The periplasmic thioredoxin SoxS plays a key role in activation *in vivo* of chemotrophic sulfur oxidation of *Paracoccus pantotrophus*

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The significance of the *soxS* gene product on chemotrophic sulfur oxidation of *Paracoccus pantotrophus* was investigated. The thioredoxin SoxS was purified, and the N-terminal amino acid sequence identified SoxS as the *soxS* gene product. The wild-type formed thiosulfate-oxidizing activity and Sox proteins during mixotrophic growth with succinate plus thiosulfate, while there was no activity, and only traces of Sox proteins, under heterotrophic conditions. The homogenote mutant strain GB Ω S is unable to express the *soxSR* genes, of which *soxR* encodes a transcriptional regulator. Strain GB Ω S cultivated mixotrophically showed about 22 % of the specific thiosulfate-dependent O₂ uptake rate of the wild-type, and when cultivated heterotrophically it produced 35 % activity. However, under both mixotrophic and heterotrophic conditions, strain GB Ω S formed Sox proteins essential for sulfur oxidation *in vitro* at the same high level as the wild-type produced them during mixotrophic growth. Genetic complementation of strain GB Ω S with *soxS* restored the activity upon mixotrophic and heterotrophic growth. Chemical complementation by reductants such as L-cysteine, DTT and tris(2-carboxyethyl)phosphine also restored the activity of strain GB Ω S in the presence of chloramphenicol, which is an inhibitor of *de novo* protein synthesis. The data demonstrate that SoxS plays a key role in activation of the Sox enzyme system, and this suggests that SoxS is part of a novel type of redox control in *P. pantotrophus*.

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INTRODUCTION

Reactions of the oxidative half of the global sulfur cycle are mediated by various aerobic lithotrophic and anaerobic phototrophic prokaryotes. *Paracoccus pantotrophus* is a Gram-negative, neutrophilic, facultatively chemolithoautotrophic bacterium that aerobically oxidizes thiosulfate, hydrogen sulfide and sulfur (Rainey *et al.*, 1999; Robertson & Kuenen, 1983). The gene region of *P. pantotrophus* encoding chemotrophic sulfur oxidation comprises 15 genes, namely *soxSR* and *soxVWXYZABCDEFGH*. Seven genes encode the proteins SoxYZ, SoxB, SoxCD and SoxXA. Together, these proteins catalyse hydrogen sulfide-, sulfur-, thiosulfate- and sulfite-dependent cytochrome *c* reduction *in vitro* (Friedrich *et al.*, 2001, 2005; Rother *et al.*, 2001).

The *soxSR* genes are transcribed divergently to the other *sox* genes of the cluster. *soxR* encodes the repressor SoxR, which is a member of the ArsR family that binds to two intergenic regions (*soxS*–*soxV* and *soxW*–*soxX*) of the *sox* locus (Rother

et al., 2005), and *soxS* encodes the periplasmic thioredoxin SoxS (Rother *et al.*, 2001).

The *soxS* gene is disrupted by the Ω -kanamycin-resistance-encoding interposon Tn5 in the homogenote mutant *P. pantotrophus* GB Ω S, and this strain is also unable to express *soxR*. Strain GB Ω S has about 22 % of the specific thiosulfate-dependent O₂ uptake rate of the wild-type level under mixotrophic growth conditions with succinate plus thiosulfate. Complementation of strain GB Ω S with *soxR* restores repression of the specific thiosulfate-oxidizing activity under heterotrophic conditions, suggesting that SoxR is a repressor protein (Rother *et al.*, 2005). The position of the *soxS* gene upstream of *soxR*, and the low thiosulfate-oxidizing activity of GB Ω S, seemed to suggest a function of the putative thioredoxin SoxS in the regulation of expression of chemotrophic sulfur oxidation.

In this study, we report the isolation of the periplasmic thioredoxin SoxS, and that it is encoded by the *soxS* gene. We demonstrate that a mutant unable to express *soxS* forms high levels of Sox proteins that are not active, and that SoxS is involved in activation of the thiosulfate-oxidizing enzyme

Abbreviation: TCEP, tris(2-carboxyethyl)phosphine.

system, and we exclude the involvement of SoxS in the expression of the *sox* genes.

METHODS

Bacterial strains and plasmids. Strains and plasmids used and constructed in this study are listed in Table 1. The DNA sequence of the *sox* gene region is accessible under GenBank/EMBL accession number X79242.

Media and growth conditions. *Escherichia coli* strains were cultivated in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) at 37 °C. *P. pantotrophus* strains were cultivated under mixotrophic growth conditions in mineral medium, initial pH 7.2, with 20 mM thiosulfate plus 20 mM disodium succinate (Bardischewsky & Friedrich, 2001), unless otherwise stated. Heterotrophic growth was performed in mineral medium at an initial pH of 6.8, with 20 mM succinate at 30 °C. L-Cysteine, DTT and tris(2-carboxyethyl)phosphine (TCEP) were added as specified in the text. Antibiotics were added where appropriate (for *E. coli*: 100 µg ampicillin ml⁻¹, 50 µg chloramphenicol ml⁻¹, and 50 µg kanamycin ml⁻¹; for *P. pantotrophus*: 300 µg kanamycin ml⁻¹, 10 µg chloramphenicol ml⁻¹, and 40 µg rifampicin ml⁻¹).

DNA techniques. Standard DNA techniques were applied (Sambrook *et al.*, 1989). Plasmids were prepared in small scale from *E. coli*, as described by Kieser (1984). For DNA sequencing, plasmid DNA was prepared with the Qiagen Plasmid Midi Kit (Qiagen). Starting from the known sequence, 2674 bp of pOG7 were further sequenced downstream of *soxR*. DNA sequencing was done by Invitac. Restriction enzymes, T4 DNA ligase, alkaline phosphatase and Klenow polymerase were obtained from Fermentas, Promega or Roche, and used as recommended by the manufacturer. Agarose gel electrophoresis was done in TAE (Sambrook *et al.*, 1989). DNA fragments were eluted from agarose gels using the QIAquick gel extraction kit (Qiagen).

Construction of pRIsoxR, pRIsoxS and pRIsoxSR. The *soxS* gene was disrupted by inserting an Ω-kanamycin interposon by gene replacement, yielding the homogenote mutant *P. pantotrophus* GBΩS, as described by Rother *et al.* (2005). Strain GBΩS was complemented with *soxR* by plasmid pRIsoxR (Rother *et al.*, 2005). Plasmid pRIsoxS was constructed for complementation of strain

GBΩS with *soxS*. Plasmid pOG7 (Table 1) was cut with *Bam*HI and *Kpn*II, and the generated 608 bp fragment containing *soxS* was cloned into pRII, resulting in the plasmid pRIsoxS. For complementation with SoxR and SoxS, pOG7 was cut with *Kpn*II and *Sal*I. The generated 3192 bp fragment containing *soxSR* was cloned into pRII, resulting in the plasmid pRIsoxSR. *E. coli* strains were transformed as described by Chung *et al.* (1989). *E. coli* S17-1 was used to mobilize the plasmids pRIsoxR, pRIsoxS and pRIsoxSR into *P. pantotrophus* GB17 and GBΩS (Simon *et al.*, 1983).

Preparation of cell fractions. Periplasmic proteins were extracted from the cells by osmotic shock according to the *QIAexpressionist* (2nd edn, Protocol 4; Qiagen). Tris/HCl was added to the extract to give a final concentration of 25 mM.

Cell-free extracts were prepared by passing cell suspensions (0.1 g dry cell weight ml⁻¹) twice through a French pressure cell at 150 MPa, and subjecting the extracts to differential centrifugation. The 200 000 g supernatant was used for ammonium sulfate fractionation. Proteins precipitating between 44 and 65 % ammonium sulfate saturation were dialysed against 25 mM sodium/potassium phosphate buffer, pH 6.5, and designated as the A65 fraction (Friedrich *et al.*, 2000).

Purification of the thioredoxin SoxS. SoxS was identified by antibodies raised against the immunogenic oligopeptides OP-S with the amino acid sequences LQMRDPLPPGLELAR and DVESGRLEGYPGED, as deduced from the *soxS* nucleotide sequence. SoxS was purified from the A65 fraction of cells, which were cultivated mixotrophically with succinate plus thiosulfate, using similar methods to those described for the Sox proteins that constitute the Sox enzyme system (Rother *et al.*, 2001). The protein was subjected to chromatography on Q-Sepharose Fast Flow, and eluted in the 0.10 M sodium chloride fraction of the respective step gradient. The SoxS-containing fractions were identified by Western blot analysis (Towbin *et al.*, 1979), and then combined, concentrated by ultrafiltration and subjected to gel-filtration chromatography on Sephacryl S200 HR. For further purification, SoxS-containing fractions were subjected to chromatography on Phenyl Sopharose High Performance, which is a hydrophobic interaction matrix. This step yielded a partially purified preparation of SoxS that was analysed by immunoblotting.

Analytical procedures. Denatured proteins were separated by SDS-PAGE according to Laemmli (1970). Proteins were stained

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference or source
<i>E. coli</i>		
JM 109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F'[traD36 proAB⁺ lac^r lacZAM15]</i>	Yanisch-Perron <i>et al.</i> (1985)
S17-1	<i>recA pro thi hsdS RP4-tra-functions supE44</i>	Simon <i>et al.</i> (1983)
<i>P. pantotrophus</i>		
GB17	Sox ⁺	Rainey <i>et al.</i> (1999)
GBΩS	Homogenote mutant of GB17 with Ω-kanamycin-resistance cassette in <i>soxS</i> (<i>soxS</i> ::Ω-Km ^r)	Rother <i>et al.</i> (2005)
Plasmids		
pOG7	4 kb <i>Eco</i> RI– <i>Kpn</i> I fragment <i>soxR-orfV'</i> in pUC19	Rother <i>et al.</i> (2005)
pRII	Cm ^r , Sm ^r	Pfützner <i>et al.</i> (1998)
pRIsoxR	1942 bp <i>Sma</i> I fragment of pOG7 containing <i>soxR</i> -' <i>soxS</i> in pRII	Rother <i>et al.</i> (2005)
pRIsoxS	608 bp <i>Kpn</i> II– <i>Bam</i> HI fragment of pOG7 containing <i>soxS</i> in pRII	This study
pRIsoxSR	3192 bp <i>Kpn</i> II– <i>Sal</i> I fragment of pOG7 containing <i>soxSR</i> in pRII	This study

with Coomassie blue, as described by Weber *et al.* (1972). Protein from cell-free extracts was determined by the method of Bradford (1976).

Immunoblots (Western blots) (Towbin *et al.*, 1979) were performed according to the 'semi-dry' procedure, using the multiphor electrophoresis system (Pharmacia) and antibodies raised in rabbits, at the facilities of Eurogentec. SoxYZ antigens were detected with polyclonal antibodies raised against homogeneous SoxYZ. SoxCD antigens were detected with polyclonal antibodies raised against homogeneous SoxCD. Antibodies raised against the cytochrome complex SoxXA of *P. pantotrophus* were used to detect SoxXA. SoxB antigens were detected with antibodies against SoxB antigens expressed in *E. coli* (Friedrich *et al.*, 2000).

The N-terminal amino acid sequence was determined from purified SoxS, which was separated by SDS-PAGE, and transferred to ProBlot membranes. The major protein band of 11 kDa was cut out of the membrane, and subjected to automated Edman degradation using the protein sequencer system model 494A/190A (Applied Biosystems), as detailed previously (Fischer *et al.*, 1996). The signal peptide cleavage site was predicted by the PSORT program package (Nakai & Kaneshisa, 1991).

The PHYLIP package (Felsenstein, 1989) was used to calculate protein distance matrices, and to construct phylogenetic trees. For settings, standard options were used. Trees were drawn using TREEVIEW, version 1.6.6. (Page, 1996).

Enzyme assays. The thiosulfate oxidation rate of whole cells was determined with an oxygen electrode (Rank Brothers). The assay (3 ml) contained 50 μ l cell suspension (OD_{436} 30, equivalent to 150 μ g protein) and 100 μ mol sodium/potassium phosphate buffer (pH 8.0). Reactions were started with 30 μ l 0.20 M sodium thiosulfate. The specific thiosulfate-dependent O_2 uptake rate was measured as μ mol O_2 consumed min^{-1} (mg protein) $^{-1}$.

RESULTS AND DISCUSSION

Expression of *soxS*

The *soxSR* genes are oriented divergently to the other genes of the *sox* cluster. Expression of *soxS* was analysed by immunoblot analysis of periplasmic extracts using the SoxS-specific OP-S-antibodies. SoxS antigens were detected in periplasmic extracts of wild-type cells cultivated mixotrophically with thiosulfate, while no SoxS antigens were detected from heterotrophically grown cells (Fig. 1a). As expected, no SoxS antigens were detected from the A65 fraction of extracts of the homogenate mutant strain GB Ω S (Fig. 1b) harbouring a *soxS::* Ω -disruption (Rother *et al.*, 2005). To complement strain GB Ω S with *soxS* or *soxSR*, the two plasmids pRIsoxS and pRIsoxSR (Table 1) were constructed, and conjugated into strain GB Ω S. In both strains GB Ω S(pRIsoxS) and GB Ω S(pRIsoxSR), SoxS antigens were present after mixotrophic growth (Fig. 1b). In periplasmic extracts, the signal of SoxS antigens was stronger, as observed from the A65 fraction of extracts of the wild-type GB17 that indicated the selectivity of the procedure. The faint signal of SoxS in the A65 fraction of strain GB17 indicated the low concentration of SoxS *in vivo*, and the stronger signals observed from the respective extracts of the complemented strains of GB Ω S showed the

Fig. 1. Detection of SoxS in strains of *P. pantotrophus* by Western blot analysis. (a) Periplasmic fractions (30 μ g protein per well) of GB17 after mixotrophic cultivation with thiosulfate (lane 1), and after heterotrophic cultivation (lane 2). (b) A65 fractions (20 μ g protein per well) of cells grown mixotrophically with thiosulfate. Lanes: 1, GB Ω S; 2, GB Ω S(pRIsoxS); 3, GB17; 4, GB Ω S(pRIsoxSR); 5, GB Ω S(pRIsoxSR).

higher rate of expression of the *soxS* gene from the plasmids pRIsoxS and pRIsoxSR (Fig. 1b).

Purification of the thioredoxin SoxS

SoxS, as deduced from the *soxS* nucleotide sequence, consists of 130 aa, with a molecular mass of 14 467 Da. The mature SoxS is predicted to contain 99 aa, with a molecular mass of 11 077 Da. SoxS was purified as detailed in Methods, and the OP-S antibodies identified an 11 kDa protein (data not shown). Ten amino acids of the N-terminus of SoxS were analysed to be AELRLMSEQ. This sequence was identical to that deduced from *soxS*. Also, the N-terminus Ala-32 was identical to that predicted for the signal peptide cleavage site. These data identified *soxS* as encoding the periplasmic protein SoxS, and this result matched well with the periplasmic location of SoxS antigens (Fig. 1a).

Sequence analysis of SoxS

The low molecular mass (11 077 Da) and the CXXC motif were diagnostic for thioredoxins. *Paracoccus denitrificans* 1222 is the closest relative of *P. pantotrophus* GB17 (Rainey *et al.*, 1999), which was previously classified as *P. denitrificans* GB17 (Ludwig *et al.*, 1993). From the genome sequence of *Paracoccus denitrificans* 1222 (accession no. AAIT00000000), eight proteins with characteristics of thioredoxins were identified. Of these, six were predicted to be located in the periplasm, and two in the cytoplasm (Fig. 2), all being rather diverse, and suggesting different functions and partner proteins. The amino acid sequences of thioredoxins predicted from the genomes of other bacteria were aligned together with those for which redox function has been shown. The alignment revealed that SoxS-homologous thioredoxins clustered in a distinct group differentiated from the other thioredoxins (data not shown). The BLAST search demonstrated that proteins highly homologous to SoxS were exclusively present in sulfur-oxidizing chemotrophic and phototrophic *Alphaproteobacteria*, which harbour *sox* gene clusters (data not shown). These data revealed that thioredoxins of the SoxS-type were distinct

Fig. 2. Phylogenetic tree of putative thioredoxins of *P. denitrificans* PD1222 and the relationship to SoxS of *P. pantotrophus*. The tree was constructed with the software specified in Methods. The accession numbers of the proteins are given in parentheses.

from other periplasmic thioredoxins, and from those located in the cytoplasm or attached to the cytoplasmic membrane. This analysis suggests a specific function for SoxS thioredoxins in chemotrophic sulfur metabolism.

Analysis of the SoxS function

SoxR was shown to bind to the intergenic regions *soxS*–*soxV* and *soxW*–*soxX*, and it was identified as a repressor of the transcription of the respective *sox* genes (Rother *et al.*, 2005). To analyse whether SoxS was involved in the regulation of the transcription of the *sox* genes, immunoblot analysis was performed to detect the Sox-relevant proteins in cell-free extracts of strains GB17, GBΩS, GBΩS(pRIsoxR), GBΩS(pRIsoxS) and GBΩS(pRIsoxSR). The proteins SoxXA, SoxYZ, SoxB and SoxCD were present at high concentrations in cell-free extracts of the wild-type GB17 grown mixotrophically with thiosulfate, while only traces of the proteins were detected in wild-type cells cultivated heterotrophically (Fig. 3). Mixotrophically, as well as heterotrophically, cultivated cells of strain GBΩS and GBΩS(pRIsoxS) formed the Sox proteins at the same level as the wild-type when it was cultivated under mixotrophic conditions. However, expression of *soxR* *in trans* by plasmids pRIsoxR and pRIsoxSR restored the wild-type phenotype in the complemented strains of GBΩS (Fig. 3). Therefore SoxR, but not SoxS, was involved in repression of the transcription of the *sox* genes under heterotrophic growth conditions.

Fig. 3. Sox protein formation in strains of *P. pantotrophus*. SoxXA (a), SoxYZ (b), SoxB (c) and SoxCD (d) were detected by Western blot in A65 fractions (20 µg protein per well) from cells grown mixotrophically with thiosulfate (lanes 1–5), and heterotrophically (lanes 6–10). Lanes: 1 and 6, GB17; 2 and 7, GBΩS; 3 and 8, GBΩS(pRIsoxR); 4 and 9, GBΩS(pRIsoxS); 5 and 10, GBΩS(pRIsoxSR).

After mixotrophic cultivation, the specific thiosulfate-oxidizing activity of whole cells of strain GBΩS was about 22 % of that of the wild-type. Moreover, *trans*-complementation of strain GBΩS with *soxR* had no effect on the activity of whole cells (Rother *et al.*, 2005).

To determine if SoxS affected the activity of the Sox enzyme system *in vivo*, the specific thiosulfate-oxidizing activity was determined from mixotrophically cultivated cells of the wild-type GB17, and strains GBΩS, GBΩS(pRIsoxR), GBΩS(pRIsoxS) and GBΩS(pRIsoxSR).

As expected, under mixotrophic growth conditions, the wild-type formed a high specific thiosulfate-oxidizing activity of $0.57 \mu\text{mol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$. Strain GBΩS exhibited a low specific thiosulfate-oxidizing activity of $0.13 \mu\text{mol O}_2 \text{ min}^{-1} (\text{mg protein})^{-1}$ (Table 2), while the Sox proteins were present at the same concentration as in the wild-type (Fig. 3).

Complementation of strain GBΩS with the *soxS* gene resulted in a full restoration of the specific thiosulfate-oxidizing activity, and complementation to *soxSR* increased the specific thiosulfate-oxidizing activity of whole cells to about 48 %, as compared with the wild-type. However, expression of *soxR* alone had no effect on the specific thiosulfate-dependent O_2 uptake rate (data not shown). This result strongly suggests that SoxS plays a key role in activation of one or more proteins of the Sox enzyme system of *P. pantotrophus*.

Table 2. Activation of thiosulfate oxidation in strain GBΩS by reductants

Cells were cultivated mixotrophically with succinate plus thiosulfate, and samples were taken after 30 min aerobic incubation at 30 °C. ND, Not determined.

Reductant	Concn (mM)	Specific thiosulfate-dependent O ₂ uptake rate [μmol O ₂ min ⁻¹ (mg protein) ⁻¹]	
		Wild-type GB17	GBΩS
None	–	0.57	0.13
L-Cysteine	5	0.61	0.38
L-Cysteine	1	0.52	0.21
DTT	1	0.61	0.46
DTT*	1	ND	0.46
DTT†	1	ND	0.61
TCEP	1	0.54	0.29

*Chloramphenicol (10 μg ml⁻¹) was also added.

†Rifampicin (40 μg ml⁻¹) was also added.

Reductants activate the Sox enzyme system *in vivo*

The membrane protein SoxV is essential for the function of the Sox enzyme system in *P. pantotrophus* and the phototroph *Rhodovulum sulfidophilum* (Bardischewsky *et al.*, 2006; Appia-Ayme & Berks, 2002). However, the function of SoxV can be chemically compensated by addition of the reductant DTT, resulting in the transient restoration of the specific thiosulfate-oxidizing activity under aerobic conditions (Bardischewsky *et al.*, 2006). SoxV of *P. pantotrophus* is a paralogue of the DsbD/CcdA family, which also transports electrons from the cytoplasm to the periplasm. However, DsbD/CcdA proteins exclusively function in reduction of apocytochromes to enable addition of the haem moiety. Also, the phenotypes of some *dsbAB*- and *ccdA/dsbD*-defective strains can be restored by addition of thiol compounds to the medium (Bardwell *et al.*, 1993; Sambongi & Ferguson, 1994). Therefore, the effect of reductants on the specific thiosulfate-oxidizing activity of strain GBΩS when cultivated mixotrophically with succinate plus thiosulfate was examined.

Addition of 1 mM L-cysteine to the medium led to a 1.6-fold increase in specific thiosulfate-oxidizing activity of strain GBΩS, but no increase was seen for the wild-type. The activity increase depended on the concentration of L-cysteine, and 5 mM L-cysteine increased the activity threefold (Table 2). The increase occurred within 30 min, and was maintained at the high level for at least 3 h (data not shown). Also, addition of DTT (1 mM) and the non-sulfur reductant TCEP (1 mM) increased the specific thiosulfate-oxidizing activity of whole cells. Inclusion of chloramphenicol, an inhibitor of translation, or rifampicin, an inhibitor of transcription, prior to the addition of DTT, did not affect the increase in activity (Table 2). From these studies, and the

rapid effect of the various reductants, it was concluded that one or more Sox proteins present in strain GBΩS were activated by reduction, while *de novo* protein synthesis was not involved in the observed increase in specific thiosulfate-oxidizing activity.

The Sox enzyme system is active in extracts of strain GBΩS

Immunoblot analysis of strain GBΩS revealed a high concentration of the Sox proteins (Fig. 3). However, strain GBΩS exhibited a low *in vivo* specific thiosulfate-dependent O₂-uptake rate (Table 2). For high activity, strain GBΩS required either genetic complementation to produce SoxS, or chemical complementation by reductants (Table 2). The *in vitro* specific thiosulfate-dependent cytochrome *c* reduction rate, as examined from the ammonium sulfate fraction of the cell-free extract of strain GBΩS, was 2.2 mU (mg protein)⁻¹, and thus similar to that of the wild-type [1.9 mU (mg protein)⁻¹]. From these data, SoxS appears to be required for the activation of one or more proteins of the specific thiosulfate-oxidizing enzyme system *in vivo*. The data do not suggest that SoxS is involved in the oxidation mechanism of thiosulfate, since the reconstituted Sox enzyme system functioned well without SoxS. This was evident from the *in vitro* studies of strain GBΩS specified above, and from biochemical studies (Rother *et al.*, 2001). The membrane-bound protein SoxV is specifically required for sulfur oxidation *in vivo*, while the periplasmic thioredoxin SoxW is not. Therefore, one partner of SoxS might be SoxV, while the other partner is very likely to be at least one of the Sox proteins that constitute the Sox enzyme system. Future studies using site-directed mutagenesis will aim to identify the redox partners of SoxS.

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