Differential activities of the SoxR protein of *Escherichia coli*: SoxS is not required for gene activation under iron deprivation

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Abstract

When *Escherichia coli* cells are under superoxide stress, proteins SoxR and SoxS, acting sequentially, control the expression of a set of repair and defense genes. One of these genes, *fumC*, encoding fumarase C, was reported to be also activated by iron deprivation in a soxRS-dependent manner. However, the same condition failed to induce the expression of a *soxS*:lacZ fusion. The expression of *acnA* (aconitase A) is also activated by SoxR alone when under iron deprivation, but not of *sodA* (Mn-superoxide-dismutase). SoxR completely inhibited the migration of a DNA fragment containing the promoter region of *fumC*, in gel-shift experiments. SoxR might bind to a different region than SoxS within the *fumC* promoter, or an unknown intermediate other than SoxS might be acting. It is possible that the regulatory role of SoxR is more complex than previously considered. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: *soxRS*; Stress response; *Escherichia coli*

1. Introduction

The *soxRS* regulon of *Escherichia coli* includes defense and repair mechanisms, that increases cell resistance to oxidants and other xenobiotics, including antimicrobial drugs [1]. Its expression is activated by increased intracellular superoxide concentration [2,3] and, directly or indirectly, by a number of other agents (e.g. Hg2+ [4], H2O2 [5]). The regulon is controlled by [2Fe–2S]SoxR, which activates the transcription of *soxS*. SoxS, in turn, is a transcription factor for the members of the regulon [6,7]. In addition to increased resistance to antibiotics, the activation of the *soxRS* regulon also increases resistance to activated macrophages; therefore, *soxRS* has been considered a virulence locus [8].

The lack of iron seems to be among the *soxRS* activating conditions. Gunsalus and Park reported a *soxRS*-dependent induction of *fumC* in cells treated with 2,2-dipiridyl (Dip), an iron chelator [9]. *fumC* encodes a non-iron-containing fumarase, which replaces superoxide-labile fumarase A, an iron-containing enzyme. *fumC* is a known member of the *soxRS* regulon, and its expression is increased when cells are treated with superoxide-generating agents [10]. Here, we explored the induction of *fumC* and *sodA* and *acnA*, other members of the *soxRS* regulon also encoding ‘replacement’ enzymes elicited by the lack of iron. Apparently, when deprived of iron, SoxR can induce by itself the expression of some of the genes of the *soxRS* regulon. SoxR is therefore a key regulator of responses to diverse environmental stress, acting differentially upon several promoters.

2. Materials and methods

2.1. Strains and culture conditions

Strains, phages and plasmids used in this study are described in Table 1. Cells were cultured in LB medium. Strains GC4468 and DJ901 were transfected with phage λSJP25, resulting in strain AF1010.
2.2. soxS and fumC expression analyses

To assess the effect of iron deprivation upon the expression of soxS and fumC, overnight cultures were diluted 1:100 in fresh media and incubated for 90 min at 37°C/200 rpm. Dip (Aldrich) was added to a final concentration of 150 μM (paraquat, PQ, 50 μM was used as positive control; 1 mM IPTG was used to induce the expression of plasmid-carried genes under ptac control), and incubation was continued for 30 min more. β-Galactosidase activity resulting from the expression of lacZ fusions was assayed as previously described [11].

2.3. acnA expression analysis

The effect upon the expression of acnA was assessed by detection of acnA mRNA by RT-PCR. After treatment, RNA was extracted as previously described [12], treated with DNase, and then RT-PCR was performed using buffer and Mn-acetate Tth DNA polymerase (Roche) using buffer and Mn-acetate Tth with DNase, and then RT-PCR was performed using RNA was extracted as previously described [12], treated acnA detection of 150W200 rpm. Dip (Aldrich) was added to a final concentration of 150 μM (paraquat, PQ, 50 μM was used as positive control; 1 mM IPTG was used to induce the expression of plasmid-carried genes under ptac control), and incubation was continued for 30 min more. β-Galactosidase activity resulting from the expression of lacZ fusions was assayed as previously described [11].

2.4. SOD activity measurement

After Dip treatment, cells were disrupted using a Bead-Beater, and the crude extract applied to a superoxide-dismutase-activity gel, as previously described [13].

2.5. Gel-shift experiments

32P-labeled PCR-amplified fragments, one of 179 bp (primers: 5'-CCTGATTAAAAAGCTGACTAC and 5'-TCATCGTTTCGTACGCAGTTTAAGTTTG) containing the promoter region of soxS, and another of 427 bp (primers: 5'-ATTCCACTTACGTGTTATATAA and 5'-ACCCCTCGAT-ACGTCGTTCCTGCC) containing the promoter region of fumC, were incubated with 5–25 ng of SoxR protein (a kind gift from V. Le Faúndal, Harvard School of Public Health, purified as reported [6], in DNA binding buffer as previously described [14], with or without 150 μM Dip. DNA–protein mixes were loaded into a 12% non-denaturing polyacrylamide gel and run for 2.5 h at 6 V cm⁻¹. Gels were dried, and the DNA was visualized by autoradiography.

3. Results

When treated with Dip, strains carrying an active soxR gene and a soxS⁻:lacZ fusion did not show increased β-galactosidase activity, although PQ treatment induced a ~3-fold increase (Fig. 1). β-Galactosidase activity was not detected in soxR⁻ strains, regardless of the treatment (not shown). Also, ΔsoxRS strains carrying a fumC⁻:lacZ fusion did not show changes in β-galactosidase activity if treated with Dip. However, the sole presence of the soxR gene (under the control of a tac promoter, within plasmid pSXR) induced a slight increase; further treatment with Dip, IPTG, or both, caused a 2.5-, 3- and 4-fold increase,
respectively, in β-galactosidase levels (Fig. 1). Crude extracts (20 μg of total protein) of DJ901 cells, bearing pSXS or pSXR, treated with 50 μM PQ or 150 μM Dip, were run in an SOD activity gel. Fe-SOD, a constitutively expressed enzyme, appears at the bottom. A negative image of the gel is shown.

Fe-SOD

Mn-SOD

pSXR

pSXS

Dip

PQ

control

Fig. 2. Mn-superoxide-dismutase expression under iron deprivation. Crude extracts (20 μg of total protein) of DJ901 cells, bearing pSXS or pSXR, treated with 50 μM PQ or 150 μM Dip, were run in an SOD activity gel. Fe-SOD, a constitutively expressed enzyme, appears at the bottom. A negative image of the gel is shown.

SOD activity was slightly diminished. Amplification products from an RT-PCR reaction targeting the acnA mRNA were detected ΔsoxRS cells bearing pSXS regardless the treatment (PQ or Dip), and in cells bearing pSXR, except for those treated with PQ (Fig. 3). acnA mRNA was not detected in cells carrying only the vector plasmid.

The addition of SoxR protein retarded the migration of a DNA fragment spanning the promoter region of the soxS gene in a polyacrylamide gel, as previously reported [14]. A complete retardation of a DNA fragment containing the promoter region of fumC was achieved at a higher concentration of SoxR, or when Dip was added to the

Fig. 4. Band retardation caused by the presence of SoxR. PCR-amplified DNA fragments containing the promoter region of soxS or fumC (location of each fragment as depicted in the bottom; sox and fum genes are not represented at the same scale) were incubated with different amounts of SoxR protein, treated or not with 150 μM Dip, and then run into a polyacrylamide gel (see text).
mixture (Fig. 4). The addition of Dip to DNA fragments alone or to psoxS+SoxR did not modify their migration (not shown).

4. Discussion

The ‘soxRS-dependent’ induction of fumC caused by iron deprivation was concluded from experiments comparing wild-type soxRS and ΔsoxRS strains [9]. However, the independent effect of each sox gene upon the regulation of fumC was not explored. We failed to detect induction of the expression of soxS’:::lacZ after treatment with the iron chelator Dip. This clearly indicates that the overexpression of fumC caused by the same treatment, occurring on soxRS+ but not on ΔsoxRS strains, follows a pathway different from the well-known mini-cascade elicited by superoxide stress. The sole presence of soxR within a ΔsoxRS background, slightly increases the expression of fumC’:::lacZ fusion; iron deprivation, soxR overexpression, or both, causes an increase in fumC’:::lacZ expression. This indicates that soxR alone, or through another unknown intermediate product, should exert this regulatory effect. This also seems to be the case of another TCA-cycle enzyme substitute, aconitase A. However, ΔsoxRS strains bearing a soxR-containing plasmid, did not modify their expression of sodA when treated with Dip, indicating that SoxR can not affect the promoters of all soxRS regulon genes.

Our preliminary attempts to demonstrate the binding of the SoxR protein to the promoter region of fumC yielded atypical results. Gel-shift experiments resulted in a retardation of a labeled DNA fragment containing the fumC promoter, but the effect was so dramatic that the possible DNA–protein complex was trapped on the gel well. Such effect was not observed when mixing SoxR and a DNA fragment containing the soxS’ promoter; if a large protein complex is responsible for the inability to migrate into the gel, its formation is pfumC-dependent, and it binds to pfumC-containing DNA. This effect required more SoxR protein than that required to retard the migration of the DNA fragment containing the soxS’ promoter; further treatment with the iron chelator enhanced the ability of SoxR to prevent the migration of the pfumC DNA fragment. Although this could be evidence of the binding of SoxR to the promoter of fumC, further experiments are needed in order to establish if such binding actually occurs, the DNA region involved, and the affinity of the SoxR protein towards it. It is important to stress here that binding of both, SoxS and SoxR to the same DNA region, is not entirely unusual: SoxS and SoxR bind to the soxS’ promoter region. Comparison of the predicted amino acid sequences of SoxS, MarA and Rob (all homologous regulatory proteins that bind to the promoters of a number of genes, including fumC and acnA), with SoxR, showed a similar helix–turn–helix DNA binding motif.

However, the amino acid residues that seem to be responsible for sequence identification [15], are not present in the SoxR protein. This suggests that SoxR might bind to a DNA region different from the one recognized by SoxS and other similar regulatory proteins.

Whether SoxR regulates fumC and acnA by direct binding to their promoters, when under low iron availability, or through an intermediate other than SoxS, remains to be established. It is interesting to note that predicted amino acid sequences homologous to both SoxS and SoxR were found only in Salmonella enterica serovar Typhimurium. However, in the chromosomes of Pseudomonas aeruginosa and Vibrio cholerae there are open reading frames encoding putative proteins homologous to SoxR of E. coli, but not to SoxS. Putative SoxR proteins deduced from the genome sequence of P. aeruginosa and V. cholerae are of 156- and 148-amino acid length, with a 62 and 54% identity, respectively, both containing the characteristic cysteine cluster at the C-terminal end [16]. This suggests that SoxR might play an independent regulatory role. The soxRS regulon was recently shown to furnish clinically relevant antibiotic resistance to a Salmonella strain [17], and also can provide some protection against ozone toxicity [18], in addition to the role in virulence associated to superoxide and nitric oxide resistance [8]. Defining the precise mechanisms of activation of SoxR can provide further insights into the control of virulence in Gram-negative pathogens.

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