a redox-regulated transcriptional switch. *Proc. Natl Acad. Sci. USA*, **94**, 8445–8449.

Ding,H., Hidalgo,E. and Demple,B. (1996) The redox state of the [2Fe–2S] clusters in SoxR protein regulates its activity as a transcription factor. *J. Biol. Chem.*, **271**, 33173–33175.

Gaudu,P. and Weiss,B. (1996) SoxR, a [2Fe–2S] transcription factor, is active only in its oxidized form. *Proc. Natl Acad. Sci. USA*, **93**, 10094–10098.

Gaudu,P. and Weiss,B. (2000) Flavodoxin mutants of *Escherichia coli* K-12. *J. Bacteriol.*, **182**, 1788–1793.

Gaudu,P., Moon,N. and Weiss,B. (1997) Regulation of the *soxRS* oxidative stress regulon. Reversible oxidation of the Fe–S centers of SoxR *in vivo*. *J. Biol. Chem.*, **272**, 5082–5086.

Gifford,C.M. and Wallace,S.S. (2000) The genes encoding endonuclease VIII and endonuclease III in *Escherichia coli* are transcribed as the terminal genes in operons. *Nucleic Acids Res.*, **28**, 762–769.

Gilbert,H.F. (1990) Molecular and cellular aspects of thiol–disulfide exchange. *Adv. Enzymol. Relat. Areas Mol. Biol.*, **63**, 69–172.

Hausladen,A., Privalle,C.T., Keng,T., DeAngelo,J. and Stamler,J.S. (1996) Nitrosative stress: activation of the transcription factor OxyR. *Cell*, **86**, 719–729.

Hidalgo,E. and Demple,B. (1994) An iron–sulfur center essential for transcriptional activation by the redox-sensing SoxR protein. *EMBO J.*, **13**, 138–146.

Hidalgo,E. and Demple,B. (1997) Spacing of promoter elements regulates the basal expression of the soxS gene and converts SoxR from a transcriptional activator into a repressor. *EMBO J.*, **16**, 1056–1065.

Hidalgo,E., Bollinger,J.M.,Jr, Bradley,T.M., Walsh,C.T. and Demple,B. (1995) Binuclear [2Fe–2S] clusters in the *Escherichia coli* SoxR protein and role of the metals in transcription. *J. Biol. Chem.* **270**, 20908–20914.

Hidalgo,E., Ding,H. and Demple,B. (1997) Redox signal transduction: mutations shifting [2Fe–2S] centers of the SoxR sensor-regulator to the oxidized form. *Cell*, **88**, 121–129.

Hwang,C., Lodish,H.F. and Sinskey,A.J. (1995) Measurement of glutathione redox state in cytosol and secretory pathway of cultured cells. *Methods Enzymol.*, **251**, 212–221.

Johnson,M.K., Morningstar,J.E., Cecchini,G. and Ackrell,B.A. (1985) *In vivo* detection of a three iron cluster in fumarate reductase from *Escherichia coli*. *Biochem. Biophys. Res. Commun.*, **16**, 131–136.

Jeong,H.S. and Jouanneau,Y. (2000) Enhanced nitrogenase activity in strains of *Rhodobacter capsulatus* that overexpress the *rnf* genes. *J. Bacteriol.*, **182**, 1208–1214.

Jouanneau,Y., Jeong,H.-S., Hugo,N., Meyer,C. and Willison,J.C. (1998) Overexpression in *Escherichia coli* of the genes from *Rhodobacter capsulatus*. Characterization of two-membrane-bound iron–sulfur protein. *Eur. J. Biochem.*, **251**, 54–64.

Kleckner,N., Benger,J. and Gottesman,S. (1991) Uses of transposons with emphasis on Tn*10*. *Methods Enzymol.*, **204**, 139–181.

Kobayashi,K. and Tagawa,S. (1999) Isolation of reductase for SoxR that governs an oxidative response regulon from *Escherichia coli*. *FEBS Lett.*, **451**, 227–230.

Koh,Y.S. and Roe,J.H. (1995) Isolation of a novel paraquat-inducible (*pqi*) gene regulated by the soxRS locus in *Escherichia coli*. *J. Bacteriol.*, **177**, 2673–2678.

Koo,M.S. (2001) Characterization of protein factor regulating superoxide sensor SoxR in *Escherichia coli*. PhD Thesis, Seoul National University, Seoul, Korea.

Kumagai,H., Fujiwara,T., Matsubara,H. and Saeki,K. (1997) Membrane localization, topology and mutual stabilization of the *rnfABC* gene products in *Rhodobacter capsulatus* and implications for a new family of energy-coupling NADH oxidoreductases. *Biochemistry*, **36**, 5509–5521.

Lazazzara,B.A., Beinert,H., Khoroshilova,N., Kennedy,M.C. and Kiley,P.J. (1996) DNA binding and dimerization of the Fe–S-containing FNR protein from *Escherichia coli* are regulated by oxygen. *J. Biol. Chem.*, **271**, 2762–2768.

Liochev,S.I. and Fridovich,I. (1992) Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the *soxRS* regulon. *Proc. Natl Acad. Sci. USA*, **89**, 5892–5896.

Miller,J.H. (1972) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Missiakas,D. and Raina,S. (1997) Protein folding in the bacterial periplasm. *J. Bacteriol.*, **179**, 2465–2471.

Missiakas,D., Mayer,M.P., Lemaire,M., Georgopoulos,C. and Raina,S. (1997) Modulation of the *Escherichia coli* σ^E (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins. *Mol. Microbiol.*, **24**, 55–71.

Nunoshiba,T. and Demple,B. (1994) A cluster of constitutive mutations affecting the C-terminus of the redox-sensitive SoxR transcriptional activator. *Nucleic Acids Res.*, **22**, 2958–2962.

Nunoshiba,T., de Rojas-Walker,T., Wishnok,J.S., Tannenbaum,S.R. and Demple,B. (1993) Activation by nitric oxide of an oxidative-stress response that defends *Escherichia coli* against activated macrophages. *Proc. Natl Acad. Sci. USA*, **90**, 9993–9997.

Outten,C.E., Outten,F.W. and O'Halloran,T.V. (1999) DNA distortion mechanism for transcriptional activation by ZntR, a Zn(II)-responsive MerR homologue in *Escherichia coli*. *J. Biol. Chem.*, **274**, 37517–37524.

Pomposiello,P.J. and Demple,B. (2001) Redox-operated genetic switches; the SoxR and OxyR transcription factors. *Trends Biotechnol.*, **19**, 109–114.

Popescu,C.V., Bates,D.M., Beinert,H., Munck,E. and Kiley,P.J. (1998) Mossbauer spectroscopy as a tool for the study of activation/inactivation of the transcription regulator FNR in whole cells of *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **95**, 13431–13435.

Rah,S.Y. (2002) Characterization of *resPABCDGE* operon regulating SoxR in *Escherichia coli*. MSc. Thesis, Seoul National University, Seoul, Korea.

Sääf,A., Johansson,M., Wallin,E. and von Heijne,G. (1999) Divergent evolution of membrane protein topology: the *Escherichia coli* RnfA and RnfE homologues. *Proc. Natl Acad. Sci. USA*, **96**, 8540–8544.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schmehl,M., Jahn,A., Meyer zu Vilsendorf,A., Hennecke,S., Masepohl,B., Schuppler,M., Marxer,M., Oelze,J. and Klipp,W. (1993) Identification of a new class of nitrogen fixation genes in *Rhodobacter capsulatus*: a putative membrane complex involved in electron transport to nitrogenase. *Mol. Gen. Genet.*, **241**, 602–615.

Simons,R.W., Houman,F. and Kleckner,N. (1987) Improved single and multicopy lac-based cloning vectors for protein and operon fusions. *Gene*, **53**, 85–96.

Sternberg,N.L. and Mauer,R. (1991) Bacteriophage-mediated generalized transduction in *Escherichia coli* and *Salmonella typhimurium*. *Methods Enzymol.*, **204**, 18–43.

Storz,G. and Imlay,J.A. (1999) Oxidative stress. *Curr. Opin. Microbiol.*, **2**, 188–194.

Tsaneva,I.R. and Weiss,B. (1990) *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. *J. Bacteriol.*, **172**, 4197–4205.

Wu,J. and Weiss,B. (1991) Two divergently transcribed genes, *soxR* and *soxS* control a superoxide response regulon of *Escherichia coli*. *J. Bacteriol.*, **173**, 2864–2871.

Wu,J., Dunham,W.R. and Weiss,B. (1995) Overproduction and physical characterization of SoxR, a [2Fe–2S] protein that governs an oxidative response regulon in *Escherichia coli*. *J. Biol. Chem.*, **270**, 10323–10327.

Yu,D., Ellis,M.H., Lee,E.-C., Jenkins,N.A., Copeland,N.G. and Court,D.L. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **97**, 5978–5983.

Received October 17, 2002; revised February 19, 2003; accepted March 28, 2003