SoxV transfers electrons to the periplasm of *Paracoccus pantotrophus* – an essential reaction for chemotrophic sulfur oxidation

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The *soxVW* genes are located upstream of the *sox* gene cluster encoding the sulfur-oxidizing ability of *Paracoccus pantotrophus*. SoxV is highly homologous to CcdA, which is involved in cytochrome *c* maturation of *P. pantotrophus*. SoxV was shown to function in reduction of the periplasmic SoxW, which shows a CysXaaXaaCys motif characteristic for thioredoxins. From strain GBΩV, which carries an Ω-kanamycin-resistance-encoding interposon in *soxV*, and complementation analysis it was evident that SoxV but not the periplasmic SoxW was essential for lithoautotrophic growth of *P. pantotrophus* with thiosulfate. However, the thiosulfate-oxidizing activities of cell extracts from the wild-type and from strain GBΩV were similar, demonstrating that the low thiosulfate-oxidizing activity of strain GBΩV *in vivo* was not due to a defect in biosynthesis or maturation of proteins of the Sox system and suggesting that SoxV is part of a regulatory or catalytic system of the Sox system. Analysis of DNA sequences available from different organisms harbouring a Sox system revealed that *soxVW* genes are exclusively present in *sox* operons harbouring the *soxCD* genes, encoding sulfur dehydrogenase, suggesting that SoxCD might be a redox partner of SoxV. No complementation of the *ccdA* mutant *P. pantotrophus* TP43 defective in cytochrome *c* maturation was achieved by expression of *soxV* *in trans*, demonstrating that the high identity of SoxV and CcdA does not correspond to functional homology.

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INTRODUCTION

The reversible formation of protein-disulfide bonds plays an important role in transport of electrons, protein biogenesis, protein stability and enzyme catalysis (Akyama & Ito, 1993; Yamanaka *et al.*, 1994; Okamoto *et al.*, 1995; Missiakos & Raina, 1997; reviewed by Fabianek *et al.*, 2000; Ritz & Beckwith, 2001). Different thiol:disulfide oxidoreductases have been identified which are involved in the formation, isomerization and reduction of disulfide bonds in different bacteria. All these oxidoreductases share a conserved CysX-aaXaaCys motif and a common tertiary structure known as the thioredoxin-like fold (Martin, 1995). Members of the DsbD and CcdA families are involved in the transport of electrons from the cytoplasm to the periplasm for reduction of apocytochromes to enable addition of the haem moiety (reviewed by Thöny-Meyer, 1997). The electrons are transferred from a cytoplasmic thioredoxin first to the central transmembrane β-domain of DsbD then to the C-terminal thioredoxin-like γ-domain and therefrom to the N-terminal α-domain, which then reduces either DsbC or

CcmG (Rietsch *et al.*, 1997; Stewart *et al.*, 1999; Chung *et al.*, 2000; Katzen & Beckwith, 2000).

The Gram-negative, facultatively chemolithoautotrophic bacterium *Paracoccus pantotrophus* grows under aerobic conditions lithotrophically with molecular hydrogen and thiosulfate as energy source for autotrophic carbon dioxide fixation (Robertson & Kuenen, 1983; Rainey *et al.*, 1999). In *P. pantotrophus* the *soxVWXYZABCDEFGH* genes constitute two transcriptional units, *soxVW* and *soxX–H* (Rother *et al.*, 2005). In the homogenote mutant GBΩV carrying the *soxV::Ω*-kanamycin interposon *soxX–H* are expressed upon growth with thiosulfate as in the wild-type GB17 (Bardischewsky & Friedrich, 2001b).

SoxV predicts a protein with six transmembrane helices which is closely related with respect to its primary and secondary structure to CcdA of *P. pantotrophus* and other bacteria involved in cytochrome *c* biogenesis (Bardischewsky & Friedrich, 2001a). SoxW predicts a periplasmic protein with characteristics of members of the thioredoxin superfamily (Bardischewsky & Friedrich, 2001b). SoxV and CcdA of *P. pantotrophus* are 42% identical. Despite the high degree of identity the two proteins display completely different functions. Inactivation of *ccdA* of *P. pantotrophus* causes

Abbreviations: AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; Sox, sulfur oxidation; TCEP, tris(2-carboxyethyl)phosphine; TMPD, *N,N,N',N'*-tetramethyl-1,4-benzenediamine.

pleiotropic effects due to the inability to mature *c*-type cytochromes which are involved in different metabolic pathways such as dissimilatory nitrite reduction, and hydrogen and thiosulfate oxidation. Therefore, for CcdA of *P. pantotrophus* a similar role was suggested as shown for DsbD of *Escherichia coli* (Crooke & Cole, 1995; Bardischewsky & Friedrich, 2001a) and CcdA of *Bacillus subtilis* (Schiött *et al.*, 1997), which transport reductant from the cytoplasm into the periplasm, for re-reduction of the cytochrome *c* apoprotein, an essential step for binding of the haem moiety.

Disruption of *soxV* of *P. pantotrophus* using the Ω -Km interposon does not affect the cytochrome *c* biogenesis or autotrophic carbon dioxide fixation (Bardischewsky & Friedrich, 2001b). However, strain GB Ω V is unable to grow lithotrophically with thiosulfate. Thus, the function of SoxV differs from that of CcdA.

In this study we analysed the *soxV*:: Ω -Km disruption with respect to the activity of the thiosulfate-oxidizing system *in vivo* and *in vitro*. Whole cells of strain GB Ω V oxidized thiosulfate at a rate of about 2% as compared to the wild-type, whereas the rates of thiosulfate oxidation of cell extracts of strain GB Ω V and of the wild-type were similar. We have identified SoxW to be maintained in the reduced state by SoxV. These results and complementation analysis suggest that SoxV transfers electrons via SoxW or another thioredoxin to an as yet unknown periplasmic target involved in thiosulfate oxidation *in vivo* and that SoxW is not essential and possibly substituted by other thioredoxins.

METHODS

Bacterial strains and plasmids. The *E. coli* strains used were S17-1 (*recA pro thi hsdS*, RP4 *tra*-functions *supE44*) (Simon *et al.*, 1983) and XL-1 Blue (*hsdR17 recA1 endA1 gyrA46 thi relA1 lac* [*F'**proAB, lacZ* Δ M15 Tn10 (Tet^r)] (Bullock *et al.*, 1987; Stratagene). *Paracoccus pantotrophus* strains used were GB17 (Sox⁺ Hox⁺) (Robertson & Kuenen, 1983; Rainey *et al.*, 1999), GB Ω V (Sox⁻ Hox^L, Ω -Km interposon integrated in *soxV*) (Bardischewsky & Friedrich, 2001b), GB Ω X (Sox⁻, Ω -Km interposon integrated in *soxX*) (Bardischewsky *et al.*, 2005) and TP43 (Sox⁻ *cyt c*⁻, Tn5-*mob* integrated in *ccdA*) (Chandra & Friedrich, 1986; Bardischewsky & Friedrich, 2001a). Plasmid pBHP6 carrying the *soxVW* genes (Bardischewsky & Friedrich, 2001b), pRIN2.6 the *soxW* gene, and pRIPV*phoA* the *soxV* gene (this study) were used for complementation studies. Plasmid pRIN2.6 was constructed from the 2.6 kb *NruI* fragment of pBSP3.4 (Bardischewsky & Friedrich, 2001b) containing *soxV'* WXYZA'. The gene region was cloned into pJOE733.2 (Ap^r, *lacZ* α) (Altenbuchner *et al.*, 1992), resulting in pJOEN2.6. The 2.6 kb DNA fragment containing *sox* genes was isolated from pJOEN2.6 after *PstI* restriction, and it was then cloned into the *PstI* site of pRI1 (Sm^r Cm^r Mob⁺) (Pfützner *et al.*, 1998) resulting in pRIN2.6. Construction of plasmid pRIPV*phoA* was done in two steps. First, the *phoA* gene was isolated from pPHO7 (Gutierrez & Devedjian, 1989) by restriction with *Bam*HI and *Xba*I, and cloned into pRI1, resulting in pRI*phoA*. Then the *soxV* region was amplified by PCR using primers PV1 (5'-CGGGATCCCGGGCCTGG-GCAGGGAGATTCCA-3') and PV2 (5'-CGGGATCCCGGGCGGCT-GCGGGTGCTGCCG-3'), which both introduced a restriction site for *Bam*HI (underlined). Plasmid pBSP3.4 (Bardischewsky & Friedrich,

2001b) was used as template. The 940 bp amplified DNA fragment containing the *soxV* gene region was restricted with *Bam*HI and cloned into the *Bam*HI restriction site of pRI*phoA*, resulting in pRIPV*phoA*.

Media and growth conditions. Strains were cultivated at 30 °C. Mineral media were identical for heterotrophic, for mixotrophic and for lithotrophic growth of *P. pantotrophus*, and were described previously (Chandra & Friedrich, 1986). For lithotrophic growth with thiosulfate, mineral media contained 20 mM sodium thiosulfate at a final pH of 8.0. For mixotrophic growth, mineral media contained 20 mM sodium succinate and 20 mM sodium thiosulfate at a final pH of 7.2. *E. coli* was cultivated in Luria-Bertani medium (Sambrook *et al.*, 1989). The following antibiotics were used when appropriate: for *P. pantotrophus*, 300 μ g kanamycin (Km) ml⁻¹ and 10 μ g chloramphenicol (Cm) ml⁻¹; for *E. coli*, 30 μ g Cm ml⁻¹, 100 μ g ampicillin (Ap) ml⁻¹ and 12.5 μ g tetracycline (Tc) ml⁻¹.

DNA techniques. Standard DNA techniques (Sambrook *et al.*, 1989) were used. Plasmid DNA was isolated according to the procedure of Birnboim & Doly (1979). Restriction enzymes, T4 DNA ligase and Klenow polymerase were obtained from Gibco-BRL and used as recommended by the manufacturer. DNA fragments were eluted from agarose gels using the QIAquick gel extraction kit (Qiagen).

Preparation of cell fractions. The A65 fraction and the membrane fraction were prepared from cells disrupted with a French press (Quentmeier *et al.*, 2000). The extract was subjected to differential centrifugation at 4 °C. Whole cells and cell debris were separated at 10 000 g for 20 min. The resulting cell-free extract was subjected to centrifugation at 200 000 g for 2 h to separate the soluble periplasmic and cytoplasmic proteins from the membranes. Proteins of the supernatant were precipitated at 65% saturation ammonium sulfate as described by Quentmeier *et al.* (2000) and designated the A65 fraction. The 200 000 g pellet was washed twice with 50 mM sodium phosphate buffer, pH 7.4, and designated the membrane fraction.

Periplasmic proteins were specifically extracted from the cells by osmotic shock according to the protocol of Qiagen (The QIAexpressionist, protocol 4, 2nd edition). Tris/HCl pH 6.5 was added to the extract to give a final concentration of 25 mM.

Enzyme assays. Enzyme activities were determined from whole cells and cell-free extracts. The thiosulfate-dependent oxygen uptake rate of whole cells was determined using a polarographic oxygen electrode (Rank Brothers) as described by Wodara *et al.* (1997). The thiosulfate-dependent oxygen uptake rate of cell-free extracts was determined with the oxygen electrode similarly as for whole cells. The assay (3.0 ml) contained 10 mg protein of the membrane fraction and 30 mg protein of the A65 fraction. Thiosulfate-dependent cytochrome *c* reducing activity of the A65 fraction and of periplasmic extracts was determined spectrophotometrically at 550 nm as described by Quentmeier *et al.* (2000). One unit (U) of enzyme activity was defined as 1 μ mol cytochrome *c* reduced or O₂ utilized per minute at 30 °C. Whole cells were screened for cytochrome *c* oxidase by verifying their capability to oxidize *N,N,N',N'*-tetramethyl-1,4-benzenediamine (TMPD). TMPD oxidation was monitored spectrophotometrically at 611 nm. The assay (1.0 ml) contained phosphate buffer (36 mM, pH 8.0), 50 μ g (dry weight) of whole cells and 2 mM TMPD.

Analytical procedures. SoxW was detected in cell-free extracts of *P. pantotrophus* strains by immunoblot analysis using a semidry procedure (Towbin *et al.*, 1979). Antibodies against the immunogenic oligopeptide DDGLHKPTWLRETFK as deduced from the *soxW* nucleotide sequence were raised in rabbits at the facilities of Eurogentec (Seraing, Belgium). Ribulose-bisphosphate carboxylase

(RubisCO) is a cytoplasmic enzyme and was used as marker protein to indicate the purity of the periplasmic extracts. Antibodies against RubisCO of *Ralstonia eutropha*, which were also active against the enzyme of *P. pantotrophus* (Bowien *et al.*, 1976), were obtained from B. Bowien, Göttingen, Germany.

Thiosulfate was quantified according to Sørbo (1957).

Protein from cell-free extracts was quantified according to Bradford (1976).

Determination of the redox states of SoxW. The redox states of SoxW *in vivo* were determined by electrophoretic mobility after linking free thiols of proteins with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) according to Kobayashi *et al.* (1997) and by immunochemical identification of SoxW. Proteins of whole cells were precipitated by addition of trichloroacetic acid to the culture to a final concentration of 5% (w/v). The precipitate was collected by centrifugation and washed twice with acetone. The pellet was dissolved in freshly prepared 50 mM Tris/HCl buffer, pH 7.5, containing 15 mM AMS and 1% SDS. Reduced SoxW was prepared by incubation of periplasmic extract with 10 mM tris(2-carboxyethyl)-phosphine (TCEP) for 20 min at 30 °C. Proteins were then precipitated with trichloroacetic acid and washed twice with acetone as described above to remove TCEP. The precipitate was resuspended in freshly prepared 50 mM Tris/HCl buffer, pH 7.5, containing 1% SDS and 15 mM AMS. Proteins were separated by SDS-PAGE in the absence of reductants. SoxW was detected by immunoblot analysis as described above.

Sequence analysis. Multiple alignments of amino acid sequences were performed using ClustalView (Thompson *et al.*, 1994). The PHYLIP package (Felsenstein, 1989) was used to determine the phylogenetic relationships of CcdA and ShxV. The treefile was viewed using TreeView (Page, 1996).

RESULTS

Complementation analysis of strain GBΩV

Strain GBΩV does not express *soxVW* and is unable to grow lithoautotrophically with thiosulfate, whereas heterotrophic growth is unaffected. Complementation of strain GBΩV with *soxVW* *in trans* by plasmid pBHP6 resulted in the wild-type phenotype (Bardischewsky & Friedrich, 2001b). To analyse if both gene products were required for chemotrophic growth with thiosulfate, strain GBΩV was complemented either with *soxV* located on plasmid pRIPV*phoA* or with *soxW* located on plasmid pRIN2.6. The amount of SoxW in cells of strain GBΩV harbouring *soxW* *in trans* was about 50% as compared to the wild-type as verified by immunoblot analysis (data not shown). The resulting trans-conjugant GBΩV(pRIPV*phoA*), expressing only *soxV*, was able to grow lithoautotrophically with thiosulfate, whereas strain GBΩV(pRIN2.6), expressing only *soxW*, was not (data not shown). Therefore, exclusively *soxV* but not SoxW was required for autotrophic growth with thiosulfate in *P. pantotrophus*. This result suggested that SoxW was not the exclusive partner for electron transfer of SoxV. These results were in accordance with those obtained for *Rhodovulum sulfidophilum* (Appia-Ayme & Berks, 2002).

The redox state of SoxW

The direction in which SoxV transferred electrons was examined from the redox state of SoxW *in vivo*. The redox state of proteins of whole cells was trapped upon their precipitation with trichloroacetic acid, and free thiol groups were then alkylated with AMS as described in Methods. Alkylation of SoxW with AMS causes an increase of the molecular mass, resulting in a slightly decreased mobility in non-reducing SDS-PAGE. Immunoblotting of AMS-treated proteins isolated from the wild-type revealed a slightly decreased mobility of SoxW, demonstrating that SoxW was present in the reduced state in the wild-type. Immunoblotting of AMS-treated proteins of strain GBΩV(pRIN2.6), which expressed *soxW* but not *soxV*, demonstrated that SoxW was present in the oxidized state in this strain (Fig. 1). No SoxW antigens were detected from strains GBΩV and GBΩV(pRIPV*phoA*), confirming previous results (data not shown). From these data it was concluded that SoxW of *P. pantotrophus* was involved in a reduction reaction and that SoxV was required to maintain SoxW in the reduced state. A similar conclusion was drawn for the SoxVW system of the anaerobic phototrophic sulfur-oxidizing bacterium *Rhodovulum sulfidophilum* (Appia-Ayme & Berks, 2002).

Chemical complementation of strain GBΩV

The phenotypes of some *dsbAB*- and *ccdA/dsbD*-defective strains could be restored by addition of thiol compounds to the medium (Bardwell *et al.*, 1993; Sambongi & Ferguson, 1994, 1996). Since the direction of the electron transfer from the cytoplasm to the periplasm appeared to be identical with that of the DsbD system of *E. coli* (Rietsch *et al.*, 1997; Stewart *et al.*, 1999; Chung *et al.*, 2000; Katzen & Beckwith, 2000) the effect of reductants on the thiosulfate-oxidizing activity of whole cells of the mutant strain was examined. Indeed, addition of reductant (1 mM DTT) to mixotrophically cultivated strain GBΩV in the early stationary phase resulted in a 50-fold increase in formation of the thiosulfate-oxidizing activity of whole cells (Fig. 2) while addition of DTT had no effect on the thiosulfate-oxidizing activity of whole cells of the wild-type (data not shown).

Fig. 1. Electrophoretic separation of oxidized and reduced SoxW after modification with AMS. Cell extracts were subjected to immunoblot analysis after SDS-PAGE under non-reducing conditions. GBΩV (lane 1), GBΩV(pRIN2.6) (lane 2) and GB17 (lane 3) were cultivated mixotrophically with thiosulfate. As a control, 10 µg TCEP-reduced (lane 4) and air-oxidized (lane 5) periplasmic proteins were treated with AMS.

Fig. 2. Specific thiosulfate-dependent oxygen uptake rate of whole cells of the mutant strain GBΩV cultivated mixotrophically in the presence (◆) and absence (◇) of 1 mM DTT.

This result differed from that reported for a SoxVW-deficient mutant of the anaerobic phototrophic thiosulfate-oxidizing bacterium *Rhodovulum sulfidophilum* (Appia-Ayme & Berks, 2002). A chemical complementation of cell extracts with DTT was not possible since low-molecular-mass thiols interfere with the assay system by chemically reducing horse cytochrome *c*.

Thiosulfate oxidation in strain GBΩV

Expression of the *sox* genes and stability of the respective structural Sox proteins was not affected in mutant GBΩV. Since CcdA homologues are involved in the maturation of *c*-type cytochromes it was proposed for *Rhodovulum sulfidophilum* that SoxV could be involved in the maturation of one of the Sox proteins (Appia-Ayme & Berks, 2002). To uncover the specific function of SoxVW, oxidation of thiosulfate by the wild-type and strain GBΩV was examined *in vivo* and *in vitro*. Cells were cultivated mixotrophically with succinate plus thiosulfate as strain GBΩV is unable to grow lithoautotrophically with thiosulfate (Bardischewsky & Friedrich, 2001b). Wild-type cells exhibited a high thiosulfate-dependent oxygen uptake rate after the culture entered the stationary phase, whereas from cells of strain

Fig. 3. Specific thiosulfate-dependent oxygen uptake rate of *P. pantotrophus* strains GB17 and GBΩV during mixotrophic growth. Wild-type GB17 (◆, ◇) and mutant strain GBΩV (●, ○) were cultivated mixotrophically with succinate plus thiosulfate. Cell growth was monitored by measuring the OD₄₃₆ (filled symbols). The thiosulfate-dependent oxygen uptake rate of whole cells was determined with an oxygen electrode (open symbols).

GBΩV only a minor activity of about 14 % of that determined from the wild-type was transiently determined, which, however, was distinct (Fig. 3). These differences in thiosulfate-oxidizing activities of whole cells prompted us to examine the activities of cell extracts. Surprisingly, the thiosulfate-dependent cytochrome *c* reduction rate of the periplasmic fraction from cells of strain GBΩV was almost identical to that observed from the wild-type while the thiosulfate-dependent cytochrome *c* reduction rate of the A65 fraction of strain GBΩV was about half of that of the wild-type. Furthermore, the thiosulfate-dependent electron yields of A65 extracts from GBΩV and the wild-type were almost identical and amounted to 81–86 % of the theoretical yield (Table 1). Thus, the proteins of the sulfur-oxidizing system were fully functional in extracts from the mutant strain GBΩV. Therefore, the low thiosulfate-oxidation rate of whole cells of the mutant strain is not due to a defect in maturation of one of the Sox proteins.

Table 1. Specific activity of whole cells and A65 extracts from *P. pantotrophus* GB17 and GBΩV

Strain	Specific activity (mU)			Yield of electrons A65‡
	Whole cells*	Periplasmic fraction†	A65 extract†	
GB17	520.0	5.0	1.3	6.5
GBΩV	10.0	5.1	0.6	6.9

*Thiosulfate-dependent O₂ uptake rate [μmol O₂ min^{−1} (mg protein)^{−1}].

†Cytochrome *c* reduction rate (mU).

‡Mol cytochrome *c* reduced (mol thiosulfate)^{−1}.

The similar specific activities of thiosulfate-dependent oxygen uptake of cell fractions of strains GB17 and GBΩV suggested that SoxV affected either the electron transfer from the Sox system to the cytoplasmic membrane or a vital reaction required for maintaining the Sox system in a functional state. To discriminate between these two possibilities, the thiosulfate-oxidizing activity was examined with molecular oxygen as the terminal electron acceptor, using isolated membranes which were combined with the A65 fraction either from wild-type cells or from strain GBΩV. The similar specific thiosulfate-dependent oxygen uptake rate using the membrane fractions of both strains when mixed with the A65 fraction of the wild-type demonstrated that the electron transport via the respiratory chain was fully functional in strain GBΩV. The A65 fraction of strain GBΩV supplemented with the membrane fraction isolated from strain GBΩV resulted in an activity of 13.7 mU as compared to 22.7 mU using the A65 fraction of the wild-type (Table 2). This result was in accordance with the decreased thiosulfate-dependent cytochrome *c* reduction rate by the A65 fraction isolated from strain GBΩV (Table 1). Moreover, the result suggested SoxVW to be involved in the reaction cycle of the Sox enzyme system *in vivo*.

Complementation of the *ccdA*-negative strain TP43 with *soxV*

Strain TP43 is defective in expression of *ccdA* and therefore unable to synthesize mature *c*-type cytochromes (Bardischewsky & Friedrich, 2001a). In view of the high identity in primary structure of CcdA and SoxV, strain TP43 was complemented with pBHP6 and pRIPV*phoA* to verify if SoxV was able to substitute for CcdA. Strains TP43(pBHP6) and TP43(pRIPV*phoA*) were analysed for their TMPD-oxidizing ability, diagnostic for cytochrome *c* oxidase. Furthermore, cell extracts from both strains were analysed for the *c*-type cytochrome SoxXA by immunoblot analysis. Surprisingly, despite the high structural homology of CcdA and SoxV of *P. pantotrophus* the wild-type

phenotype was not restored in strain TP43 by *in trans* expression of *soxV* (data not shown).

DISCUSSION

In this study we have confirmed that the CcdA-like membrane protein SoxV is essential for aerobic chemotrophic sulfur oxidation of *P. pantotrophus*. We have further demonstrated that SoxV reduces the periplasmic thioredoxin SoxW, and addition of the reductant DTT to the medium resulted in a partial restoration of the thiosulfate-oxidizing activity of whole cells of strain GBΩV. The effect of DTT supports the finding that SoxVW are involved in a reducing step necessary for full activity of the thiosulfate-oxidizing system. We have demonstrated that SoxV but not SoxW is essential for lithoautotrophic growth of *P. pantotrophus* with thiosulfate. Therefore SoxV is not restricted to SoxW and appears to accept more than one reaction partner. Although *Rhodovulum sulfidophilum* is an anaerobic phototroph, the results and conclusions previously reported for this bacterium were identical to those obtained here for *P. pantotrophus* except for the effect of low-molecular-mass thiols, which did not restore thiosulfate-oxidizing activity in a *soxV* mutant of *Rhodovulum sulfidophilum* (Appia-Ayme & Berks, 2002). Although the general function of SoxV is evident from its essentiality for thiosulfate oxidation *in vivo*, its specific function within the Sox enzyme system is not fully resolved. Proteins of the CcdA and DsbD family were shown to be involved in maturation of *c*-type cytochromes by maintaining the conserved cysteines of the apocytochrome reduced prior to haem attachment. We have previously shown that neither the biosynthesis of *c*-type cytochromes nor the expression of the *sox* structural genes or the stability of the proteins of the Sox system are affected in strain GBΩV (Bardischewsky & Friedrich, 2001b).

Analysis of the activity of whole cells and cell extracts of the wild-type and of the mutant strain GBΩV allowed a deeper insight into the function of SoxV during thiosulfate oxidation. Strain GBΩV was unable to grow lithoautotrophically with thiosulfate. After mixotrophic cultivation, whole cells of the mutant strain displayed at most 14 % of the thiosulfate-dependent oxygen uptake rate as compared to the wild-type. In contrast, when analysing cell-free extracts from both strains only insignificant differences in activities and yields of electrons were observed. Therefore SoxYZ, SoxXA, SoxB and SoxCD appeared to be identically functional in strain GBΩV as in the wild-type, and the mutation in *soxVW* did not cause a defect in protein maturation or biosynthesis as in the case of DsbD and CcdA.

The mutation in *soxV* exclusively affected thiosulfate oxidation of strain GBΩV *in vivo* but not *in vitro*, suggesting that SoxV might be involved in the transport of electrons from the Sox system to the cytoplasmic membrane in *P. pantotrophus*. However, the thiosulfate-dependent oxygen-uptake rate of mixtures of cell-free extracts and membrane

Table 2. Thiosulfate-dependent activities of membranes and A65 extracts from *P. pantotrophus* GB17 and GBΩV

Membranes	A65 extract	Activity*
GB17	GB17	23.2
GBΩV	GBΩV	13.7
GB17	GBΩV	11.0
GBΩV	GB17	22.7
GB17	—	0
GBΩV	—	0
—	GB17	0
—	GBΩV	0

*Thiosulfate-dependent O₂ uptake rate [μmol O₂ min⁻¹ (mg protein)⁻¹].

Fig. 4. Phylogenetic relationship of SoxV of *P. pantotrophus* to homologous proteins of other bacteria. The accession numbers of the proteins are given in parentheses.

fractions from GBQV and the wild-type were similar, demonstrating that SoxV was not involved in the transport of electrons from the Sox system to the cytoplasmic membrane.

Thioredoxins are not only involved in maturation of proteins but they are also essential for the catalytic activity of several enzymes such as phosphoadenosine-phosphosulfate reductase and arsenate reductase (Lillig *et al.*, 1999; Shi *et al.*, 1999). An important recycling function of thioredoxins during catalysis has been described for a methionine sulfoxide reductase. This enzyme was shown to bind its substrate methionine sulfoxide via the thiol group of a highly conserved cysteine residue. After the release of methionine the active-site cysteine has to be re-reduced by a thioredoxin-regenerating system or DTT (Boschi-Muller *et al.*, 2000; Lowther *et al.*, 2000).

The overall thiosulfate-oxidizing activity of cell-free extracts from mutant strain GBQV and the wild-type was only about 1% as compared with the oxygen uptake rate of whole cells of the wild-type (Table 1). This low thiosulfate oxidation *in vitro* as compared to that *in vivo* might indicate that SoxVW are involved in the catalytic cycle, e.g. by a recurrent reduction of cysteine residues of one of the Sox proteins.

Despite the marginal identity of 24% between CcdA of *Rhodobacter capsulatus* and the β -domain of DsbD of *E. coli*

Katzen *et al.* (2002) demonstrated that these two proteins were functional homologues. The expression of *ccdA* from *Rhodobacter capsulatus* in an *E. coli dsbD*-null strain restored the wild-type phenotype as well as a *ccdA*-defective mutant of *Rhodobacter capsulatus* was complemented by the expression of the *E. coli dsbD* gene *in trans*. The amino acid sequence of CcdA and SoxV of *P. pantotrophus* is 42.3% identical and the two proteins have a similar predicted secondary structure (Bardischewsky & Friedrich, 2001b). The thioredoxin SoxW was demonstrated to be dispensable for thiosulfate oxidation in *P. pantotrophus*, and therefore SoxV appears not to be specific for a single reaction partner. However, constitutive expression of *soxV* in the mutant strain TP43 did not restore the wild-type phenotype. This functional specificity of SoxV and CcdA is also evident from the phylogenetic distance of the proteins (Fig. 4).

Genome analysis revealed that of 18 strains harbouring the *sox* gene cluster, 9 contained the *soxVW* genes and also a typical *ccdA* gene probably encoding the cytochrome *c* maturation protein CcdA, strengthening the results that demonstrate the inability of SoxV to substitute for CcdA. Furthermore, the *soxVW* genes are exclusively present in strains harbouring the *soxCD* genes, which encode sulfur dehydrogenase (Friedrich *et al.*, 2005). This correlation may point to a functional link of the respective gene products.

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