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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 *soxW* 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

†Deceased.

Abbreviation: EMSA, electrophoretic mobility shift assay.

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*. Luria–Bertani broth (LB) and Luria–Bertani agar (LA) media respectively were used for growing and maintaining strains of *E. coli* (at 37 °C) as well as *Pseudaminobacter salicylatoxidans* strain KCT001 (Mukhopadhyaya *et al.*, 2000; Deb *et al.*, 2004) and its mutant (at 30 °C). *E. coli* TOP10 used in experiments involving arabinose regulator expression was grown in RM medium (pH 7.4) containing M9 salts (Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 0.5 g and NH_4Cl 1 g per litre water), 2 % (w/v) Casamino acids and 1 mM $MgCl_2$. Filter-sterilized 0.2 % (w/v) arabinose (or glucose) was added when needed. For physiological studies related to sulfur lithotrophic functions, the cells were grown in MS medium (NH_4Cl, 1 g; Na_2HPO_4, 7.9 g; KH_2PO_4, 1.5 g; $MgSO_4$, 0.5 g and 5 ml trace metal solution per litre water; Vishniac & Santer, 1957) supplemented with filter-sterilized 40 mM thiosulfate (MST) or 18.5 mM succinate (MSS).

Construction of the *soxR*::Ω-kanamycin insertion mutant strain. *soxR* was disrupted by inserting the kanamycin cassette derived from pUC4K (Amersham) through the recombinant suicide plasmid pKSRTKm by gene replacement. A 1.5 kb PCR amplicon consisting of the *V′SRT′* genomic region was generated using the primer pair TR (5′-CGGAATTCGAAACACCCACCACGACAA-3′) and VR (5′-GCTCTAGAGGCGAGACGAATGACAGAAG-3′) with KCT001 genomic DNA by the aid of proofreading *Taq* polymerase. The purified amplicon was subjected to restriction digestion by *Eco*RI and *Xba*I, as the primer TR has an *Eco*RI and VR has a *Xba*I site in the 5′ region. The digested and subsequently purified fragment was ligated with the *Eco*RI- and *Xba*I-digested suicide vector pKAS32 (Skorupski & Taylor, 1996). The ligated mixture was transformed into the *λ-pir*-containing competent *E. coli* SY327 strain (Miller & Mekalanos, 1988). Recombinant colonies were selected and confirmed by both restriction digestion and PCR with insert-specific primer. The recombinant pKSRT suicide plasmid was digested with *Xho*I, for which there is one site within the inserted *soxR* gene of the *V′SRT′* fragment. A *Sal*I-digested kanamycin cartridge from pUC4K was introduced into the *Xho*I site of pKSRT. The resulting pKSRTKm plasmid was selected on kanamycin plates and confirmed by restriction digestion. The pKSRTKm-containing *E. coli* SY327 was conjugated with KCT001SR. The transconjugants with single crossovers were streptomycin sensitive because of the presence of *rpsL* provided with pKAS32. The transconjugants with double crossovers were selected by their streptomycin-resistant phenotype. The recombinant strain was confirmed by PCR and Southern blotting.

Substrate-dependent oxygen consumption. KCT001 (wild-type) and KCT001SR::Ω*soxR* (mutant) were grown in LB medium overnight at 30 °C. Experimental MST and MSS media were inoculated with equal amounts of overnight-grown LB culture. Growing cells were harvested at different time intervals by centrifugation, washed, and resuspended in sodium phosphate buffer (100 mM, pH 8.0). The sulfur-oxidizing activity of whole cells was determined polarographically with a biological oxygen monitor having a Clark-type oxygen

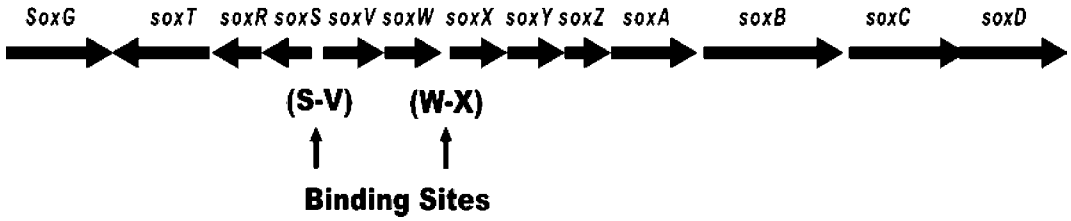

Fig. 1. Physical map of the *sox* gene cluster of *P. salicylatoxidans* KCT001. Horizontal arrows indicate the ORFs and their direction of transcription. ORFs are not in exact scale. Upward vertical arrows demarcate the two binding sites (*sv* and *wx*) of SoxR.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference/source
Escherichia coli		
XL-1 Blue	*recA1 lac endA1 gyrA46 thi hsdR17 supE44 relA1* [F′ *proAB lacI*^{q}ZΔM15 Tn*10*(Tet^{r})]	Bullock *et al.* (1987); Stratagene
TOP10	F^{-} *mcr*A Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ* ΔM15Δ*lac*X74 *deoR recA1 araD139* Δ(*ara*A-*leu*)7697 *galU galK rpsL endA1 nupG*	Invitrogen
SY327λ*pir*	Δ(*lac pro*) *argE* (Am) *recA* (Rif) *nalA* λ*pir*	Miller & Mekalanos (1988)
M15	K12 derivative	Qiagen
Sulfur chemolithotrophs		
Pseudaminobacter salicylatoxidans KCT001	Wild-type, Sox^{+}*	Deb *et al.* (2004); Mukhopadhyaya *et al.* (2000); DSM 13826
KCT001SR	Sox^{+} Sm^{r} Rif^{r}(spontaneous mutant of KCT001)	Mukhopadhyaya *et al.* (2000)
KCT001SRΩR	*soxR*::Ω Km (*soxR*-inactivated KCT001SR)	This study
Plasmids		
pUC4K	pUC vector carrying the kanamycin cassette from Tn*903*; Kan^{r} Ap^{r}	Amersham
pKAS32	Cloning vector with dominant *rpsL* gene	Skorupsky & Taylor (1996)
pKSRT	pKAS containing part of *soxV*, *soxS*, *soxR* and part of *soxT*	This study
pKSRTKm	pKSRT with *soxR* inactivated by kanamycin cassette	This study
PQE30	Ap^{r} Cm^{r} T5 promoter [His]$_{6}$	Qiagen
pQER	*soxR* ORF cloned in *Bam*HI–*Hin*dIII site of pQE30	This study
pBAD	Ap^{r} *ara* promoter	Invitrogen
pBADR	*soxR* ORF cloned in *Bam*HI–*Hin*dIII site of pBAD	This study
pSD5B	Promoter-probe vector; Km^{r}	Jain *et al.* (1997)
pSDSV	S–V intergenic region cloned in *Xba*I site of pSD5B	This study
pSDVS	S–V intergenic region cloned in reverset orientation in *Xba*I site of pSD5B	This study
pSDWX	W–X intergenic region cloned in *Xba*I site of pSD5-β-Gal	This study

*Ability to oxidize reduced sulfur compounds and chemolithotrophic growth is denoted by Sox^{+}.

electrode (Yellow Springs Instrument Co.) at 30 °C. The final assay volume was 3 ml and the cells were suspended in 100 mM phosphate buffer at pH 8.0. Calculations were made on the basis of an oxygen concentration of 236 μM in air-saturated buffer at 30 °C (Meulenberg *et al.*, 1992). Oxygen consumption rates were corrected for chemical or auto-oxidation of substrates and endogenous respiration rates.

Construction of recombinant expression plasmids. The *soxR* gene was PCR amplified for in-frame insertion into the N-terminal His-tag expression vector pQE30 (Qiagen). The forward primer (SoxRN) (5′-GTCATAGGATCCATGCATGGGAACCCGCAA-3′) that was used in the above amplification carried a unique *Bam*HI site at the 5′ end of the gene, while the reverse primer (SoxRC) (5′-GCCAGCAAGCTTCCTTGGCGGATTGTTATT-3′) carried a *Hin*dIII site located 13 nucleotides downstream from the TAA translation stop codon. The amplified DNA fragment was digested with *Bam*HI and *Hin*dIII and ligated into the same sites 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 promoter using the vector pBAD (Invitrogen). The *Bam*HI- and *Hin*dIII-digested *soxR*-containing fragment of pQER was ligated with *Bgl*II- and *Hin*dIII-digested pBAD to generate recombinant construct pBADR. The inserted DNA fragments were sequenced from the expression vectors to check the coding frame and for any misincorporation of nucleotide(s) in the course of polymerization during PCR.

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 OD_{600} 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, as in this case 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 NaH_2PO_4, 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 Ni^{2+}-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 12.5 % SDS-PAGE. Fractions containing SoxR protein were pooled and dialysed against storage buffer (50 mM NaH_2PO_4, 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 γ-^{32}P-labelled primers SR (5′-GTCGCCACCATTACCAGTG-3′)

and VR (5′-GGCGAGACGAATGACAGAAG-3′) for *sv*, and WF (5′-GCTCTAGAAGGAGTAGTTCACAGGGTTT-3′) and XR (5′-GCTCTAGATCATATCTCTGCCCCTCCA-3′) for *wx*, in PCR using the KCT001 genomic DNA as template. Primer labelling was done by kinasing 10 pmol of the desired primer with [γ-^{32}P]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 (30 µl final volume), unless mentioned otherwise, contained the desired amount of purified protein, 3 µl 10× binding buffer (100 mM Tris/HCl pH 8, 300 mM NaCl, 30 mM $MgCl_2$, 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 10 000 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/borate buffer (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/HCl 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′-GCTCTAGAGTCGCCACCATTACCAGTG-3′) and VR1 (5′-GCTCTAGAGGCGAGACGAATGACAGAAG-3′) for the *sv* operator/promoter region, and WF1 (5′-GCTCTAGAAGGAGTAGTTCACAGGGTTT-3′) and XR1 (5′-GCTCTAGATCATATCTCTGCCCCTCCA-3′) for the *wx* operator/promoter region. All the primers have *Xba*I sites in their 5′ ends. The *Xba*I-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 thus 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 (Na_2HPO_4, 16.1 g; NaH_2PO_4, 5.5 g; KCl, 0.75 g; $MgSO_4$, 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 His_6-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.

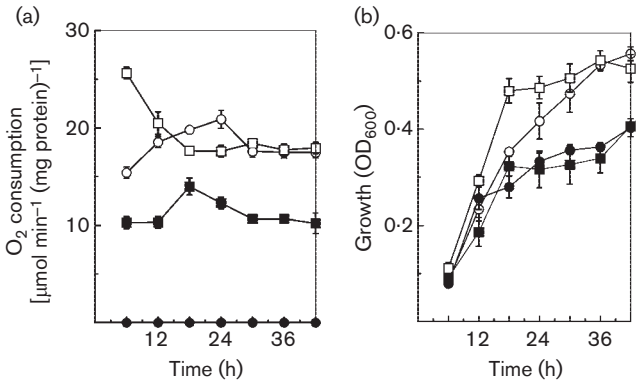

Fig. 2. Sox activity (a) and cellular yield (b) of *P. salicylatoxidans* KCT001 and the *soxR*-inactivated mutant grown in heterotrophic and autotrophic media. Overnight-grown KCT001 and its mutant were inoculated into the desired media and samples were removed at defined time intervals. The Sox activity, 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.

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:

$$F_0 = 1/(1 + A_1.X + A_2.X^2)$$

$$F_1 = A_1.X/(1 + A_1.X + A_2.X^2)$$

$$F_2 = A_2.X^2/(1 + A_1.X + A_2.X^2)$$

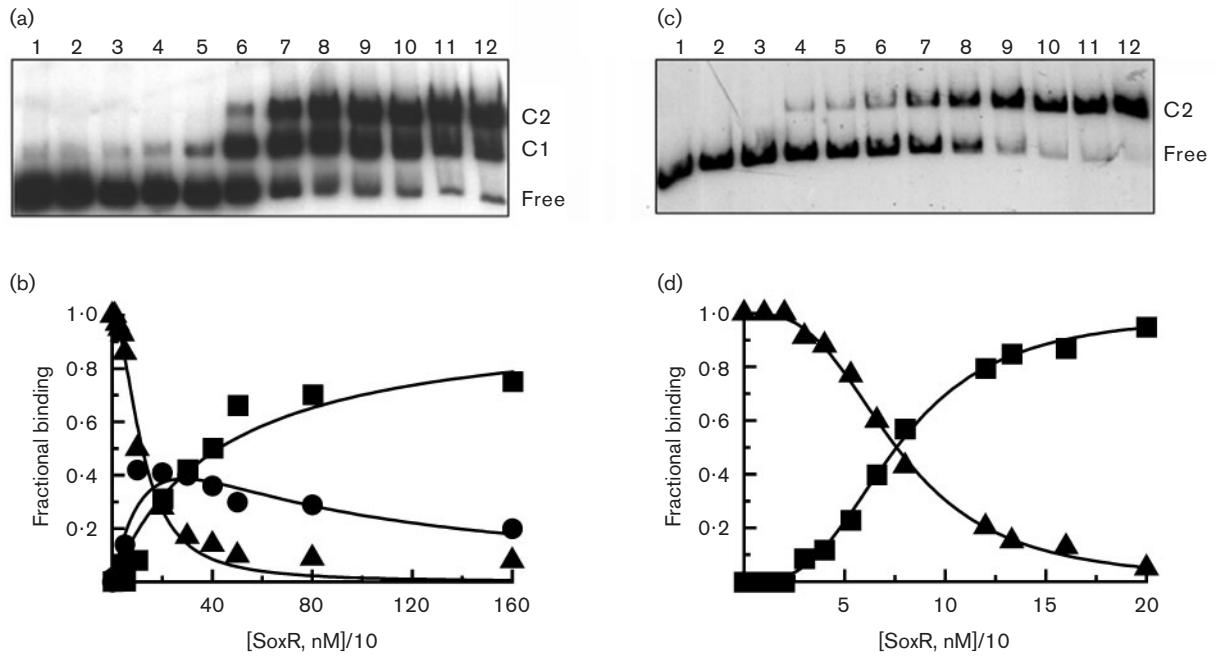

Fig. 3. Interaction of SoxR with its binding sites in the *sox* operon. (a) EMSAs with *wx* site: lanes 1–12 were supplied with increasing amounts (0, 10, 20, 30, 40, 50, 100, 300, 400, 500, 800, 1600 nM, respectively) of SoxR. (b) Binding data were fitted to a two-site model as described in Results. ▲, 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.

F_0, F_1 and F_2 represent the fraction unbound and fraction in the liganded states (1 or 2), A_1, A_2 are the macroscopic association constants and X is the concentration of free ligand. The values of A_1 and A_2 thus obtained in the case of *wx* were approximately 4×10^7 M^{-1} and 10^{16} M^{-2}, respectively. When a binding phenomenon involving two sites is non-cooperative it is expected that $A_2 = (A_1^2)/4$. However, it is apparent that $A_2 > (A_1^2)/4$; hence the phenomenon is cooperative. In the case of *sv*, which gives a single complex, curve fitting was attempted using two- as well as single-site binding models. 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. In the case of *sv* A_2 was 10^{16} M^{-2} but A_1 became vanishingly small and could not be accurately determined. The cooperativity of binding in the case of the *sv* region is thus even higher than for *wx*. 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 concentration: 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

Chemolithotrophic 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

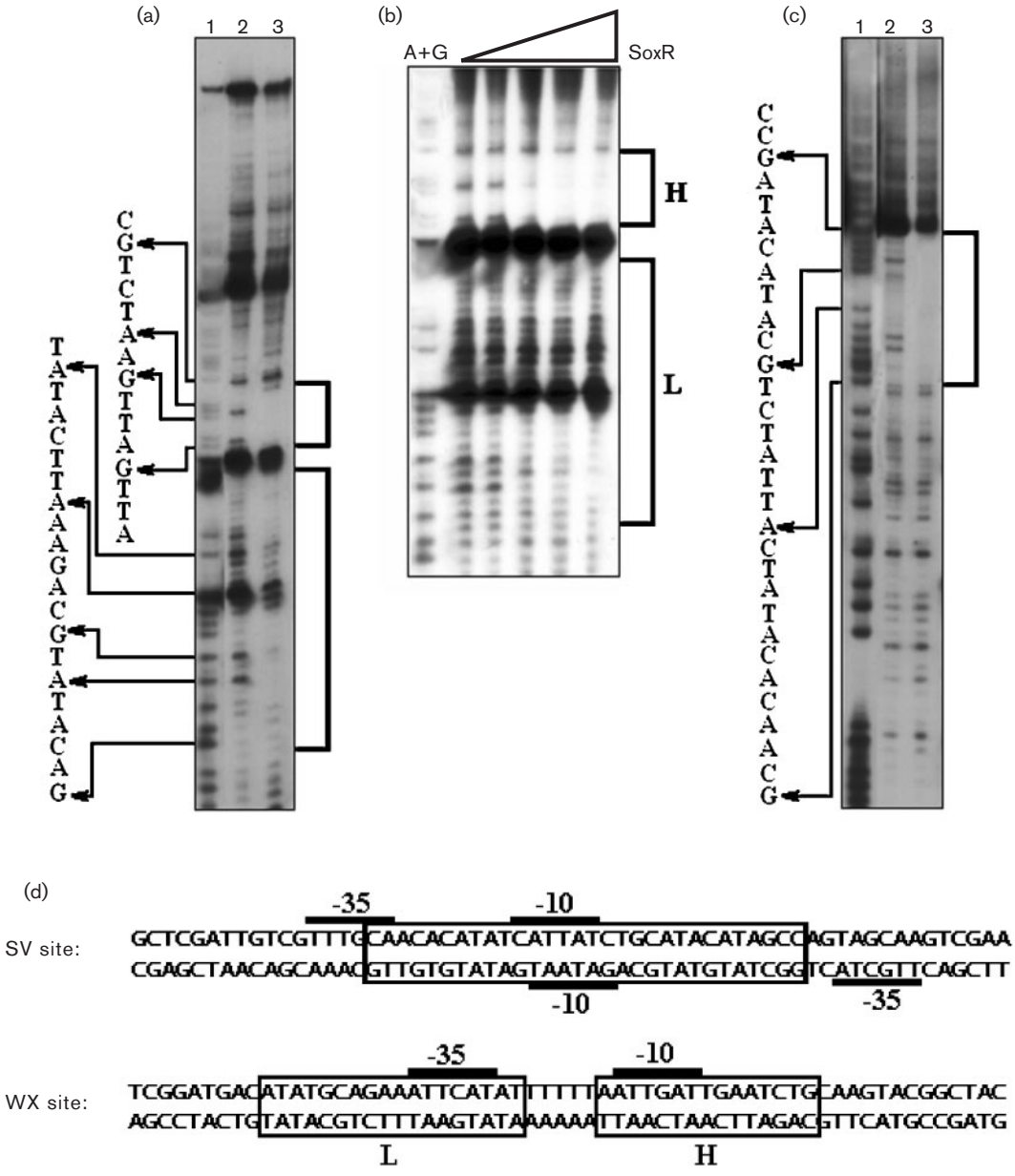

Fig. 4. DNase I protection assay to determine SoxR-binding sites in the *wx* (a, b) and *sv* (c) regions. The binding-site sequences are indicated by brackets. Footprinting was performed using 5 μM SoxR in A and C. In the case of (b) the concentration of SoxR was increased progressively (0, 1, 2, 3 and 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.

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

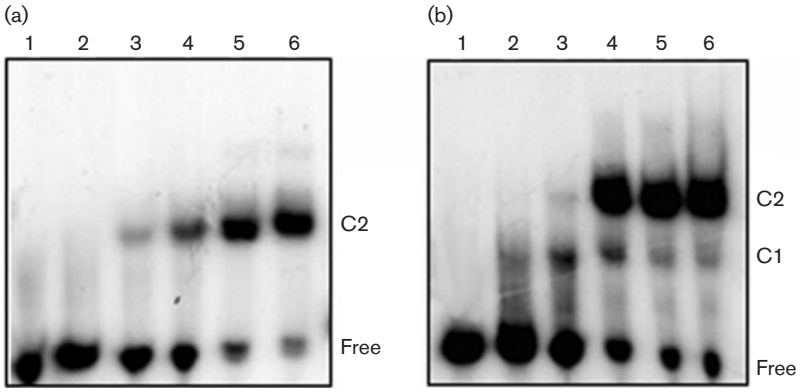

Fig. 5. EMSA of the core-binding region with SoxR. (a) Binding with the *sv* site. Binding was carried out with increasing concentrations of SoxR (lanes 1 to 6: 0, 6, 30, 45, 90 and 180 nM SoxR, respectively). (b) Binding with the *wx* site. Binding was carried out with increasing concentrations of SoxR (lanes 1 to 6: 0, 6, 30, 150, 300 and 600 nM SoxR, respectively).

(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 can not 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

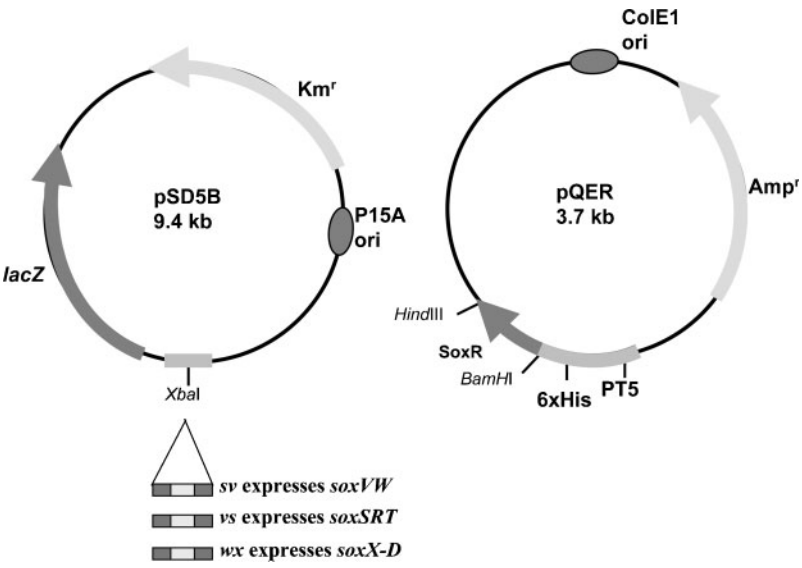

Fig. 6. Physical map of the reporter plasmid pSD5B and the recombinant SoxR expression vector pQER.

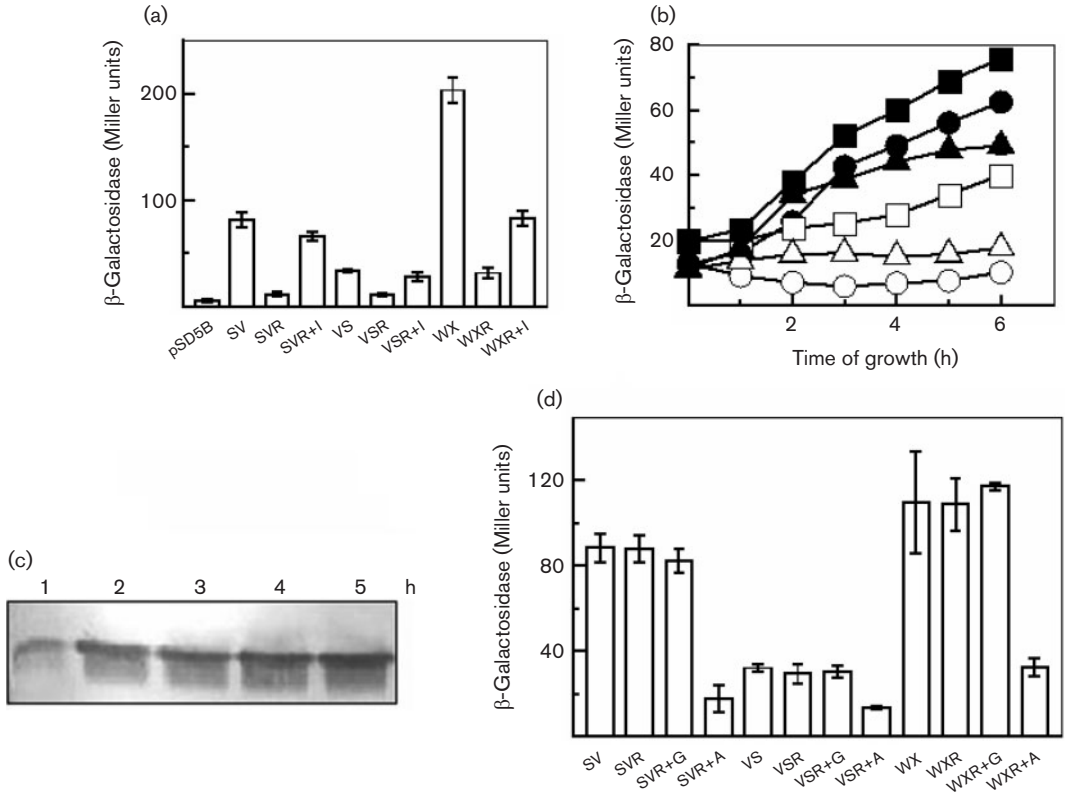

Fig. 7. Reporter assays for determining SoxR function. (a) *E. coli* XL-1 Blue transformed with promoter constructs pSDSV, pSDVS and pSDWX, either alone or in combination with SoxR expression vector pQER was assayed for the level of *lacZ* under uninducing or inducing (+I) conditions. The combinations 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 (squares), pSDVS (triangles) or pSDSV (circles) 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), SDVS+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).

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

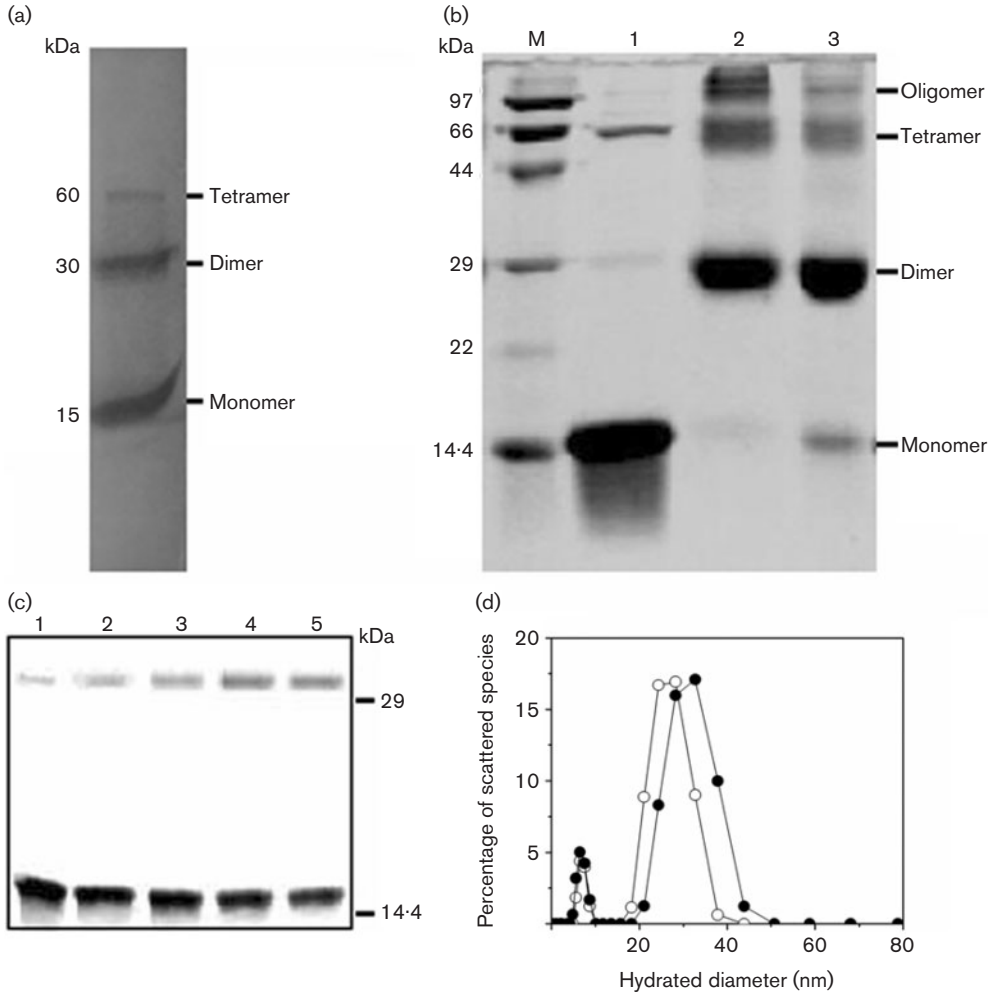

Fig. 8. Multimeric nature of SoxR. (a) Immunoblotting of purified His-tagged SoxR with anti-His antibody. (b) SDS-PAGE profile of glutaraldehyde cross-linked SoxR. Lane M, molecular mass markers; lanes 1, 2 and 3, cross-linked SoxR with 0 %, 0.01 % and 0.005 % (v/v) glutaraldehyde, respectively. (c) 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.

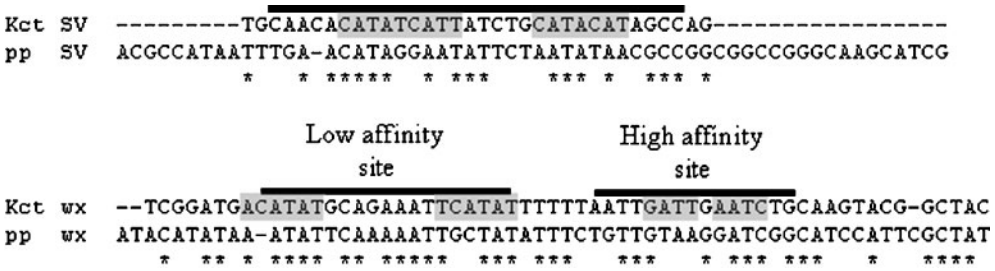

Fig. 9. Alignment of the core SoxR-binding regions of KCT001 (Kct) with those of *Paracoccus pantotrophus* (pp). The bar on the sequence represents the DNase I protected region by SoxR. The shaded sequences are near-exact repeats.

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 indeed 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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