SoxRS-mediated regulation of chemotrophic sulfur oxidation in *Paracoccus pantotrophus*

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*Paracoccus pantotrophus* GB17 requires thiosulfate for induction of the sulfur-oxidizing (Sox) enzyme system. The *soxRS* genes are divergently oriented to the *soxVWXYZA–H* genes. *soxR* predicts a transcriptional regulator of the ArsR family and *soxS* a periplasmic thioredoxin. The homogenate mutant GBtS carrying a disruption of *soxS* by the ß-kanamycin-resistance-encoding interposon expressed a low thiosulfate-oxidizing activity under heterotrophic and mixotrophic growth conditions. This activity was repressed by complementation with *soxR*, suggesting that SoxR acts as a repressor and SoxS is essential for full expression. Sequence analysis uncovered operator characteristics in the intergenic regions *soxS–soxV* and *soxW–soxX*. In each region a transcription start site was identified by primer extension analysis. Both regions were cloned into the vector pRI1 and transferred to *P. pantotrophus*. Strains harbouring pRI1 with *soxS–soxV* or *soxW–soxX* expressed the *sox* genes under heterotrophic conditions at a low rate, indicating repressor titration. Sequence analysis of SoxR suggested a helix–turn–helix (HTH) motif at position 87–108 and uncovered an invariant Cys-80 and a cysteine residue at the C-terminus. SoxR was overproduced in *Escherichia coli* with an N-terminal His<sub>6</sub>-tag and purified to near homogeneity. Electrophoretic gel mobility shift assays with SoxR retarded the *soxS–soxV* region as a single band while the *soxW–soxX* region revealed at least two protein–DNA complexes. These data demonstrated binding of SoxR to the relevant DNA. This is believed to be the first report of regulation of chemotrophic sulfur oxidation at the molecular level.

**INTRODUCTION**

The oxidation of hydrogen sulfide or sulfur to sulfionic acid represents the oxidative half of the global sulfur cycle and is mediated primarily by specialized prokaryotes. The ability to oxidize reduced inorganic sulfur compounds (via the sulfur oxidation enzyme system, Sox) is found in aerobically chemotrophic bacteria and anaerobic phototrophic bacteria. *Paracoccus pantotrophus* is an aerobic, Gram-negative, neutrophilic, facultatively autotrophic bacterium which grows with thiosulfate or molecular hydrogen as energy source and heterotrophically with a large variety of carbon sources (Ludwig *et al.*, 1993; Rainey *et al.*, 1999). This species, a member of the a-Proteobacteria, was isolated as *Thiosphaera pantotropha* (Robertson & Kuenen, 1983) and is the closest relative of *Paracoccus denitrificans* (Rainey *et al.*, 1999). The genus *Paracoccus* has been of particular interest with respect to its highly flexible energy metabolism, the alternative anaerobic respiratory growth and the electron-transport chain used for aerobic growth, which has long been used as model for mitochondrial electron transport. It shows versatility with respect not only to physiology but also to regulation and promoter structures (reviewed by Baker *et al.*, 1998; Steinrücke & Ludwig, 1993).

*P. pantotrophus* grows with thiosulfate exclusively under aerobic conditions and not anaerobically with nitrate as electron acceptor (Friedrich, 1998). The formation of proteins required for chemotrophic growth with thiosulfate is induced by thiosulfate. Thiolsulfate-induced cells are able to oxidize thiosulfate and hydrogen sulfide at a high rate, while sulfur is slowly oxidized and sulfite is not metabolized by whole cells (Chandra & Friedrich, 1986; Friedrich & Mitrenga, 1981).

The gene region of *P. pantotrophus* encoding sulfur-oxidizing ability comprises 15 genes, of which seven, *soxXYZABCD*, encode the periplasmic proteins SoxYXZ, SoxB, SoxCD and SoxXA, which catalyse hydrogen sulfide-, sulfur-, thiosulfate- and also sulfite-dependent cytochrome *c* reduction *in vitro* (Rother *et al.*, 2001). The first gene of the *sox* gene region, *soxR*, previously designated ORF1, predicts a transcriptional regulator of the ArsR family, and *soxS* (formerly ORF2) a periplasmic thioredoxin (Friedrich *et al.*, 2001). Both genes are oriented divergently to the other...
genes of the sox cluster (Fig. 1). The Sox proteins required for sulfur oxidation are expressed in the presence of thiosulfate in chemotrophic bacteria (Friedrich et al., 2001; Kelly et al., 1997) as are the respective proteins in phototrophic purple bacteria, purple non-sulfur and green bacteria (reviewed by Friedrich, 1998). Apart from marginal physiological studies no information is available on the regulation of the proteins involved in sulfur oxidation at the molecular biological level, or on the mechanisms that lead to the formation of the Sox proteins in chemotrophic or phototrophic bacteria.

In P. pantotrophus the thioredoxin-encoding soxW gene (formerly shxW) is expressed in the presence of thiosulfate (Bardischewsky & Friedrich, 2001), as are the structural genes soxXYZABCD and subsequent genes soxEFGH of unknown function (Friedrich et al., 2000; Rother et al., 2001). In the mutant strain P. pantotrophus GBOV the soxV gene (formerly shxV) is disrupted by the Ω-kanamycin interposon. In the presence of thiosulfate, strain GBOV does not express soxW due to the polarity of the interposon but fully expresses the subsequent genes soxX–H (Bardischewsky & Friedrich, 2001). This result suggested a regulatory region upstream of soxX.

In this study we report the construction of the homogenote mutant GBOV, which expresses the sox genes under heterotrophic growth conditions, and the identification of two sox promoter regions by primer extension analysis. We have expressed soxR of P. pantotrophus in Escherichia coli, isolated the His-tagged SoxR, demonstrated SoxR binding to the two promoter regions of the sox gene cluster and identified SoxR as specific repressor protein.

**METHODS**

**Bacterial strains and plasmids.** Strains and plasmids used and constructed in this study are listed in Table 1.
Media and growth conditions. E. coli was cultivated in Luria–Bertani (LB) medium (Sambrook et al., 1989) at 37°C. P. pantotrophus was cultivated mixotrophically in mineral medium, pH 7.2, with 20 mM thiosulfate plus 20 mM disodium succinate (Bardischewsky & Friedrich, 2001) unless otherwise stated, and heterotrophically in mineral medium pH 7.2 with 10 mM disodium succinate at 30°C. Antibiotics were added where appropriate (for E. coli 100 μg ampicillin ml⁻¹, 25 μg chloramphenicol ml⁻¹ or 25 μg kanamycin ml⁻¹, and for P. pantotrophus 5 μg chloramphenicol ml⁻¹).

DNA and gene transfer techniques. Standard DNA techniques were applied (Sambrook et al., 1989). Plasmid DNA was isolated according to Kieser (1984). Restriction enzymes and T4 DNA ligase were obtained from Promega, Roche or Fermentas (Oli) and used as recommended by the manufacturer. PCR reactions were performed with DyNAzyme EXT polymerase (Finnzymes) essentially as recommended by the manufacturer. DNA sequencing, plasmid DNA was prepared with the Invisorb Spin Plasmid Mini Kit (Invitec). DNA was sequenced as described previously (Rother et al., 2001). E. coli was transformed as described by Chung et al. (1989). E. coli S17-1 was used to mobilize plasmids into P. pantotrophus GB17 (Simon et al., 1983). P. pantotrophus and E. coli S17-1 were conjugated as described by Rother et al. (2001).

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>recA1 supE44 endA1 hisdR17 gyrA96 relA1 thi Δ(lac–proAB)</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>M15</td>
<td>K12 derivative</td>
<td>Qiangen</td>
</tr>
<tr>
<td>S17-1</td>
<td>recA pro thi hisD, RP4 tra functions, supE44</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>Paracoccus pantotrophus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GB17</td>
<td>Sox⁺</td>
<td>Rainey et al. (1999)</td>
</tr>
<tr>
<td>GB20V</td>
<td>soxV::Ω; Sox⁻</td>
<td>Bardischewsky &amp; Friedrich (2001)</td>
</tr>
<tr>
<td>GB20S</td>
<td>soxS::Ω</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript SK⁻</td>
<td>Ap⁺ lacZ ColE1 ori, f1 ori, T7 and T3 promoter</td>
<td>Stratagen</td>
</tr>
<tr>
<td>pEG9</td>
<td>9-5 kb EcoRI fragment soxR–soxC in pSUP202</td>
<td>Wodara et al. (1994)</td>
</tr>
<tr>
<td>pEG12</td>
<td>13 kb EcoRI sox-relevant DNA fragment in pSUP202</td>
<td>Mittenhuber et al. (1991)</td>
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<td>pHP450-Km</td>
<td>Ap⁺ Km⁻</td>
<td>Rémy et al. (1987)</td>
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<td>pJOE773.2</td>
<td>Ap⁺, lacZ⁺, positive selection vector</td>
<td>Altenbuchner et al. (1992)</td>
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<td>pJOEB9</td>
<td>8-851 bp BamHI fragment soxR–soxC of pEG12 in pJOE773.2</td>
<td>This study</td>
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<td>pOG5A</td>
<td>9-5 kb EcoRI fragment soxR–soxC in pUC19</td>
<td>This study</td>
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<td>pOG7</td>
<td>4 kb EcoRI–KpnI fragment soxR–orfV in pUC19</td>
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<td>pOG9A</td>
<td>Ω-Km interposon cloned into Oli of pOG8</td>
<td>This study</td>
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<td>pOG10A</td>
<td>7-8 kb Xhol–SacI fragment of pOG8 in pSUP202</td>
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<td>pQE30</td>
<td>Ap⁺ Cm⁺ T5 promoter [His]₆</td>
<td>Qiangen</td>
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<td>soxW–soxX as 232 bp EcoRI–Smal PCR fragment in pUC19</td>
<td>This study</td>
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<tr>
<td>pRD149.2</td>
<td>soxS–soxV as 246 bp EcoRI–Smal PCR fragment in pUC19</td>
<td>This study</td>
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<td>pRD150.6</td>
<td>soxR⁺ as BamHI–HindIII fragment in pUC19</td>
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<td>pRD151.2</td>
<td>soxR⁻ as BamHI–HindIII fragment in pUC19</td>
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<td>pRD152.4</td>
<td>Oligos S34 and S35 with enterokinase cleavage site in pRD151.2</td>
<td>This study</td>
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<tr>
<td>pRD154.7</td>
<td>Binding region soxW–soxX as 246 bp BamHI–EcoRI fragment in the EcoRV site of pRII</td>
<td>This study</td>
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<tr>
<td>pRD156.5</td>
<td>Binding region soxS–soxV as 260 bp BamHI–EcoRI fragment in the EcoRV site of pRII</td>
<td>This study</td>
</tr>
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<td>pREP4</td>
<td>Km⁺ lac repressor</td>
<td>Qiangen</td>
</tr>
<tr>
<td>pRII</td>
<td>Cm⁺ Sm⁺</td>
<td>Piitzner et al. (1998)</td>
</tr>
<tr>
<td>pRIsoxR</td>
<td>1-9 kb Smal fragment soxR–soxS in pRI1</td>
<td>This study</td>
</tr>
<tr>
<td>pSUP202</td>
<td>Cm⁺ Tc⁺, Tra⁻ Mob⁺</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap⁺ lacZ</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
</tbody>
</table>

*Sox, lithotrophic growth with thiosulfate; Tra, transfer of mobilizable plasmids; Mob, mobilizability.
Cloning of soxR. For complementation of the mutant P. pantotrophus GB28 with SoxR the plasmid pOG7 was cut with Smal and the generated 1942 bp fragment containing soxR was cloned into the shuttle vector pR11, resulting in the plasmid pRIssoxR.

Cloning of the intergenic regions soxW–soxX and soxS–soxV. The soxW–soxX region was amplified by PCR using the primers S30 (5′-AAAAACCGGCGATGGCTATGAAATGT-3′) and S31 (5′-TTTGAATTCGAAAGCAGACGGCCCG-3′), which adds a His6-tag to the N-terminus of a protein. The resulting fragment was transferred to the expression vector pQE30 (Qiagen), of pUC19, yielding plasmid pRD150.6. After verification of the 434 bp fragment was cloned between the BamHI–EcoRI site of pJOEB9 as template. Primer S32 is compatible to bp 1037 and was determined with primers pEG_7.5 (PV1; bp 1199–1221, 5′-TTTTGAATTCAAAGGACAGCAGCCCCG-3′) and pEG_7.3 (PX1; bp 2675–2696, 5′-TTTTGACATTGCACTGACGGCAGG-3′) and that of pEG_7.2 (PV2; bp 1286–1308, 5′-CCGCCACCATGCGAGGATCGAGG-3′) and pEG_7.4 (PX2; 2731–2753, 5′-CGCCTTGGACATAGAACCGCCAC-3′), one picomole of primer was annealed to 1 μg total RNA and extended for 1 h at 42°C by using 200 U Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s protocol.

Expression of soxR-his and purification of His6-SoxR. Cells of E. coli M15(pREP4, pRD152.4) were grown at 37°C in 1 L medium supplemented with 100 μg ampicillin ml⁻¹ and 25 μg kanamycin ml⁻¹. Plasmid pREP4 supplied additional lactose-repressor (Lac) to allow soxR-his expression exclusively upon induction. At an OD₆₀₀ of 0.6, IPTG was added to a final concentration of 0.4 mM and the culture was incubated for an additional 4 h. Cells were harvested by centrifugation (Sorvall GS3 rotor, 6000 r.p.m., 10 min, 4°C), washed twice with lysis buffer (50 mM Tris/HCl pH 7.5, 500 mM NaCl, 10 mM MgCl₂, 2 mM CaCl₂, 1 mM imidazole, 5 mM 2-mercaptetoanethol, 1 μM PMSF, 5%, w/v, glycerol), resuspended in 25 ml of this buffer and passed four times through a French press at 150 MPA. Cell debris was removed by centrifugation (Sorvall S53 rotor, 20 000 r.p.m., 30 min, 4°C), and the supernatant was referred to as crude extract. This extract was subjected to Ni-NTA affinity chromatography. A column filled with 4 ml Ni-NTA agarose (Qiagen) was equilibrated with lysis buffer. An aliquot of crude extract (5 ml) was applied to the column and rinsed with 20 ml lysis buffer, followed by two washing steps, each with 16 ml wash buffer (50 mM Tris/HCl pH 7.5, 500 mM NaCl, 10 mM MgCl₂, 2 mM CaCl₂, 20 mM imidazole, 5 mM 2-mercaptoethanol, 1 μM PMSF, 5%, w/v, glycerol). SoxR was eluted with elution buffer (50 mM Tris/HCl pH 7.5, 500 mM NaCl, 10 mM MgCl₂, 2 mM CaCl₂, 250 mM imidazole, 5 mM 2-mercaptoethanol, 1 μM PMSF, 5%, w/v, glycerol). Fractions (2 ml) containing His-tagged protein were pooled and concentrated with a Vivaspin concentrator (Vivasience). To remove NaCl and imidazole during concentration the protein was washed with buffer A (50 mM Tris/HCl pH 7.5, 10 mM MgCl₂, 2 mM CaCl₂, 1 μM PMSF, 5%, w/v, glycerol). This extract was applied to a 6 ml Resource Q-column equilibrated against buffer A. Protein was eluted with a linear gradient of 0–1 M NaCl. His₆-SoxR-containing fractions were pooled (40 ml), washed with SoxR buffer (100 mM Tris/HCl pH 7.5, 1 mM EDTA, 10 mM MgCl₂, 2 mM CaCl₂, 5%, w/v, glycerol, 2 mM 2-mercaptoethanol) and concentrated to 1 ml.

DNA labelling. For gel mobility shift assays the binding regions were isolated from the plasmids pRD148.3 and pRD149.2 after restriction with EcoRI and PstI. From pRD148.3 a 260 bp fragment with the binding region soxW–soxX and from pRD149.2 a 274 bp fragment with the binding region soxS–soxV were isolated by gel elution. These DNAs were labelled using the Biotin 3′ End DNA Labelling Kit (Perbio). Deoxynucleotidyl transferase (TdT) catalyses the incorporation of biotin-N4-CTP onto the 3′-OH end of DNA (Ruychoughdy & Wu, 1980; Ruychoughdy et al., 1976). As TdT exhibits a substrate preference for single-stranded DNA, the isolated fragments were denaturated at 95°C for 5 min prior to the labelling reaction, which was performed according to the manufacturer’s instructions. Subsequently the DNA single strands were renatured by hybridization.

Gel mobility shift assays. These assays were performed with the LightShift chemiluminescent EMSA Kit (Perbio). Biotin-labelled DNA was incubated with varying amounts of protein for 20 min in gel shift assay buffer (10 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 2-5%, v/v, glycerol, 50 ng poly(dIdC) μl⁻¹, 0-05% NP-40) at room temperature. DNA and DNA–protein complexes were separated in a 8% polyacrylamide gel. The gels were prerun at 100 V in Tris/ borate/EDTA (TBE) buffer (Sambrook et al., 1989) for 30 min, and

Isolation of RNA and primer extension. Total RNA was isolated from P. pantotrophus cells after mixotrophic growth with succinate plus thiosulfate with the High Pure RNA isolation kit (Roche). 5′-Fluorescently labelled primers (MWG Biotech) were used for primer extension analyses. The transcription start upstream of soxV was determined with primers pEG_7.5 (PV1; bp 1199–1221, 5′-CCCCCGATGCCTGGCGCTGCC-3′) and pEG_7.2 (PV2; bp 1286–1308, 5′-CCGGACACCATGCGAGGATCGAGG-3′) and that of pEG_7.4 (PX2; 2731–2753, 5′-CGCCTTGGACATAGAACCGCCAC-3′). One picomole of primer was annealed to 1 μg total RNA and extended for 1 h at 42°C by using 200 U Moloney murine leukemia virus reverse transcriptase according to the manufacturer’s protocol.
after samples (20 μl) had been loaded into the wells, gels were run at 100 V for 90 min. The gel was then transferred to Hybond-N + nylon membrane (Amersham). Blotting was performed by a semi-dry procedure using the Multiphor electrophoresis system (Pharmacia) in 0.5 × TBE buffer at 380 mA and 10 °C for 45 min. Biotin-labelled DNA was detected according to the manufacturer’s instructions. The membrane was exposed to Kodak BioMax light film for 2–5 min.

Analytical procedures. Denatured proteins were separated by SDS-PAGE according to Laemmli (1970). Proteins were stained with Coomassie blue as described by Weber et al. (1972). Protein from cell-free extracts was determined by the method of Bradford (1976). A ligand blotting procedure (Towbin et al., 1979) was performed by the semi-dry procedure using the Multiphor electrophoresis system (Pharmacia) with nickel-nitrilotriacetate (Ni-NTA) conjugate (Qiagen). The transfer buffer was 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, 20% (v/v) methanol. The transfer took place at room temperature for 60 min. Ni-NTA conjugate consisting of Ni-NTA coupled to calf intestinal alkaline phosphatase was used to detect the His6-tag of SoxR.

Enzyme assays. The thiosulfate oxidation rate of whole cells was determined with an oxygen electrode (Rank Brothers). The assay (3 ml) contained 50 μl cell suspension of OD436 30, equivalent to 150 μg protein, and 100 μmol sodium/potassium phosphate buffer (pH 8.0). Reactions were started with 30 μl 0-2 M sodium thiosulfate. One unit (U) of thiosulfate oxidation activity was defined as 1 μmol O2 consumed (mg protein)−1.

Sequence analyses. Database searches were performed with BLASTN and BLASTP (http://www.ncbi.nlm.nih.gov/blast/) (Altschul et al., 1997), including a CD search (Marchler-Bauer et al., 2003). Pairwise alignments were carried out with BLAST 2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) (Tatusova & Madden, 1999) or with LALIGN (http://www.fasta.bioch.virginia.edu/fasta_wwww/lalign.htm) (Huang & Miller, 1991) and multiple alignments with CLUSTALW (http://www.ebi.ac.uk/clustalw/) (Thompson et al., 1994). HTH motifs were predicted with Network Protein Sequence Analysis (http://www.npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=\!/NPSA/npsa_hth.html) (Dodd & Egan, 1990). DNA sequences were analysed with the Clone Manager 6 program (Scientific & Educational Software).

RESULTS AND DISCUSSION

Inactivation of soxS

The transposon Tn5 acts in a polar manner on the expression of sox genes located downstream of the insertion, as demonstrated for soxV::Ω-Km (Bardischewsky & Friedrich, 2001) and soxX::Ω-Km (F. Bardischewsky, unpublished data). The homogenate mutant GBØS carried the soxS::Ω-kanamycin interposon, which inactivated soxS and very likely acted in a polar manner on the expression of soxR. In contrast to the wild-type, this strain expressed thiosulfate-oxidizing activity under heterotrophic conditions, albeit at a low rate. However, also under mixotrophic growth with succinate plus thiosulfate a low thiosulfate-oxidizing activity of 18% of the wild-type was expressed (Table 2). Complementation in trans with soxR restored the repression of the formation of thiosulfate-oxidizing activity under heterotrophic growth with succinate but did not restore the full expression when thiosulfate was included in the medium (Table 2). These data suggested SoxR as repressor protein for expression of the sox genes and SoxS as an essential periplasmic component for their optimal expression.

Analysis of promoter/operator regions of the sox cluster

The mutant GBØV suggested a regulatory site upstream of soxV and of soxX (Bardischewsky & Friedrich, 2001). Sequence characteristics for operator regions were detected in the 69 bp intergenic region between soxS and soxV and in a second 114 bp intergenic region between soxW and soxX. Both regions, as well as parts of adjacent genes, contain characteristics for binding of dimeric regulatory proteins. For soxS–soxV the perfect inverted repeat CAAGCATCGGC and the perfect direct repeat TGCCGGGGC were detected. For soxW–soxX the inverted repeat TGAAAAATG and the direct repeats CAGGGAG and TGGCCAT were present for the soxS–soxV region, and TGCCGATGGC was located downstream of soxX (Fig. 1). Such sequence features are typical recognition elements of DNA-binding proteins possessing an HTH motif. Similar repeats are observed in the respective regions of Rhodovulum sulfidophilum, Rhodopseudomonas palustris, Silicibacter pomeroyi and Bradyrhizobium japonicum. One of the sequences (TGGTTCGAACA) is also present as a perfect palindromic site in soxS of S. pomeroyi and Rv. sulfidophilum with an identity of 80% (data not shown). The significance of these observations is presently unknown.

Further intergenic regions exist between soxA and soxB (119 bp) and between soxE and soxF (53 bp). No promoter/operator sites were considered for the soxA–soxB and soxE–soxF intergenic regions of P. pantotrophus since the homogenate mutant strain GBØX did not express any of the sox genes downstream of the soxX::Tn5 insertion (F. Bardischewsky, unpublished data). For Rv. sulfidophilum, however, it was concluded that soxF can be transcribed from a promoter lying between soxC and soxF (Appia-Ayme et al., 2001).

Table 2. Trans complementation of P. pantotrophus GBØS with pRIsoxR

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific thiosulfate oxidation rate [μmol O2 (mg protein)−1]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mixotrophic growth</td>
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<tr>
<td>GB17 (wild-type)</td>
<td>3.94</td>
</tr>
<tr>
<td>GB17(pRIsoxR)</td>
<td>3.37</td>
</tr>
<tr>
<td>GBØS</td>
<td>0.69</td>
</tr>
<tr>
<td>GBØS(pRIsoxR)</td>
<td>0.56</td>
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</table>
Determination of sox transcription start sites

Two transcription starts were determined by primer extension for the sox gene cluster of P. pantotrophus GB17. The existence of a transcription start site upstream of soxX was evident from strain GB0V, which expressed the sox structural genes. To identify the site upstream of soxV the 5′ ends of the sox mRNAs were determined by primer extension analysis. For the primer extension reactions two different primers were used for each transcription start site. For examination of the soxS–soxV intergenic region the primers used were complementary to a sequence upstream of the ATG codon of soxV and a region internal to soxV, and for soxW–soxX two different primers were used internal to soxX. Primer extension analysis using primers PV1 and PV2 determined the transcription start at thymine-1124 (Fig. 2a), which is 104 nt upstream of the translation start of soxV. Using the primers PX1 and PX2 a second transcription site was determined at thymine-2568 or thymine-2569 (Fig. 2b), which are 85 or 86 nt upstream of the translation start codon of soxX (Fig. 1). Thus, the nucleotide sequences suggesting operator sites for binding of regulatory proteins were located downstream of the RNA polymerase binding sites for both intergenic regions.

Expression of soxR in E. coli

To study the role of SoxR in regulation of the sox genes by in vitro gel mobility shift assays, SoxR was required in significant amounts; therefore, soxR–his was expressed in E. coli. The complementary oligonucleotides S34 and S35 completed soxR′ and introduced sequence variations at the third position of the third and the fifth codon without changing the amino acid sequence, and led to plasmid pRD152.4. In both cases the rarely used codon CCC was changed to CCG, most frequently used for proline in E. coli. This nucleotide exchange was performed since the efficiency of translation depends on the RNA sequence having optimal codons for the first five N-terminal amino acids downstream of the translational start codon as well as on the RNA sequence immediately upstream of the translation start (Degryse, 1990).

After transformation with plasmid pRD152.4, E. coli M15(pREP4, pRD152.4) cells were analysed for SoxR production by SDS-PAGE. A strong IPTG-inducible protein band of 20 kDa was observed, which was in agreement with the predicted size of 18-46 kDa of the 166 aa comprising His6-SoxR (Fig. 3a, lane 3). The protein was present in the soluble fraction of the cell-free extracts and absent from cell extracts from E. coli M15(pREP5, pQE30) not expressing soxR–his (Fig. 3a, lane 2). Ni-NTA-AP conjugate (Qiagen) identified the His-tag and demonstrated that the additional band represented the His6-tagged SoxR (Fig. 3a, lanes 7 and 8). The calculated pI of the His-tagged SoxR is 6-94, whereas for SoxR a pI of 5-71, a size of 149 aa and a molecular mass of 16-47 kDa were predicted.

SoxR binds to the intergenic regions soxS–soxV and soxW–soxX

Gel retardation assays were used to examine binding of SoxR to the intergenic regions soxS–soxV and soxW–soxX. The soxS–soxV DNA fragment formed a protein–DNA complex with His6-SoxR-containing cell extracts from E. coli M15(pREP4, pRD152.4), increasing in intensity with increasing protein concentration (Fig. 4a, lanes 4–6). The soxW–soxX DNA fragment formed three protein–DNA complexes when various amounts of cell extract were used (Fig. 4b, lanes 4–6), indicating multiple binding sites on the fragment used in the shift experiment.

The specificity of the binding was evident from gels using different concentrations of cell extract of E. coli M15(pREP4, pQE30), which is specifically devoid of SoxR. No protein–DNA complexes were formed with these extracts lacking His6-SoxR (Fig. 4a, lanes 7 and 8; Fig. 4b, lanes 7 and 8).
The specificity of the binding was further examined by addition of 40-fold excess of unlabelled fragments to the assays, which abolished the retardation of the labelled DNA (Fig. 4a, lanes 9 and 10; Fig. 4b, lanes 9 and 10). These findings demonstrated that His6-SoxR bound specifically to the intergenic regions soxS–soxV and soxW–soxX. Further evidence for the specificity of His6-SoxR regarding the DNA-binding regions was achieved by using purified His6-SoxR in the gel mobility shift assay. When 0–5 ng of DNA carrying the intergenic region soxS–soxV was used with decreasing amounts of protein the typical protein–DNA complex was seen. With crude extract, 200–500 ng of the protein was required to observe a band-shift whereas with purified SoxR protein a faint band appeared with 10 ng and a more intense band with 50 ng of His6-SoxR, demonstrating that binding of the purified SoxR protein was specific, although a slight loss of activity occurred during the purification process (Fig. 4c). As expected from the binding assays using cell-free extract of E. coli, the purified His6-SoxR also bound to the soxW–soxX promoter region (data not shown).

When thiosulfate was added to the in vitro binding assays at concentrations of 10 μM to 10 mM no notable release of

**Fig. 3.** SDS-PAGE analysis of His6-SoxR expressed in E. coli M15. (a) Coomassie stain; (b) ligand blotting of His6-SoxR with Ni-NTA conjugate. Lanes: 1, molecular mass markers; 2, crude extract from E. coli M15(pREP4, pQE30), induced, not expressing His6-SoxR, 15 μg; 3, crude extract from E. coli M15(pREP4, pRD152.4), induced, 15 μg; 4, eluate from affinity chromatography with Ni-NTA-agarose, 3 μg; 5, eluate from anionic-exchange chromatography with a Resource Q column, 3 μg; 6, crude extract from E. coli M15(pREP4, pQE30), induced, 25 μg, not expressing His6-SoxR; 7, crude extract from E. coli M15(pREP4, pRD152.4), induced, 25 μg; 8, purified His6-SoxR, 3 μg.

**Fig. 4.** Gel retardation analysis of the soxS–soxV– and the soxW–soxX–His6-SoxR complex. The amount of labelled DNA was 0–5 ng for each lane. (a, c) soxS–soxV DNA, (b) soxW–soxX DNA. For binding studies of SoxR, crude extracts from E. coli M15(pREP4, pRD152.4) (SoxR+) and E. coli M15(pREP4, pQE30) (SoxR−) (a, b) and purified His6-SoxR (c) were used. (a, b) Lanes: 1, DNA only; 2, DNA plus 100 ng extract (SoxR+); 3, DNA plus 200 ng extract (SoxR+); 4, DNA plus 500 ng extract (SoxR+); 5, DNA plus 1000 ng extract (SoxR+); 6, DNA plus 2000 ng extract (SoxR+); 7, DNA plus 1000 ng extract (SoxR−); 8, DNA plus 2000 ng extract (SoxR−); 9, DNA plus 1000 ng extract (SoxR+) and 20 ng of unlabelled competitor DNA; 10, DNA plus 2000 ng extract (SoxR+) and 20 ng unlabelled competitor DNA. (c) Lanes: 1, DNA only; 2, DNA plus 200 ng crude extract (SoxR+); 3, DNA plus 500 ng crude extract (SoxR+); 4–10, DNA plus decreasing amounts of purified His6-SoxR (4, 500 ng; 5, 200 ng; 6, 100 ng; 7, 50 ng; 8, 10 ng; 9, 5 ng; 10, 1 ng). Due to the ageing of the protein extracts the signal intensity decreases with time.
His6-SoxR was observed from the binding regions soxS–soxV and soxW–soxX (data not shown). This result indicated that thiosulfate per se was not involved in derepression of the sox genes in *P. pantotrophus* and suggested a so far unknown sulfur intermediate as the active principle in regulation of chemotrophic sulfur oxidation of *P. pantotrophus*.

**SoxR, a transcriptional repressor**

The function of SoxR as a repressor of sox gene expression would require an interaction of the protein with operator sequences. To confirm the findings from in vitro binding studies, thiosulfate-dependent oxygen consumption rates were examined from *P. pantotrophus* GB17 containing plasmid pRI1, pRD156.5 or pRD154.7 and compared to the wild-type cultivated under mixotrophic and heterotrophic growth conditions. Under mixotrophic conditions similar thiosulfate oxidation rates of 2·29–2·83 U were obtained for all four strains: the wild-type *P. pantotrophus* GB17 (2·85 U), *P. pantotrophus* GB17(pRI1) harbouring the vector without any insert (2·29 U), *P. pantotrophus* GB17(pRD156.5) carrying the intergenic region soxS–soxV (2·63 U), and *P. pantotrophus* GB17(pRD154.7) carrying the intergenic region soxW–soxX (2·75 U). As the inducer thiosulfate was not supplied under heterotrophic growth conditions absolutely no thiosulfate oxidation rates were measured for the wild-type and the control strain GB17(pRI1). In strain GB17(pRD156.5) and GB17(pRD154.7) carrying soxS–soxV and soxW–soxX low but distinct thiosulfate oxidation rates of 0·10 U and 0·19 U, respectively, were determined. These findings suggested that SoxR was titrated from the genomic binding regions, leading to a constitutive albeit low expression of the sox genes.

**Sequence analysis of SoxR and the sox regulatory regions**

The gel shift assays identified SoxR as a protein binding to the intergenic regions soxS–soxV and soxW–soxX, and the constitutive synthesis of Sox proteins upon introduction of the regions suggested the function of SoxR as repressor protein of the sox gene cluster of *P. pantotrophus*. The DNA-binding property of SoxR was in accordance with the HTH motif at position 87–106 of the deduced primary structure (Network Protein Sequence Analysis), as HTH motifs are diagnostic for DNA-binding proteins (Fig. 5). An invariant Cys-80 is located 7 aa in front of the HTH motif present exclusively in SoxR homologues (Fig. 6) but not in other ArsR transcriptional regulators (data not shown). Cys-149 is the C-terminus of SoxR of *P. pantotrophus* and cysteine residues are generally present at the C-termini of SoxR and its ArsR homologues from various sources (data not shown). *Streptomyces lividans* 1326 harbours an inducible mercury resistance for which MerR, belonging to the ArsR family, is the transcriptional regulator. The C-terminal cysteine of MerR binds the inducer mercury(II) ions (Rother, 1998). Since thiosulfate did not affect binding of SoxR to the relevant sox intergenic regions thiosulfate-dependent formation of a protein disulfide in SoxR may be involved in the regulation of sox expression.

![Fig. 5. Alignment of SoxR of *P. pantotrophus* and other bacteria. The box indicates the HTH motif. The invariant cysteine in front of the HTH motif and the cysteine in the C-terminal region are highlighted in bold.](image-url)
Database comparisons of the primary structure of SoxR showed high amino acid sequence similarities to other putative proteins of other chemo- and phototrophic bacteria. soxR of Rv. sulfidophilum predicting SoxR of 140 aa (accession no. AAA11780) is 70% identical in a 74 aa overlap. A 118 aa SoxR homologue (IGwit database RMQ02638) of Methylobacterium extorquens shows an identity of 54-3%. A SoxR homologue of 116 aa of Bradyrhizobium japonicum (accession no. BAC48771) (Kaneko et al., 2002) and the 124 aa homologue of Rps. palustris (accession no. CAE29915) (Larimer et al., 2004) have identities of 52%. *Pseudaminobacter salicylatoxidans* (accession no. AJ404005.4) has a SoxR (121 aa) with an identity of 48% of the entire primary structure to that of *P. pantotrophus*. The same degree of identity is given for SoxR (128 aa) of *Silicibacter pomeroyi* (accession no. YP_166241) (Moran et al., 2004). SoxR of *Rhodobacter sphaeroides* (127 aa) (accession no. NZ_AAAE0100156.1) (Matsuzaki et al., 2003) has a 61% identity in a 91 aa overlap (data not shown). These chemo- and phototrophic bacteria all contain essential sox genes homologous to those of *P. pantotrophus*, and the SoxR homologues may represent repressor proteins of sox expression in these strains.

The essentially invariant HTH motif of SoxR homologues led us to examine the identity of the nucleotide sequences of the two binding regions. With LALIGN a 59-4% identity in a 64 nt overlap was observed between the soxS–V and soxW–X regions of *P. pantotrophus*. Moreover, a 64-2% identity in a 81 nt overlap with the soxS–X region of the phototroph *Rv. sulfidophilum* was found. From the highly conserved HTH motif in SoxR (Fig. 5) similarly conserved DNA sequences were expected. However, although similar structural features like repeats are present in the intergenic regions of these bacteria there is no sequence similarity in any of them.

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