The dimeric repressor SoxR binds cooperatively to the promoter(s) regulating expression of the sulfur oxidation (sox) operon of *Pseudaminobacter salicylatoxidans* KCT001

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Sulfur oxidation in *Pseudaminobacter salicylatoxidans* KCT001 is rendered by the combined action of several enzymes encoded by a thiosulfate-inducible sox operon. In this study it has been conclusively demonstrated by insertional mutagenesis that the regulatory gene of this operon is soxR, which encodes a DNA-binding protein belonging to the ArsR-SmtB family. SoxR was found to bind to two promoter-operator segments within the sox cluster, of which the one (wx) located between soxV and soxX controls the expression of sulfur-oxidation genes soxX through soxD while the other, a bi-directional element (sv) located between soxS and soxV, controls the expression of soxVW in one direction and the putative regulatory cluster soxSRT in the other. In the case of the wx promoter the repressor was found to bind in a cooperative manner to two distinct binding sites having different affinities, while in the case of the sv promoter binding occurred at a symmetric dimeric site and involved a higher degree of cooperativity. The high degree of cooperativity observed in the binding of SoxR to its target sites seemed to be due to the propensity of SoxR monomers to form dimers. The apparent dissociation constants of the SoxR–operator complexes were in the nanomolar range, indicating relatively strong interactions. It was demonstrated using a reporter system in *Escherichia coli* that this high-affinity binding of SoxR led to efficient repression in *trans*. Thus the role of SoxR as a repressor of the sox operon has not only been conclusively established but it has also been shown that this repression is brought about through cooperative interactions of SoxR with dimeric binding sites that occlude the operon promoters.

INTRODUCTION

Reducing equivalents produced during the oxidation of sulfur compounds are used by chemolitho-autotrophic bacteria for aerobic respiration as well as carbon dioxide fixation, while anaerobic phototrophic bacteria utilize them primarily for carbon dioxide fixation. Thiosulfate, the only sulfur substrate that is universally oxidized by the majority of known chemolithotrophic organisms irrespective of their taxonomic identity, is oxidized directly to sulfate by the *α*-proteobacteria without the formation of any detectable intermediate sulfur compounds in the medium (Kelly, 1989). Genetic studies with both chemo- and photolitho-trophic sulfur-oxidizing *α*-proteobacteria like *Paracoccus pantotrophus*, *Pseudaminobacter salicylatoxidans* KCT001 and *Rhodovulum sulfidophilum* have recently led to the identification of a cluster of sulfur oxidation (sox) genes, viz. soxVW and soxXYZABCDEFGH (Friedrich et al., 2000; Mukhopadhyaya et al., 2000; Rother et al., 2001; Appia-Ayme & Berks, 2002). A consensus mechanism allegedly governing the complete oxidation of thiosulfate, sulfite, sulfide and elemental sulfur has been proposed with *α*-proteobacteria as model systems involving a sulfur-oxidizing multi-enzyme complex comprising the thiosulfate-induced periplasmic proteins SoxXA, SoxYZ, SoxB and SoxCD (Friedrich, 1998; Friedrich et al., 2001; Appia-Ayme et al., 2001). While the proteins SoxV and SoxW are believed to be involved in the biosynthesis or maintenance of the multienzyme complex system (Bardischewsky & Friedrich, 2001; Appia-Ayme & Berks, 2002), the genes soxEFGH, though co-expressed with the sox structural genes in *P. pantotrophus*, might not be essential for sulfur oxidation by the aforesaid mechanism (Rother et al., 2001).

Although some information is available regarding the function of the sox structural genes, there is still insufficient
knowledge about the regulation of their expression. While mutational and physiological studies with *Pseudaminobacter salicylatoxidans* KCT001 had previously indicated that the gene cluster *soxSRT* could be associated with the regulation of this operon (Lahiri *et al.*, 2006), insertional mutagenesis of *soxS* of *Paracoccus pantotrophus* resulted in low levels of constitutive expression of *sox* genes (Rother *et al.*, 2005). This low-level constitutive expression in *soxS*-inactivated mutants was however attributed to a polar effect on *soxR*, as the mutant phenotype could be suppressed by the introduction of a plasmid carrying a DNA fragment corresponding to *soxR*. Although these observations suggested a possible role for SoxR as a repressor, it is still necessary to obtain more direct evidence by inactivating *soxR* itself and investigating the resulting phenotype. Moreover, although the ability of the SoxR to bind with DNA sequences had been demonstrated earlier, no detailed analysis of binding isotherms and/or delineation of binding sites had been performed.

Previous theoretical investigations by our laboratory with the SoxR of *Pseudaminobacter salicylatoxidans* KCT001, the model organism of the present study, had revealed that the protein was capable of binding as a dimer to regulatory regions within the *sox* cluster (Bagchi *et al.*, 2005). The general lack of detailed knowledge regarding the repressor function of SoxR and the availability of a hypothetical model for the binding of *P. salicylatoxidans* KCT001 SoxR to its target site motivated us to express SoxR of this organism in *Escherichia coli* and use the purified protein to study its DNA-binding activities. The present investigation not only provides conclusive evidence for the repressor function of SoxR but also offers new insights into the understanding of the binding sites as well as the mechanism of binding of SoxR to different regions within the *sox* locus (Fig. 1).

**METHODS**

**Bacterial strains, plasmids and growth conditions.** The bacterial strains and plasmids used in this study and their sources are listed in Table 1. *E. coli* XL-1 Blue (Bullock *et al.*, 1987) or TOP10 was used for cloning experiments, expression work and promoter assays. For promoter assays a β-galactosidase-based reporter plasmid (pSD5B) was used (Jain *et al.*, 1997). Originally, the vector was constructed for studying mycobacterial promoters. However, pSD5B is equally effective as a promoter-probe vector for *E. coli*.

![Fig. 1. Physical map of the *sox* gene cluster of *P. salicylatoxidans* KCT001. Horizontal arrows indicate the ORFs and their direction of transcription. ORFs are not in exact scale. Upward vertical arrows demarcate the two binding sites (*sv* and *wx*) of SoxR.](http://mic.sgmjournals.org)
was subsequently transformed into competent pQE30 to generate the recombinant plasmid construct pQER, which carried a HinHI digest with BamHI. The amplified DNA fragment was 9 SoxRC (5′BamHI) that was used in the above amplification carried a unique (Invitrogen). The sulfonated arabinose-inducible promoter using the vector pBAD. The forward primer (SoxRR) 5′GTCATAGGATCCATGCATGGGAACCCGCAA-3′ was that used in the above amplification carried a unique BamHI site at the 5′ end of the gene, while the reverse primer (SoxRR) 5′GCCAGAAGCTTCTTGGCGGATGTATT-3′ carried a HindIII site located 13 nucleotides downstream from the TAA translation stop codon. The amplified DNA fragment was digested with BamHI and HindIII and ligated into the same site of pQE30 to generate the recombinant plasmid construct pQER, which was subsequently transformed into competent E. coli XL-1 Blue (Bullock et al., 1987). SoxR was also expressed from the tightly regulated arabinose-inducible system pBAD This study.

**Expression and purification of recombinant SoxR.** Recombinant SoxR was overproduced in E. coli M15 cells. The cells were grown at 37 °C in 500 ml LB containing appropriate antibiotic selection up to an OD600 of 0.7. Expression of SoxR was induced by adding 1 mM IPTG. In the case of expression from pBADR, induction was done by adding 0.2 % (w/v) arabinose. Whenever tight repression of expression from pBADR was necessary, 0.2 % (w/v) glucose was added in place of arabinose. For purification of recombinant SoxR, the pQE-based IPTG-inducible system (pQER) was preferred, in which the protein was tagged with six histidine residues. IPTG-induced E. coli cells harbouring pQER were grown for 4 h, after which the cells were harvested, washed with 0.9 % (w/v) NaCl, resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl and 10 mM imidazole) and lysed by sonication. The insoluble materials were separated by centrifugation (10 000 g). The soluble fraction was applied to a Ni2+-NTA agarose column (Qiagen) equilibrated with lysis buffer. The column was washed with 10 volumes of lysis buffer and the protein was eluted with a 20 ml linear gradient of imidazole (20–500 mM) in the same buffer. The fraction was assessed for its purity by SDS-PAGE. Fractions containing SoxR protein were pooled and dialysed against storage buffer (50 mM NaH2PO4, 250 mM NaCl, 0.1 mM EDTA and 10 %, v/v, glycerol) for 10 h at 4 °C.

**Gel retardation assay.** For the electrophoretic mobility shift assay (EMSA), promoter fragments derived from the intergenic region between soxS–soxV (sv) and soxW–soxX (wx) (Fig. 1) were amplified using p23P-labelled primers SR (5′-GTCGCCGATTTCGAGTG-3′) and 10 %, v/v, glycerol) for 10 h at 4 °C.

### Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>recA1 lac endA1 gyrA46 thi hsdR17 supE44 relA1 [F’ proAB lacF’/ZAM15 Tn10(Tet’)]</td>
<td>Bullock et al. (1987); Stratagene</td>
</tr>
<tr>
<td>TOP10 F- mcrA Δ(mrr-hsdRMS-mcrBC) 80lacZ AM15lacX74 dioR recA1 araD139 Δ(araA-leu)7697 galU galK rpsL endA1 supG</td>
<td>Invitrogen</td>
<td></td>
</tr>
<tr>
<td>SY327Δpir</td>
<td>Δ(lac pro) argE (Am) recA (Rif) nalA Δpir</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td>M15 KCT001SR</td>
<td>Sox R SmR Rif (spontaneous mutant of KCT001)</td>
<td>Mukhopadhyaya et al. (2000); DSM 13826</td>
</tr>
<tr>
<td>KCT001S</td>
<td>soxR::Ω Km (soxR-inactivated KCT001SR)</td>
<td>This study</td>
</tr>
<tr>
<td>pBADR</td>
<td>soxR ORF cloned in BamHI–HindIII site of pQE30</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD</td>
<td>Ap′ ara promoter</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBADR</td>
<td>soxR ORF cloned in BamHI–HindIII site of pBAD</td>
<td>This study</td>
</tr>
<tr>
<td>pSD5B</td>
<td>Promoter-probe vector; Km′</td>
<td>Jain et al. (1997)</td>
</tr>
<tr>
<td>pSD5V</td>
<td>S–V intergenic region cloned in Xbal site of pSD5B</td>
<td>This study</td>
</tr>
<tr>
<td>pSVDVS</td>
<td>S–V intergenic region cloned in reverse orientation in Xbal site of pSD5B</td>
<td>This study</td>
</tr>
<tr>
<td>pSDWX</td>
<td>W–X intergenic region cloned in Xbal site of pSD5–β-Gal</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Ability to oxidize reduced sulfur compounds and chemolithotrophic growth is denoted by Sox+.*
and VR (5′-GGCGAGACGTAAGACGAAAG-3′) for sv, and VF (5′-GCTCTAGAGGAGTAGTTCAAGGTTT-3′) and XR (5′-GCTCTAGATCTCTCTGCCCTCCCA-3′) for wx, in PCR using the KCT001 genomic DNA as template. Primer labelling was done by cinasing 10 pmol of the desired primer with [γ-32P]ATP (BRIT, Bombay, India) and T4 polynucleotide kinase (New England Biolabs), the product being used directly in PCR after heat inactivation. The PCR product was purified using a PCR purification kit (Qiagen). The binding reaction mixture contained 30 µl final volume, unless mentioned otherwise, contained the desired amount of purified protein, 3 µl 10× binding buffer (100 mM Tris/Cl pH 8, 300 mM NaCl, 30 mM MgCl2, 1 mM EDTA, 20%, v/v, glycerol) and 1 µg salmon sperm DNA. Reaction mixtures were preincubated for 10 min followed by a further 10 min incubation on ice after adding 10000 c.p.m. of labelled DNA amplicon. The reaction mixtures were separated on a 5% native polyacrylamide gel (following pre-run at 100 V for 1 h) by electrophoresis in 0.5× Tris/Cl borate (50 mM Tris/borate, 1 mM EDTA) at 200 V for 3–4 h at 4°C. Following electrophoresis, gels were vacuum dried and the bands were visualized by autoradiography. For quantification, intensities of bands corresponding to SoxR-bound and free DNAs were densitometrically estimated using an imaging densitometer (Bio-Rad GS-700).

**DNase I footprint assay.** DNase I footprint analysis was performed with the above-mentioned probe. Approximately 0.31 pmol labelled DNA was incubated with SoxR protein for 20 min at room temperature. Then 50 ng DNase I (Sigma) was added and incubated for 3 min at room temperature. Digests were stopped with DNase I stop solution (50 mM Tris/Cl pH 8, 50 mM EDTA, 2%, w/v, SDS and 0.4 mg proteinase K ml⁻1). Digested DNA fragments were resuspended in loading buffer [98% (v/v) deionized formamide, 10 mM EDTA, 0.025% (w/v) xylene cyanol and 0.025% (w/v) bromophenol blue], boiled for 5 min followed by rapid chilling and separated by gel electrophoresis on an 8% (w/v) urea/Tris/borate/EDTA sequencing gel at 1200 V for 3.5–4 h. The gel was dried on Whatman paper and exposed to Kodak BioMax film. An A–G ladder was prepared with 0.31 pmol labelled DNA according to standard protocol (Sambrook & Russell, 2001) and analysed along with the digested DNA.

**Promoter construct and co-transformation.** The sv and wx intergenic regions were amplified from KCT001SR genomic DNA with primer pairs SR1 (5′-GCTCTAGATCCGCAATTTAC-CAGT-3′) and VR1 (5′-GCTCTAGAAGGGCGAAGATGACAG-GAAG-3′) for the sv operator/promoter region, and WF1 (5′-GCTCTAGAAGGAGTAGTTCACAGGGTTT-3′) and XR1 (5′-GCTCTAGATCATATCTCTGCCCTCCCA-3′) for the wx operator/promoter region. All the primers have XbaI sites in their 5′ ends. The XbaI-digested amplicons were cloned upstream of the lacZ cartridge in the promoter-probe vector pSD5B, resulting in the recombinant plasmid pSDSV (sv promoter which expresses soxVW), pSDVS (containing the sv region in the opposite orientation, soxSRT direction) and pSDWX (wx promoter which expresses soxX–D). To set up complementation experiments, promoter constructs (see Fig. 6) based on pSD5B (Jain et al., 1997) were cotransformed into E. coli along with either an IPTG- (pQER) or arabinose (pBADR)-inducible SoxR construct. The cotransformed vector systems are compatible, as pSD5B replicates using a p15A origin and has kanamycin as a selectable marker, whereas pQER or pBADR uses a ColE1 origin and has ampicillin as marker. Cotransformed cells were then selected on LB agar plates containing kanamycin and ampicillin (50 µg ml⁻1 each). Transformed colonies were grown in LB with kanamycin and ampicillin for promoter assays.

**β-Galactosidase assay.** Overnight cultures of E. coli XL-1 Blue harbouring promoter constructs with or without soxR coexpression were grown under specified conditions and subjected to promoter assays as described by Sambrook & Russell (2001). One millilitre of the culture was pelleted by centrifugation and suspended in 0.5 ml Z buffer (Na2HPO4, 16.1 g; NaH2PO4, 5.5 g; KCl, 0.75 g; MgSO4, 0.24 g and 50 mM mercaptoethanol per litre water). The cells were permeabilized by adding one drop of 0.1% (w/v) SDS and two drops of chloroform with mixing. The reaction mixture contained 0.2 ml of cells, 0.1 ml of 8 mg ml⁻¹ ONPG and 0.7 ml of Z buffer. Enzyme activity was estimated from the release of nitrophenol, which was detected spectrometrically at 420 nm and was expressed in Miller units (Miller, 1972).

**Immunological analysis.** To monitor the induced synthesis of SoxR in E. coli Western blot analysis using anti-His antibody (Qiagen) was performed. Equal amounts of cytosolic proteins extracted from E. coli cells expressing His₆-tagged SoxR were resolved on 12.5% polyacrylamide gels and subsequently transferred electrophoretically to Nytran membrane at 80 mA constant current for 50 min according to standard protocol (Towbin et al., 1979). The membranes were probed with anti-His antibody as primary antibody and anti-rabbit IgG–alkaline phosphatase conjugate as the secondary antibody. A chromogenic (NBT-BCIP) method was used to detect the desired band, following the instruction manual (Roche Applied Science).

**Protein cross-linking assays.** Multimer formation was studied using glutaraldehyde cross-linking assays (Randell & Coen, 2004). Purified SoxR protein was incubated for 10 min with 0.005% and 0.01% (v/v) glutaraldehyde and analysed by SDS-PAGE. To investigate the effect of SoxR binding to DNA sequences on the multimerization of the protein, PCR-amplified DNA fragments representing SoxR-binding sites were incorporated in increasing concentrations in the cross-linking assay. The protein profile was analysed by SDS-PAGE and visualized by staining with Coomassie blue.

**Dynamic light scattering on SoxR.** The dynamic light scattering experiment was performed in a Zetasizer Nano ZS instrument (Malvern Instruments). The measurements were carried out in 50 mM phosphate buffer (pH 8.0) containing 100 mM NaCl and 10% (v/v) glycerol. The purified protein sample was passed twice through a filter membrane of 0.22 µm pore size. The protein concentration of the sample was then measured by the Lowry method and the sample diluted accordingly during the light-scattering measurements. All the light-scattering measurements were performed at 25°C. A single run represents an average of 20 independent 10 s runs.

**RESULTS**

**Effect of soxR inactivation on expression of the sox locus**

soxR of P. salicylatoxidans KCT001 was insertionally inactivated, and the sulfur utilization capability and growth of the resulting mutant were compared with those of the wild-type. The cells were grown in either heterotrophic medium (MSS) or autotrophic medium (MST). At defined intervals, cells were harvested and the level of Sox activity monitored by incubating the cells transiently in the presence of thiosulfate. The µmol oxygen consumed per minute per mg total protein in the process of oxidizing thiosulfate was used as the parameter for Sox activity. The wild-type showed no activity in heterotrophic medium, indicating tight repression of the operon in the absence of thiosulfate (Fig. 2a). In contrast, the mutant showed high level of activity in heterotrophic medium, comparable to the activity observed when the wild-type was grown under autotrophy (Fig. 2a). These results indicate clearly that SoxR acts as the repressor.
DNA-binding activity of SoxR

The recombinant SoxR was overexpressed and subsequently purified for investigating its DNA-binding properties. Two SoxR-binding regions were chosen based on the DNA-binding studies performed with *Paracoccus pantotrophus*. The binding regions were designated either sv or wx, representing the intergenic regions between soxS–soxV and soxW–soxX, respectively. Concentration-dependent increase in SoxR binding to sv and wx regions was observed (Fig. 3). In the case of wx and sv, 50% of the probe was bound at SoxR concentrations of 150 nM and 70 nM, respectively. These values therefore represent approximate dissociation constants for SoxR binding to the respective sites. The binding was analysed by fitting the data into a two-site binding model represented by the following equations:

\[
F_0 = \frac{1}{1 + A_1 \cdot X + A_2 \cdot X^2}
\]

\[
F_1 = A_1 \cdot X / (1 + A_1 \cdot X + A_2 \cdot X^2)
\]

\[
F_2 = A_2 \cdot X^2 / (1 + A_1 \cdot X + A_2 \cdot X^2)
\]

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**Fig. 2.** Sox activity (a) and cellular yield (b) of *P. salicylatoxidans* KCT001 and the soxR-inactivated mutant grown in heterotrophic and autotrophic media. Overnight-grown KCT001 and its mutant were inoculated into the desired media and samples were removed at defined time intervals. The Sox activity, expressed as oxygen consumption, was measured by incubating the harvested cells in an assay buffer containing thiosulfate for 5 min. O_2_ consumption (a) and growth (b) of heterotrophically grown (○) and autotrophically grown (△) wild-type and heterotrophically grown (●) and autotrophically grown (□) mutant.

**Fig. 3.** Interaction of SoxR with its binding sites in the sox operon. (a) EMSAs with wx site: lanes 1–12 were supplied with increasing amounts (0, 10, 20, 30, 40, 50, 100, 300, 400, 500, 800, 1600 nM, respectively) of SoxR. (b) Binding data were fitted to a two-site model as described in Results. ▲, Free probe; □, C1 complex; ●, C2 complex. (c) Interaction of SoxR with sv binding sites. EMSAs with sv intergenic region. Binding was carried out with increasing concentrations of SoxR (lanes 1 to 12: 0, 8, 16, 25, 33, 44, 55, 66, 100, 111, 132 and 166 nM SoxR, respectively) (d) Binding data were fitted to a two-site model as described in Results. ▲, Free probe; □, C2 complex.
regions of Reporter assays for Sox promoter activity in the even more cooperative, as indicated by the strong suppression of the C1 complex. The data could be fitted to the two-site model, described above (Fig. 3b, d), but not adequately to the single-site model (analysis not shown). This suggests that binding to sv, like that to wx, also involves two sites.

The complex that is visible in the case of sv is possibly C2. The C1 complex is not detectable in the case of sv, apparently due to the phenomenon being strongly cooperative, resulting in suppression of the C1 complex.

Defining the core binding sequences

To identify the exact binding sites of the transcription regulator SoxR within the sv and wx promoter regions, DNase I footprinting experiments were performed using the PCR amplicons used in the EMSA. Addition of SoxR to the sample resulted in distinct DNase I footprints. In the case of wx two distinct but closely juxtaposed regions could be identified (Fig. 4a). However, the two sites seem to have different affinities (Fig. 4b). The high-affinity site gave footprints at relatively low concentrations. In contrast to wx the sv region gave a single footprint (Fig. 4c). This is consistent with the single complex demonstrated in the EMSA. The core elements (Fig. 4d) thus identified were then synthesized and SoxR binding with these segments was tested. Both the core elements were bound to SoxR (Fig. 5a, b). The binding patterns were similar to those of the larger probes used in the initial EMSA experiments. However, it may be noted that in the case of wx core the binding became even more cooperative, as indicated by the strong suppression of the intermediate complex C1 (Fig. 5b).

Reporter assays for Sox promoter activity in the presence of SoxR

Reporter constructs were made in which the promoter regions of wx and sv were fused in-frame to lacZ in a vector which replicates utilizing the p15A origin of replication, giving rise to pSDWX and pSDSV or pSDVS respectively (Table 1, Fig. 6). To supply SoxR in trans the IPTG-inducible SoxR expression plasmid pQER was cotransformed along with the promoter constructs. Reporter gene expressions were then assayed in the absence and the presence of pQER. In the absence of SoxR a high level of activity was observed in the case of sv and wx and a moderate level in the case of vs. When reporter gene expression was monitored after cotransformation of pQER, it was observed that without addition of IPTG, where expression is expected at the basal level, efficient repression (10–20-fold) occurred. Introduction of IPTG should have given further repression but in fact the opposite happened. Repression seemed to be relieved as increasing SoxR accumulated in the system (Fig. 7a). The phenomenon was also observed in the case of a time-course experiment (Fig. 7b), where it can be seen that following addition of IPTG, a time-dependent derepression was observed with increasing amount of SoxR (Fig. 7c). The results show that a basal level of expression apparently due to leaky protein expression was sufficient to repress promoter activity. To further verify that the efficient repression observed above was indeed due to leaky expression, a parallel experiment was performed using the tightly regulated promoter provided in the pBAD series of vectors. In this case leaky expression can be completely eliminated by the use of glucose in the absence of arabinose. The results show that under such tightly regulated conditions there is no repression (Fig. 7d), which confirms that the repression observed in the case of pQER was indeed due to basal expression of SoxR. However, addition of arabinose did show strong repression. The SoxR protein therefore is most active at low concentrations and seems to lose repressor activity at higher concentrations.

Dimerization of SoxR

Initial investigations using SDS-PAGE analyses and subsequent immunoblotting indicated the existence of a band corresponding to the dimer (Fig. 8a). To investigate the ability of SoxR to form dimers and multimers a glutaraldehyde cross-linking experiment was performed. The results showed efficient cross-linking and the formation of multimers, of which the dimeric form was predominant (Fig. 8b). Considering that SoxR interaction with sv is highly cooperative, the possibility that sv promotes dimerization was tested by incorporating increasing doses of sv DNA in a cross-linking experiment. The results showed a dose-dependent increase in the intensity of cross-linked dimer (Fig. 8c). This gradual increase of SoxR dimer in the presence of the binding sequence demonstrated that repressor SoxR interacts with it as a dimer.

The process of multimerization was also examined using dynamic light-scattering experiments. Fig. 8(d) shows the distribution of species having a different hydrated diameter at two protein concentrations: 37 μM and 150 μM. Two peak hydrated diameter values were obtained, with values of approximately 8 nm and 32 nm. The results indicate the presence of two populations with respect to multimerization.

DISCUSSION

Chromolithotrophic sulfur oxidation is induced by reduced sulfur compounds like thiosulfate, in the absence of which (heterotrophic growth) no sulfur-oxidizing activity is detectable. On the other hand, autotrophically grown cells show high levels of sulfur-utilizing activities. Thus any
mutant that becomes constitutive must possess a level of activity under heterotrophic conditions that can match the level obtained by the wild-type under autotrophic conditions. In a previous study using Paracoccus pantotrophus, it was shown that an insertion within soxS, the gene immediately upstream of soxR, resulted in a low level of constitutive expression (about 10%) under heterotrophic conditions (Rother et al., 2005). Since this low level of constitutive expression could be repressed by complementation with soxR, it was concluded that the phenotype was due to a polar effect of the soxS mutation on soxR. In this study, it has been demonstrated that direct inactivation of soxR resulted in a relatively higher (greater than 60%) level of constitutive expression under heterotrophic conditions. The slight reduction in activity relative to the wild-type is possibly due to marginal retardation in growth rate.

**Fig. 4.** DNase I protection assay to determine SoxR-binding sites in the wx (a, b) and sv (c) regions. The binding-site sequences are indicated by brackets. Footprinting was performed using 5 μM SoxR in A and C. In the case of (b) the concentration of SoxR was increased progressively (0, 1, 2, 3 and 5 μM). Lane 1 in each case represents A+G ladder, 2 is DNase I ladder of free probe and 3 is DNase I ladder SoxR-bound probe. (d) Location of the binding sequences (boxes) in the sv and wx regions, H and L stand for high- and low-affinity site. The black bars represent the putative −35 and −10 sequences.
(Fig. 2b). Under autotrophic (and also mixotrophic, data not shown) conditions, nearly 100% wild-type level of activity was observed in the soxR mutant as compared to only 18% in the case of the soxS mutant. These observations suggest that the phenotype reported earlier probably arises from partial impairment of soxR function whereas in this study the inactivation appears to be complete. In the case of the mutant reported here, it is unlikely that insertion in soxR had any effect on soxS, since it is upstream of soxR. Hence the constitutive phenotype of the KCT001 soxR mutant appears, by and large, to be soxS independent. The same argument cannot be applied to the downstream gene soxT, which could potentially be affected due to insertion in soxR. However, it may be noted that the mutant did not show the delayed Sox phenotype (Lahiri et al., 2006) and therefore apparently there is no polar effect on soxT.

SoxR was earlier demonstrated to bind to two intergenic regions within the sox locus of Paracoccus pantotrophus. In this study the binding of Pseudaminobacter salicylatoxidans KCT001 SoxR to the corresponding sites was examined. The binding isotherms presented in this study support a two-site model, as in both cases the data could be fitted to two-site binding equations. The binding was cooperative in both cases but in the case of sv cooperativity was significantly greater than for wx, as is evident from the complete suppression of an intermediate complex in this case. The cooperativity may also be dictated by DNA conformation. It is interesting to note that when the minimal core sequence was used the cooperativity seemed to increase in the case of wx, as is indicated by the substantially diminished intensity of the intermediate band. The size of the core sequence (44 bp) is below the persistent length of DNA, which is considered to be about 100 bp (Shore et al., 1981), and hence the core sequence is likely to be more rigid than the longer sequence. The rigidity may result in facilitated interactions between bound monomer, causing increased cooperativity. These differences indicate that binding could be potentially regulated by the flexibility of the DNA.

The two-site model that has been proposed on the basis of mathematical derivations is supported strongly by footprinting data, particularly in the case of wx, where two distinct footprints were visible, one of which represented a high-affinity and the other a low-affinity interaction site. In the case of sv only one footprint was obtained, which is
consistent with the single complex observed in the EMSA. It is however most likely that the binding at sv, like that at wx, represents a dimeric complex. This is evident not only from the binding curve but also from theoretical modelling studies (Bagchi et al., 2005), which show that this region can form a stable dimeric complex. In addition, the observation that sv DNA promotes dimerization of SoxR, as evident from the cross-linking studies, gives further support to a dimeric site model. It is interesting to note that the sv promoter-operator appears to have a degree of symmetry, with two CATA sequences being positioned at equivalent sites on either side of the centre of symmetry (Fig. 9). A similar organization was also shown in the case of Paracoccus pantotrophus. This symmetrical disposition is probably necessary as the promoter is a bi-directional one. Hence, although SoxR binds to both the loci sv and wx, the binding patterns are dissimilar; this is probably a reflection of the fact that the two promoters sv and wx function in different contexts. The wx promoter drives the expression of genes directly involved in sulfur oxidation. In this case a relatively more subtle regulation is perhaps required as the gene products perform an intricate metabolic function under conditions of autotrophy. In contrast, in the case of sv, which controls the expression of genes encoding auxiliary proteins required for Sox function, the regulation need not be "rheostatic"; on the other hand an abrupt repression or derepression may be required. Cooperative mechanisms generally cater to such abrupt situations and therefore it

### Fig. 7. Reporter assays for determining SoxR function.

(a) E. coli XL-1 Blue transformed with promoter constructs pSDSV, pSDVS and pSDWX, either alone or in combination with SoxR expression vector pQER was assayed for the level of lacZ under uninducing or inducing (+I) conditions. The combinations tested were pSD5B, pSDSV (SV), pSDSV + pQER (SVR), pSDSV + pQER induced by IPTG (SVR+I), pSDVS (VS), pSDVS + pQER (VSR), pSDVS + pQER induced by IPTG (VSR+I), pSDWX (WX), pSDWX + pQER (WXR) and pSDWX + pQER induced by IPTG (WXR+I). (b) β-Galactosidase assay at different times of growth of E. coli cells harbouring either pSDWX (circles), pSDVS (triangles) or pSDSV (squares) in combination with pQER. Solid symbols are for IPTG-induced and open symbols are for non-induced cells. (c) Immunoblot analysis of SoxR present in equal amounts of cell-free extract of IPTG-induced E. coli cells harbouring both reporter and expression plasmids at different time points. (d) E. coli strain TOP10 transformed with promoter constructs pSDSV, pSDVS and pSDWX, either alone or in combination with SoxR expression vector pBADR was assayed for the level of lacZ under uninducing, repressing (+G) or inducing (+A) conditions. The combinations tested were pSDSV (SV), pSDSV + pBADR (SVR), pSDSV + pBADR repressed by glucose (SVR+G), pSDSV + pBADR induced by arabinose (SVR+A), pSDVS (VS), pSDVS + pBADR (VSR), pSDVS + pBADR repressed by glucose (VSR+G), pSDVS + pBADR induced by arabinose (VSR+A), pSDWX (WX), pSDWX + pBADR (WXR), pSDWX + pBADR repressed by glucose (WXR+G) and pSDSV + pBADR induced by arabinose (WXR+A).
Fig. 8. Multimeric nature of SoxR. (a) Immunoblotting of purified His-tagged SoxR with anti-His antibody. (b) SDS-PAGE profile of glutaraldehyde cross-linked SoxR. Lane M, molecular mass markers; lanes 1, 2 and 3, cross-linked SoxR with 0%, 0.01% and 0.005% (v/v) glutaraldehyde, respectively. (c) Glutaraldehyde cross-linking of SoxR in the presence of increasing amount of sv DNA. SoxR was incubated for 10 min with the DNA; 0.01% (v/v) glutaraldehyde was added with the reaction mixture just before sample buffer was added. Samples were boiled for further SDS-PAGE analysis. Lanes 1–5 represent cross-linking in the presence of 0, 0.5, 1, 1.5, 2 μg sv DNA, respectively. (d) Dynamic light-scattering experiments carried out at 37 μM (○) and 150 μM (●) of SoxR, respectively. The percentage of scattered species was plotted against the hydrated diameter of the protein. In each experiment, the output of a single run is an average of 20 independent scans of 10 s duration. The data presented are means of five independent measurements.

| Kct SV | --------------TCACGTGACATATCTTTGCGCATAAGCCAG------------- |
| pp SV  | ACCCATATTGATTCATATCCACCCCTGCGCATAAGCCAG |

Low affinity site

High affinity site

Fig. 9. Alignment of the core SoxR-binding regions of KCT001 (Kct) with those of Paracoccus pantotrophus (pp). The bar on the sequence represents the DNase I protected region by SoxR. The shaded sequences are near-exact repeats.
probably makes sense that the binding to sv is highly cooperative.

The various pieces of evidence reported in this study and the previous attempts to derive a model for SoxR binding suggest the importance of dimerization. Using several methods the efficient dimerization of SoxR has been demonstrated here for the first time. Cross-linking experiments clearly indicated the formation of dimer. Light-scattering experiments indicated the presence of two species, the larger one having a four times larger diameter. Considering that the volume should be proportional to the diameter cubed and that mass is proportional to volume, it appears, with some approximation, that a fourfold increase in diameter reflects a twofold increase in mass. The process of dimerization, although it takes place spontaneously, is further stimulated in the presence of binding sequences, sv DNA in particular. The differences in the binding patterns to sv and wx can also be explained on the basis of dimerization. It may be noted that in the case of wx two sites, a high-affinity and a low-affinity one, can be clearly demarcated. The two sites, although located side by side, are separated by a couple of nucleotides. In such a situation it appears that the high-affinity site is engaged first followed by the low-affinity site. Once the two sites are occupied the monomers interact with each other, resulting in cooperativity. The sv site on the other hand appears symmetrical, with two sites located side by side without any apparent gap. It is extremely difficult to understand the exact sequence of events in such cases of highly cooperative interaction, but the present account of the sv site binding is consistent with earlier theoretical findings (Bagchi et al., 2005).

The dissociation constants appeared to be in the nanomolar order, which may be considered as sufficiently strong. Such strong interactions mean that the amount of SoxR required for saturation binding is extremely small. That SoxR is an efficient repressor at low concentrations is evident from the reporter assays performed in E. coli. The basal level of expression produced due to leaky expression was enough to bring about substantial repression. Interestingly, repression was reversed as the level of SoxR was increased, thereby indicating that SoxR could become inactive at higher concentrations. This could either be due to the formation of inactive aggregates or have other causes not clear at present.

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