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Regulation of SoxR, the superoxide-sensory regulator in *Escherichia coli.*

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Abstract

In order to find out SoxR-reducing system in *E. coli*, we generated Tn10-insertion mutants and screened for constitutive expression of SoxS in a soxS-lacZ fusion strain. One mutation was mapped in rseB, a gene in rseABC (Regulation of SigmaE) operon. The constitutive soxS-expressing phenotype was due to the polar effect on the downstream gene, rseC. RseC is likely to function as a component of SoxR reduction system because SoxR was kept in oxidized form to activate soxS expression in rseC mutant. RseC is an integral membrane protein with an N-terminal cysteine-rich domain in the cytoplasm. The functionally critical cysteines were determined by substitution mutagenesis. The truncated N-terminal domain of RseC reduced the soxS transcription by 50% as judged by in vitro transcription assay. Currently RseC is believed to be a reducing factor for SoxR. However, the mechanism for the reduction needs further investigation.

Introduction

Aerobes are in the challenge of reactive oxygen species from respiration, radiation, redox-cycling agents, or macrophages at bacterial invasion, and so have developed some kind of defense systems to the oxidative stress. *E. coli* has two defense regulons, one for H_2O_2 (*oxyR* regulon) and the other for superoxide radical (*soxRS* regulon). In the *oxyR* regulon, at least 10 genes including *katG*, *ahpCF*, *gorA*, *grxA*, *fur* and *oxyS* are under the positive control of OxyR. In the *soxRS* regulon, at least 13 genes including *nfo*, *zwf*, *sodA*, *fumC*, *micF*, *acnA*, *fpr*, *ribA*, and *pqi5* are under the positive control of SoxRS.

Although SoxRS regulon is a well-characterized system, many questions still remained unrevealed. One of these questions is the reduction mechanism of SoxR. This is very closely related to the redox-sensing mechanism of SoxR. In the activation of target genes by SoxRS, SoxR is a redox-sensor containing 2Fe-2S cluster, which is oxidized upon exposure to superoxide radical and make SoxR its activated form. Reversibly, SoxR must return to its inactivated form on the removal of oxidative stress by uncharacterized factors, hypothetically termed "SoxR reductase". Activated SoxR binds to the *soxS* promoter, and induces SoxS expression and in turn, induced SoxS activate the transcription of the target genes. So far, SoxR reductase has been hypothesized but not characterized. Here, we will report our trial exploring the trans-acting factors affecting SoxR-mediated *soxS* induction, including putative SoxR reductase. We isolated two candidates using Tn10 random mutagenesis and characterized one of them, *rseC*.

Results

Screening of trans-acting mutants affecting *soxS* expression. To search for the unknown factors modulating the SoxR activity containing its reduction system, we screened trans-acting factors affecting *soxS* expression. Screening strategy is presented in Fig. 1A. Simply, to monitor the *soxS* expression, we constructed *soxS'-lacZ* single copy fusion strain (MS1343) by amplifying *soxS* promoter region by PCR, cloning it onto the promoterless *lacZ* fusion vector, pRS415, and introducing it into the chromosome in single lysogen state through λRZ5. Independently, we constructed the random insertional mutation pool using miniTn10 in the *soxRS* deletion strain to avoid the mutation of *soxRS* itself. The pooled 200,000 independent mutations were transduced into MS1343 by P1 transduction and subsequently screened by color on the MacConkey-containing plate. Non-inducible mutation of *soxS* can be selected by white color on the paraquat-containing plate and constitutive mutations can be selected by red on the paraquat-free plate. Finally we could select five constitutive mutants, but no non-inducible mutant (Fig. 1B). Here, only one of them, MS1306 was further analyzed. To discriminate the true mutants from false mutants, such as the second site mutation, double mutation, or a mutation of *soxS-lacZ* fusion site itself, we retransduced the markers of mutants into fresh MS1343 by P1. MC1306 was a true mutant, constitutively expressing SoxS 6-8 folds higher compared with MS1343. MS1306 also increased the expression of other *soxRS* regulon genes, such as *rfaY*, *ribA*, and *fpr*, about two folds (data not shown), which ensured that MC1306 mutation made *soxRS* regulon constitutively activated.

Mutation locus was cloned using the tetracycline resistance and Southern hybridization with tet^r gene of Tn10 as probe. tet^r gene-containing 9.5 kb *Xho*I-*Sal*I fragment was cloned into pUC18 plasmid and the Tn10-insertion region in the 9.5 kb fragment was mapped by restriction mapping and Southern. The most precise insertion site was determined by sequencing, which showed the just chromosome-Tn10 junction point. From the results, MC1306 mutation locus was mapped in *rseB*, a gene of *rseABC* operon, which regulates SigE, an ECF (extracytoplasmic function) sigma factor (Fig. 1C).

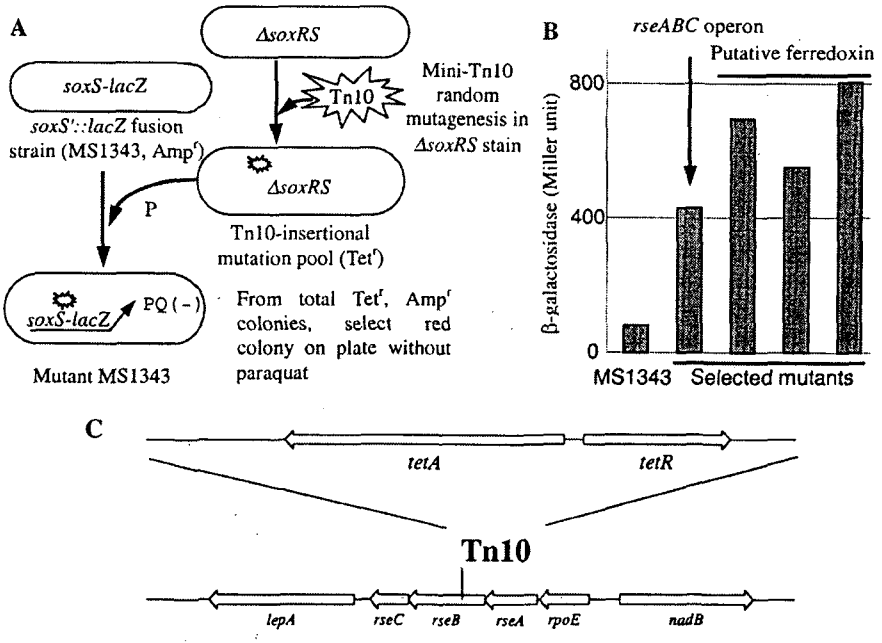

Fig. 1. Screening (A, B) and localization (C) of Tn10-insertion mutants activating SoxS

***soxS* upregulation by MC1306 is not due to the SigE pathway.** The regulation mechanism of SigE and *rseABC* is well characterized. The misfolded proteins by heat shock or Ethanol and the misassembly of the overproduced porins or outer membrane proteins induce the conformational change of RseA, the anti-sigma factor of SigE. Then, SigE is released and activates the SigE-regulon genes. RseB is a periplasmic protein binding to RseA and probably strengthens the anti-sigma activity of RseA. The function of RseC is unknown, yet. Because MC1306 was mapped in *rseB*, we suspected that the elevation of *soxS* expression by MC1306 could be due to the constitutive activation of SigE pathway. To activate the SigE pathway, 42°C heat shock and ethanol were challenged to MS1343, MS1343+MC1306, and *rfaY-lacZ* fusion strain, respectively. Heat and ethanol shock had no effect on soxS expression, implying that the effect of MC1306 is not due to the activation of SigE pathway (data not shown). 50°C heat shock had no effect, too (data not shown).

MC1306 effect is due to *rseC* mutation. To know which of *rseA*, *B*, and *C* is responsible for the MC1306 phenotype each mutation allele were introduced into MS1343. Unexpectedly, when *rseC*⁻ was introduced, the expression of *soxS* increased similar to MC1306. With *rseA*⁻, *soxS* expression increased slightly, while *rseB*⁻ had no effect on *soxS* (data not shown). We had to confirm that *rseC* is really responsible for the *soxS* upregulation, because MC1306 was originally mapped into *rseB* and *rseBC* mutation showed MC1306 phenotype, too. However, *rseB* mutation could give rise to polar effect on *rseC* expression. So, *rseC*⁻ and *rseBC*⁻ mutations were complemented with the episomally expressed RseB and RseC. Only RseC could complement the mutation phenotype. Finally, we directly confirmed the constitutive elevation of *soxS* expression level in *rseC*⁻ strain by Northern hybridization with *soxS* specific probe (data not shown). It excluded the artifact from the mutation of *soxS-lacZ* fusion moiety itself. From our results, MC1306 effect is due to *rseC* mutation

Although SoxR is a sole factor to regulate SoxS expression so far, we had to exclude the possibility that *rseC* mutation effect may be the phenotype by other factor than SoxR. For this, we investigated whether *rseC* mutation effect is SoxR-mediated or not. When the *ΔsoxR* mutation was introduced into *rseC* and *rseBC* mutants, *soxS*-constitutive expression of both mutants disappeared, indicating that SoxR is required for *rseC* mutation phenotype (data not shown). On the contrary, when we introduced *ΔsoxR* into *rseA* mutant, *rseA* mutant still had a constitutive effect on *soxS* expression, indicating that SoxR has nothing to do with *soxS* upregulation in *rseA* mutant. So, we concluded that *rseC* mutation effect is independent of RseA/RpoE pathway and related to the redox state of SoxR, different from *rseA* mutation. So, our further study was concentrated on only RseC.

If *rseC* mutation should be related to the redox state, it could have an effect on OxyR, another redox regulator. To test the SoxR-specificity, we constructed *oxyS-lacZ* single copy fusion strain and introduced *rseC* mutation into it. *oxyS* expression decreased to the half level rather than constitutively increased, implying that *rseC* mutation does not drive OxyR to its oxidized state. So, *rseC* mutation effect is oxR-specific (data not shown).

SoxR is constitutively oxidized in *rseC* mutant. Crucially, we had to detect directly that SoxR is really oxidized in *rseC* stain. EPR (Electron Paramagnetic Resonance) spectroscopy of SoxR was monitored in the whole wild type and *rseC* mutant cell. When the equal amount of cell with equally expressed SoxR was applied to EPR spectroscopy (Fig. 2B), the intensity of signal significantly reduced in *rseC* mutant (Fig. VI-6A). Since only reduced [2Fe-2S] cluster of SoxR can give the EPR signal, It is apparent that *rseC* mutation makes SoxR oxidized and so activated form. From this result, we suggest that the function of RseC is related to the reduction of SoxR; RseC is a SoxR reductase or a component of SoxR reduction system.

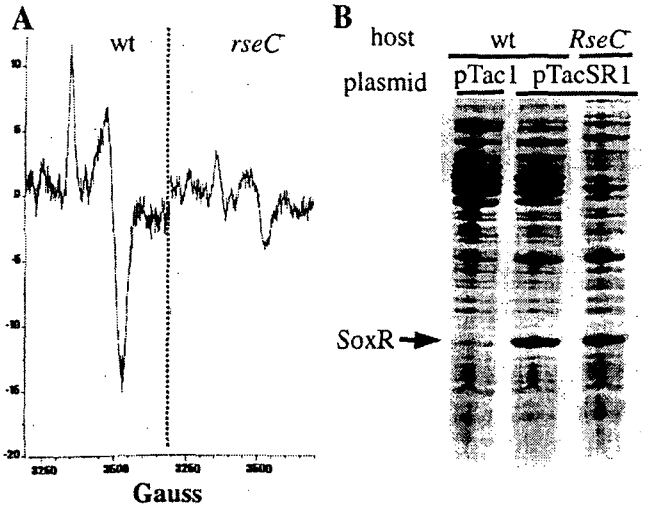

Fig. 2. Whole cell EPR in *rseC* mutant; SoxR in *rseC* is more oxidized

Primary structure of RseC protein. RseC is a small protein whose deduced molecular weight is 17 kD. Although RseC was originally named as regulatory factor of SigE (regulation of sigmaE), there is no evidence that RseC has any regulatory role in the SigE regulon. Moreover, Because RseC has not been studied at all with relation to redox, so far, we could not take any information about it. Instead, we found out one special feature of RseC from its primary amino acid sequence. RseC is cysteine-rich considering its size (total 6 cysteines) and has a CXXC motif in its N-terminus (Fig. 3). Generally, cysteine residues are important in redox change of proteins and especially CXXC is a very important motif in redox regulation of many proteins, such as thioredoxin, glutaredoxin, and Dsb proteins, which are categorized as thioredoxin superfamily. Interestingly, 5 cysteines of total 6 cysteines of RseC are localized in the N-terminus half region, composing the CX_5CXXCX_5C motif. These characters of RseC implied that RseC might be a redox protein to regulate the redox state of SoxR, probably acting as the hypothetical protein, SoxR reductase. To obtain some information from protein database, we searched the RseC-homologous proteins. 3 proteins were found out with significant homology (Fig. 3). Two of them were hypothetical proteins, P44020 and P44060 of *Haemaphilus influenza*, and other one was a RnfF, a *rnfF* gene product of *fdxC-fdxN-rnfF-orf10* gene cluster of *Rhodobacter capsulatus*. *fdxC-fdxN-rnfF-orf10* gene cluster is functionally related to the nitrogen fixation of *R. capsulatus*. Interestingly, all cysteines of CX_5CXXCX_5C motif were conserved in P44020 and P44060 of *H. influenza* and latter three cysteines of them were conserved in RnfF of *R. capsulatus* (Fig. 3), suggesting their crucial role in redox function.

RseC is a membrane protein facing the cytoplasm. Because RseC has many hydrophobic amino acid residues in C-terminal half region, We examined whether RseC had a transmembrane domain, or not. RseC was predicted with high possibility to have two transmembrane domains in its C-terminal region. We also tried to predict the topology of RseC in many web sites and the most representative one of many similar results is presented here, which was predicted by the TMHMM (version 0.1, http://www.cbs.dtu.dk/services/TMHMM-1.0/). This server is for prediction of transmembrane helices in proteins. From the prediction, the possible primary and tertiary structure and topology of RseC are presumed (Fig. 4). For the confirmation of the membrane localization of RseC, we prepared the anti-RseC antibody for Western analysis. After the overproduction of RseC on the multicopy plasmid, pTrc99A, whole cell extract was separated into the soluble and membrane fraction and each fraction was electrophoresed in SDS-PAGE and applied to the Western analysis. RseC was enriched only in the membrane fraction showing that it is a integral membrane protein (data not shown). To verify the predicted topology, we made the LacZ- and PhoA-fused RseC chimeric proteins. LacZ has its activity only in the reduced form without disulfide bond and PhoA has its activity only in the oxidized form with disulfide bond. Because cytoplasm is maintained reduced state (about -260 ~ -

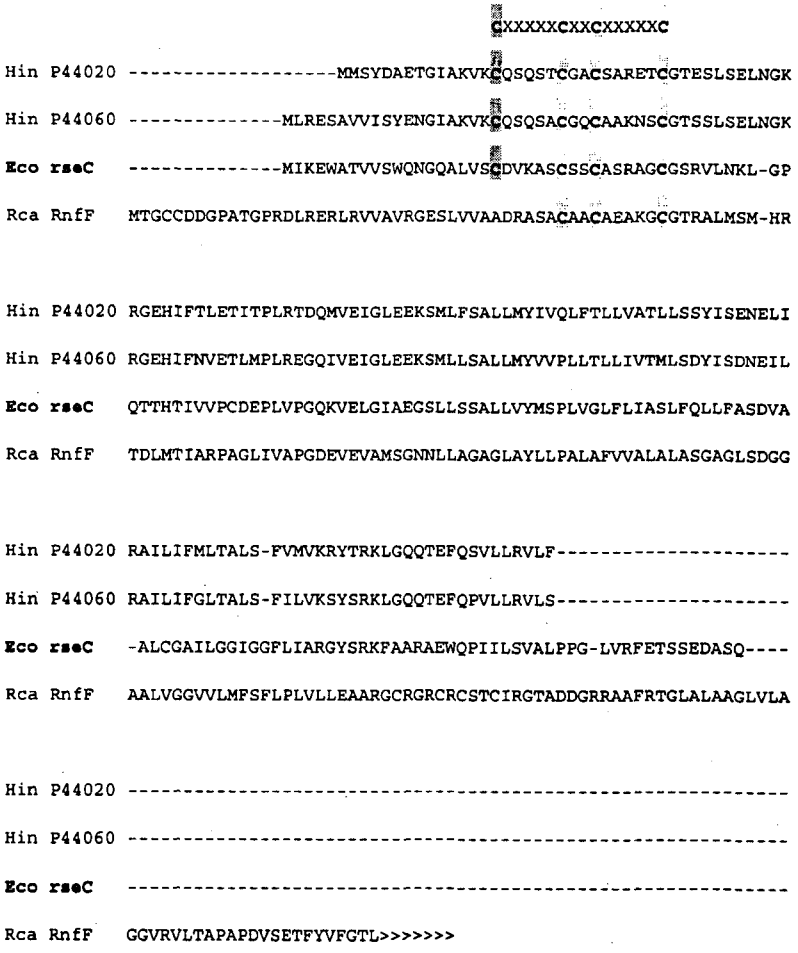

Fig. 3. Alignment of RseC and homologous proteins. Hin, *Haemaphilus influenza*, Rca, *Rhodobacter capsulatus*, Eco, *Escherichia coli*. Rnf, *Rhodobacter* nigrogen fixation

280 mV) by Thioredoxin/Glutaredoxin system and periplasm is generally maintained oxidized state by Dsb system, LacZ has its activity only in cytoplasm and PhoA has its activity only in periplasm. Since PhoA has the signal peptide in its N-terminal region for the secretion, Truncated PhoA was used for fusion, which lacks only the coding region for the signal peptide and five additional amino acid residues. All fusions were constructed on pTrc99A plasmid. The LacZ or PhoA moiety of the chimeric protein will have its activity only in its appropriate localization, cytoplasm or periplasm. With LacZ fusions, LacZ fused at 35th and 130th amino acid of RseC had its activity, but LacZ fused at 98th amino acid had no activity (data not shown). It means that N-terminal domain and C-terminal end of RseC face the cytoplasm and the predicted topology is right (Fig. 4).

The conserved 3 cysteines of $CX_5CX_2CX_5C$ motif and transmembrane domains are essential for the RseC activity. To investigate that the membrane localization of RseC is important for its activity to reduce SoxR, we made a transmembrane domain deletion construct. Because RseC has two transmembrane domains in its C-terminus, we deleted the C-terminal region of RseC serially and fused to trc promoter on pTrcApr. It was investigated whether *rseC*$^-$ phenotype could be complemented with the serial deletion constructs. Only deletion construct with all two transmembrane domains could complement the *rseC*$^-$ phenotype. From this result, we concluded that both transmembrane domains are functionally crucial, but C-terminal end region latter 130th amino acid was partially important. There were two possibilities about the function of transmembrane domain; one is a just structural role to anchor RseC to membrane and the other is a functional role to make RseC functionally active. To answer this question, we constructed two chimeric proteins, $RseC_{N59}$ and $RseC_{N74}$ fused by transmembrane domain of EnvZ to swap the transmembrane domains. When the chimeric proteins were introduced into *rseC*$^-$ mutant, they could not complement the *rseC*$^-$ phenotype, indicating that the transmembrane domains has a functionally important role.

Since cysteine residues are generally important in redox regulation and the cysteines of $CX_5CX_2CX_5C$ motif are conserved in other organisms, we examined that the cysteines are essential for the activity of RseC. For this purpose,

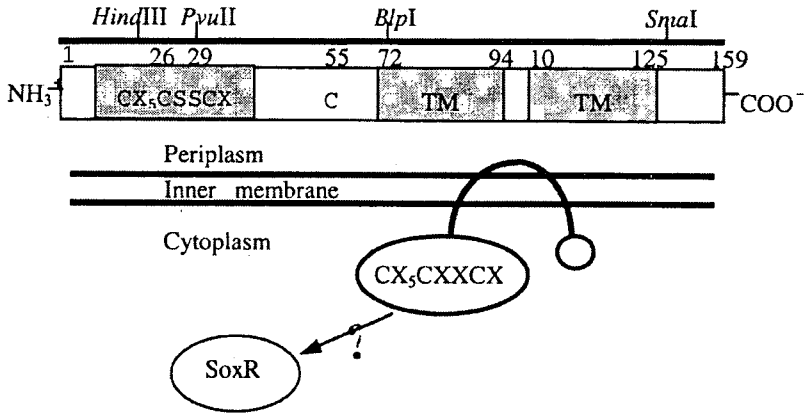

Fig. 4. RseC is a membrane protein facing the cytoplasm

the substitution mutagenesis of the cysteines to serines was done. We constructed all possible combinations of the cysteine-to-serine substitutions of the $CX_5CX_2CX_5C$ motif on the pTrcApr. The complementation test was done to monitor the activities of the plasmid-carrying mutants of RseC in *rseC*⁻ strain. The result showed that two cysteines of CX_2CX_5C, the latter part of $CX_5CX_2CX_5C$ motif are essential because the substitution of more than 2 cysteines of the CX_2CX_5C made RseC lose its SoxR reduction activity (Fig. 5).

UV-visible spectrum of the purified $RseC_{N75}$. While two electrons are generally transferred by disulfide bond exchange, other redox moieties, such as Fe-S cluster, flavins, quinones, and metal centers excel at univalent electron transfers and are therefore plausible one electron donors. Since SoxR has [2Fe-2S] cluster, which is oxidized or reduced by one electron transfer and DTT, the most representative thiol reductant cannot reduce SoxR *in vivo* (data not shown), it is reasonable that RseC has a certain cofactor, such as metal center and reduce SoxR by the cofactor rather than simple disulfide exchange. In this case, essential cysteines of RseC are likely to act as ligands for unknown cofactor. However, in the protein data base, we could not find any motif for cofactor binding in RseC. We investigated the UV-visible spectrum of purified $RseC_{N75}$. $RseC_{N75}$ had a characteristic peak at 320 nm. We don't know what provoke this peak but it strongly implies the existence of a certain cofactor. Metal analysis is required. When $RseC_{N75}$ was reduced by DTT or dithionite, there was no change in the peak (data not shown).

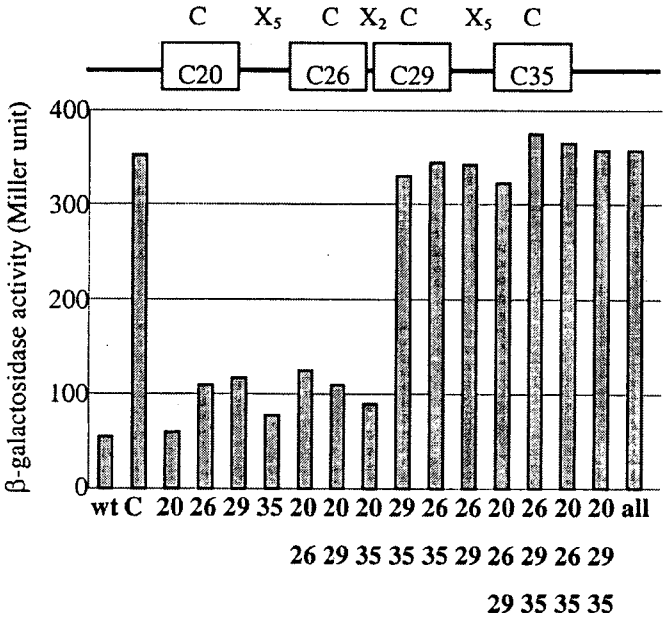

Fig. 5. Cysteine to serine substitution mutagenesis

Inactivation kinetics of SoxR in *rseC* mutant. The activated SoxR returns to the basal reduced state very rapidly at the withdrawal of redox cycling agent, probably by its reductase. If RseC is a SoxR reductase or a component of SoxR reduction system, inactivation rate of SoxR will be retarded in *rseC* mutant. From this presumption, we measured the inactivation kinetics of SoxR in wild type, *rseC* mutant, and *resB* mutant, which is an another candidate of SoxR reductase. *resB* gene was found out with *rseC* from the same screening and encodes unknown ferredoxin like protein. In *rseC* mutant, inactivation rate of SoxR was slightly retarded while it was significantly retarded in *resB* mutant (Fig. 6). This result strongly implies that ResB is also related to SoxR reduction system more closely than RseC. If both of RseC and ResB are involved in SoxR reduction, are they in the same lineage of the reaction, or different pathway? To answer this question, the double knock-out mutant of *rseC* and *resB* was constructed in MS1343 strain. The expression level of *soxS* was same as a single mutant, implying that they are in the same pathway of SoxR reduction (data not shown). Probably SoxR must be reduced *in vivo* not by sole factor, SoxR reductase, but by multiple factors, SoxR reduction system, including both of RseC and ResB.

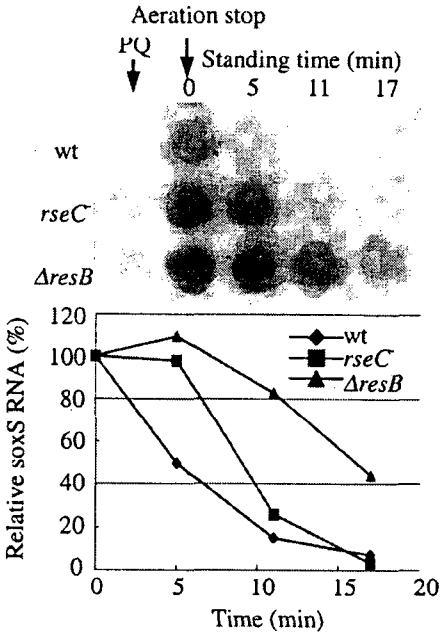

Fig. 6. Inactivation kinetics of SoxR

RseC reduce *soxS* transcription *in vitro*. Because ResB is also involved in the SoxR reduction, *in vitro* reconstitution experiment was required to know the minimal set of factors necessary for SoxR reduction. First of all, for the direct detection of SoxR activity, we tried the *in vitro* transcription assay with $RseC_{N75}$ as preliminary test. The purified RNAP (containing σ^{70}), SoxR, and $RseC_{N75}$ were reconstituted with *soxS* promoter as template and amount of *soxS* transcript was measured by phosphoimager (Fig. 7). Since we didn't know exactly what is the electron donor for RseC activity, we used NADPH as potential electron donor. With increasing amount of $RseC_{N75}$, SoxR activity significantly decreased, compared with the decrease by chemical reductant, dithionite (DTN). Without NADPH, SoxR activity still decreased by $RseC_{N75}$, but extent of decrease was smaller than that with NADPH (data not shown). For the precise estimation of decrease of SoxS transcript, we used the tac promoter as internal control. When it was normalized with the transcript from tac promoter, the *soxS* transcription decreased by 50% with 16/19 molar ratio of $RseC_{N75}$ to SoxR, in proportion to the amount of $RseC_{N75}$. To confirm this result, same assay was repeated with 70 bp longer *soxS* promoter as template and showed the consistent result (data not shown).

RseC has a peroxiredoxin activity. We speculated that RseC might have peroxiredoxin activity from the clues following; 1) RseC is multimerized in the oxidized condition, which is a property of peroxiredoxin, 2) it has the conserved and crucial cysteine residues, and 3) it is resistant to the oxidation by H_2O_2 but not diamide. To test the peroxiredoxin activity, DNA cleavage assay in the iron-catalyzed oxidation (MCO) system was employed, which contained supercoiled DNA (pUC18), 10 mM DTT, 0.4 mM Fe^{3+}. Without protectant, supercoiled DNA would be cleaved to open circle or linear DNA by the hydroxyl radical generated from MCO system. With purified $RseC_{N75}$ as a protectant protein, supercoiled DNA could be protected from the degradation in MCO system (data not shown). When bovine serum albumin (BSA) and inactivated $RseC_{N75}$ with anti-RseC antibody were used as protectant to

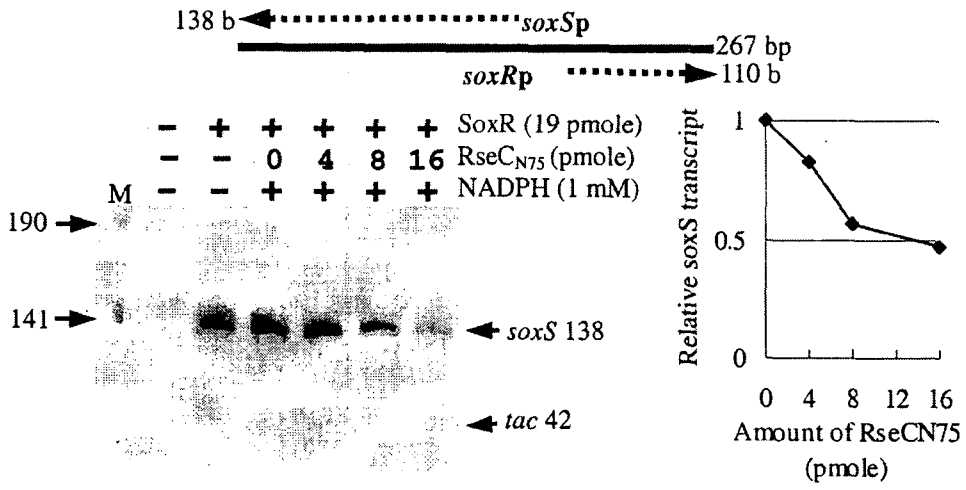

Fig. 7. Repression of SoxR activity by $RseC_{N75}$; *in vitro* transcription assay

confirm the specificity of $RseC_{N75}$ for the protection, no protection activity was observed. The protection activity of $RseC_{N75}$ was fairly specific. This means that RseC has a peroxiredoxin activity *in vitro*.

To certify the antioxidant activity of RseC *in vivo*, we examined the resistance of RseC-overexpressing cell to the H_2O_2 and cumene hydroperoxide. Wild type RseC- and $RseC_{N75}$-overproducing cells were cultured on the various oxidant-containing plates, such as menadione, plumbagin, paraquat, H_2O_2, cumene hydroperoxide, and etc. Wild type RseC and $RseC_{N75}$ were episomally overexpressed on the multicopy plasmid, pTrcApr. *rseC* mutant was slightly sensitive to high concentration of menadione and plumbagin, but not to H_2O_2, and cumene hydroperoxide (data not shown). On the contrary, wild type RseC- and $RseC_{N75}$-overproducing cell were resistant to H_2O_2, and cumene hydroperoxide, but not to menadione and plumbagin (data not shown). It is consistent with *in vitro* result that RseC has a peroxiredoxin activity, because RseC-overproducing cell became resistant to peroxide, such as H_2O_2 and cumene hydroperoxide.

Discussion

In this study, we report the isolation and characterization of RseC as the candidate of SoxR reductase. So far, SoxR reductase has been hypothesized but not characterized. We isolated the candidates, RseC with genetic method, random mutagenesis with miniTn10 and screening the mutants elevating the *soxS* expression. In *rseC* mutant, SoxR is more oxidized form and so activate *soxS* expression constitutively. RseC has conserved cysteine residues and tr ansmembrane domains, which are functionally crucial. N-terminal domain of RseC faces cytoplasm and redu ced the transcription of *soxS in vitro*. *rseC* mutant is slightly sensitive to high concentration of redox cycli ng agent. RseC has peroxiredoxin activity *in vitro* and *in vivo*. *rseC* mutant has some differences from wil d type in stationary phase.

Although SoxRS regulon of *E. coli* is a well-characterized system, many questions still remained unrevealed. One of these questions is about the redox-sensing mechanism of SoxR. The *soxRS* regulon is induced by redox-cycling agents. This process is accompanied by the consumption of cellular reductants, raising the question as to whether the SoxR protein responds directly to superoxide or indirectly to the depletion of NADPH. Experimental results supporting both views have been reported, but no consensus has been reached. In addition, the activation of the SoxR by nitric oxide might occur through another different mechanism that remains to be understood, Although some evidences of the direct nitrosylation of [2Fe-2S] center of SoxR by nitric oxide were recently suggested.

One important question that has not been resolved is how the reduced state of SoxR is maintained to counter auto-oxidation during normal aerobic growth. This is a question about SoxR reductase or reduction system. The redox potential of the [2Fe-2S] cluster of SoxR was determined to be -285 mV, suggesting that the reduction of SoxR may be linked to the NADPH/$NADP^+$ redox pool, which has an estimated redox potential of -340 mV. But no gene or protein related to electron transfer between NADPH/$NADP^+$ redox pool and SoxR was found.

Here, we intended to isolate the unknown intermediate factors by screening the transacting factors affecting SoxR activation. We showed some plausible results reflecting that RseC has SoxR-reducing activity. From the results we suggest that the function of RseC is related to the SoxR reduction. With the same purpose, some groups used a biochemical approach. Since *fpr* (NADPH:flavodoxin oxidoreductase), *fldA* (flavodoxin 1, an iron-sulfur protein reductase), and *zwf* (glucose 6-phosphate dehydrogenase, and NADPH-generating enzyme) are targets of SoxRS

regulation, it is attractive to speculate that the oxidized SoxR is reduced by the NADPH:flavodoxin oxidoreductase / flavodoxin 1 couple with the consumption of NADPH, making SoxRS regulation autoregulated. It is also reasonable approach but so far, no clue has been obtained. More work is required to elucidate the oxidation and reduction mechanisms of SoxR.

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