The *shxVW* locus is essential for oxidation of inorganic sulfur and molecular hydrogen by *Paracoccus pantotrophus* GB17: a novel function for lithotrophy

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Abstract

The *shxVW* genes of *Paracoccus pantotrophus* were identified to be essential for lithotrophic oxidation of sulfur and hydrogen. *shxV* predicts a membrane protein which is 42\% identical to CcdA of *P. pantotrophus* essential for cytochrome *c* biogenesis. *shxW* predicts a periplasmic thioredoxin. Disruption of *shxV* by an \(\Omega\)-kanamycin interposon disabled the resulting mutant GB\(6\)\(V\) to grow with thiosulfate or molecular hydrogen and to express ShxW while cytochrome *c* formation was not affected. Mixotrophic growth with thiosulfate or molecular hydrogen revealed 2\% of the thiosulfate-dependent oxygen uptake rate as compared to the wild-type while antigens of proteins essential for sulfur oxidation were present in both strains. Mixotrophic growth of strain GB\(6\)\(V\) with succinate and molecular hydrogen revealed neither hydrogenase activity nor antigens. Complementation analysis with plasmid pBHP6 carrying the *shxVW* genes revealed the wild-type phenotype of strain GB\(6\)\(V\)(pBHP6). **\(\odot\)** 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Hydrogen oxidation; Interposon mutagenesis; ShxV protein; Sulfur oxidation; Thioredoxin; *Paracoccus pantotrophus* GB17

1. Introduction

The oxidation of hydrogen sulfide to sulfuric acid is a major reaction of the global sulfur cycle and mediated by different litho-, methylo- and phototrophic prokaryotes [1–3].

*Paracoccus pantotrophus* GB17 is a Gram-negative, neutrophilic facultatively lithotrophic bacterium able to grow with thiosulfate or with molecular hydrogen as energy source, and with a large variety of carbon sources [4]. The sulfur-oxidizing (Sox) system of *P. pantotrophus* is located in the periplasm and requires four proteins, Sox-XA, SoxYZ, SoxB, and SoxCD, for oxidation of reduced inorganic sulfur compounds to sulfuric acid [5,6]. *P. pantotrophus* forms a heterodimeric membrane-bound hydrogenase with subunits of 30 and 60 kDa [7].

Four open reading frames (ORF) of unknown function, ORF1, ORF2, *shxV* and *shxW*, are located upstream from the *sox* structural genes (Fig. 1 [6]). ORF1 predicts a 149 amino acid polypeptide with identities of over 40\% to transcriptional regulators. ORF2 predicts a periplasmic thioredoxin of 99 amino acids for the mature polypeptide. Both ORFs are located in opposite direction to the other genes of the cluster. *shxV* predicts a 245 amino acid protein with six transmembrane helices. ShxV is closely related to proteins involved in transport of reductant from the cytoplasm to the periplasm for heme coupling in cytochrome *c* biogenesis of different bacteria (reviewed in [8]). *shxW* predicts a periplasmic thioredoxin of 186 amino acid residues [6].

ShxV is 42\% identical to CcdA of *P. pantotrophus* and the presence of two CcdA homologues in one strain is so far unprecedented. Disruption of *ccdA* causes complete deficiency in *c*-type cytochromes in this strain [9] and the presence of *shxV* posed the question of similar functions, a Sox specific function as plausible from the location of *shxVW* adjacent to the *sox* gene cluster, or a general function.

We here report a novel function of the CcdA homologue ShxV and of ShxW: their involvement in lithotrophic oxidation of reduced sulfur compounds and of mo-
molecular hydrogen but not in cytochrome c biogenesis of \textit{P. pantotrophus}.

2. Materials and methods

2.1. Bacterial strains and plasmids, media and growth conditions

Strains and plasmids used and constructed in this study are listed in Table 1.

\textit{P. pantotrophus} was cultivated at 30°C. Mineral media were identical for heterotrophic and for lithotrophic growth of \textit{P. pantotrophus} \cite{10}. The atmosphere for growth with molecular hydrogen contained 85% (v/v) hydrogen, 10% (v/v) carbon dioxide, and 5% (v/v) oxygen. For growth with thiosulfate mineral media contained 20 mM sodium thiosulfate at a final pH of 8.0. For mixotrophic growth with thiosulfate mineral media contained 20 mM sodium sulfate and 20 mM sodium thiosulfate at a final pH of 8.0. For anaerobic growth bacteria were cultured in mineral medium containing 0.2% (w/v) glucose and 0.1% (w/v) potassium nitrate. \textit{Escherichia coli} was cultivated in Luria–Bertani medium \cite{11}. The following antibiotics were used when appropriate for \textit{P. pantotrophus}: 300 μg of kanamycin (Km) ml⁻¹, 5 μg of tetracycline (Tc) ml⁻¹, and 5 μg of chloramphenicol (Cm) ml⁻¹; for \textit{E. coli}: 50 μg of Km ml⁻¹, 50 μg of ampicillin (Ap) ml⁻¹, 12.5 μg of Tc ml⁻¹, and 30 μg Cm ml⁻¹. Cellular yields of \textit{P. pantotrophus} were determined as described for \textit{Ralstoniaeutrophus} \cite{12}.

Table 1

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant geno- or phenotype*</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. coli}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1</td>
<td>recA pro thi hsdS, RP4-tra functions supE44</td>
<td>[25]</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td>hsdR17 recA1 endA1 gyrA46 thi relA1 lac [F'proAB, lacF6 ZnM15 Tn10(tet')]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>\textit{P. pantotrophus}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GB17</td>
<td>Sox⁺, Hox⁺</td>
<td>[4]; L.A. Robertson</td>
</tr>
<tr>
<td>GBX2IV</td>
<td>Sox⁺, Hox⁺, Km⁺, Ap⁺, heterogenote of GB17</td>
<td>This study</td>
</tr>
<tr>
<td>GB2IV</td>
<td>Sox⁺, Hox⁺, ω-Km interposon integrated in shxV</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript SK⁻</td>
<td>Ap⁺, lacZ, f1 ori, T7 Phil 10 promoter</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pSUP202</td>
<td>Cm⁺, Tc⁺, Trα⁺, Mob⁺</td>
<td>[25]</td>
</tr>
<tr>
<td>pBHR1</td>
<td>Km⁺, Cm⁺, Mob⁺</td>
<td>MobiTec</td>
</tr>
<tr>
<td>pJ0E773.2</td>
<td>Ap⁺, lacZα</td>
<td>[27]</td>
</tr>
<tr>
<td>pHP45Ω-Km</td>
<td>Ap⁺, Km⁺</td>
<td>[28]</td>
</tr>
<tr>
<td>pEG12</td>
<td>12-kb Sox relevant DNA in pSUP202</td>
<td>[5]</td>
</tr>
<tr>
<td>pBS1P3.4</td>
<td>3.4-kb Sox relevant \textit{SalI-} fragment of pEG12 containing \textit{shxW} in pBluescript SK</td>
<td>This study</td>
</tr>
<tr>
<td>pBS1P3.4ΔW</td>
<td>pBS1P3.4 with \textit{Ω-Km} interposon integrated in \textit{shxV}</td>
<td>This study</td>
</tr>
<tr>
<td>pSSP3.4ΔW</td>
<td>5.4-kb \textit{SalI-} insert of pBS1P3.4 Ω V in pSUP202</td>
<td>This study</td>
</tr>
<tr>
<td>pBHP6</td>
<td>6-kb DNA fragment of pEG12 containing \textit{shxW} in pBHR1</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Sox, lithotrophic growth with thiosulfate; Hox, lithotrophic growth with molecular hydrogen; Tra, transfer of mobilizable plasmids; Mob, mobilizability.

2.2. Analytical procedures

Total cytochromes were quantified from cell free extracts according to the pyrimidine extraction procedure. Cytochromes of the \textit{e}·\textit{type} of \textit{P. pantotrophus} GB17 and strain GB2IV were analyzed from cell free extracts by heme staining and by redox difference spectroscopy \cite{9} and references therein).

Enzyme activities were determined from whole cells. Hydrogenase activity was followed spectrophotometrically at 546 nm by hydrogen-dependent reduction of methylene blue \cite{12}. Thiosulfate oxidizing activity was determined with an oxygen electrode as described \cite{13}. One unit (U) of enzyme activity is defined as 1 μmol of substrate utilized per min at 30°C.

Cell free extracts were prepared, and hydrogenase and Sox specific antigens were detected from cell free extracts of \textit{P. pantotrophus} GB17 and strain GB2IV by immunoblot analysis as previously described \cite{13}. Antibodies against ShxW, SoxXA, SoxB, SoxC, and SoxG were raised in rabbits at the facilities of Eurogentec (Seraing, Belgium). Antibodies against SoxB were raised against SoxB heterologously expressed in \textit{E. coli} and purified by metal chelate chromatography as described previously \cite{5}. Antibodies against SoxXA were raised against SoxXA purified from \textit{P. pantotrophus} cultivated autotrophically with thiosulfate \cite{5}. Antibodies against ShxW, SoxC and SoxG were raised against the immunogenic oligopeptides \textit{DDGLHKPTWLRETFKC}, \textit{ARAAGTPDPLITEI} and \textit{PFIGYHLPGGIGR} deduced from the \textit{shxW}, \textit{soxC} and the \textit{soxG} nucleotide sequences respectively. Hydroge-
nase of \textit{P. pantotrophus} was detected with antibodies against the membrane bound hydrogenase of \textit{R. eutropha} [14] which is immunochemically closely related to the enzyme of \textit{P. pantotrophus} [15].

Thiosulfate was quantified according to Sorbo [16]. Protein from cell free extracts was quantified according to Bradford [17].

2.3. DNA techniques and sequence analysis

Standard DNA techniques [11] were used. Plasmid DNA was isolated using the high pure plasmid isolation kit (Boehringer Mannheim, Germany). Chromosomal DNA was isolated according to Ausubel et al. [18]. Amino acid homology searches were performed with the BLAST algorithm [19]. Higher order structures of primary sequences were analyzed by PROSIS (Hitachi Software Engineering, San Bruno, CA), PSORT and PROSCAN software packages.

2.4. Construction of the \textit{shxV}:\textit{Ω}-kanamycin insertion

\textit{shxV} was disrupted by inserting the \textit{Ω}-Km interposon of pHP45\textit{Ω}-Km by gene replacement using the suicide plasmid pSSP3.4\textit{Ω} (Table 1). Plasmid pSSP3.4\textit{Ω} was constructed from the 3.4-kb \textit{SalI}-\textit{PstI} fragment of pEG12 containing \textit{shxV}. The gene region was cloned into pBSK\textsuperscript{−} resulting in pBSP3.4. The \textit{Ω}-Km fragment was isolated from pHP45\textit{Ω}-Km by \textit{BamHI} restriction and then treated with mung bean nuclease to obtain blunt ends. This fragment was cloned into the \textit{StuI} site of pBS3.4 which was located within the \textit{shxV} gene region resulting in pBSP3.4\textit{Ω}. The insert carrying the \textit{Ω}-Km interposon was isolated from pBSB3.4\textit{Ω} by \textit{XbaI} and \textit{XhoI}, and then cloned into the \textit{EcoRV} site of pSUP202 after mung bean nuclease treatment resulting in plasmid pSSP3.4\textit{Ω}. Plasmid pSSP3.4\textit{Ω} was transformed into \textit{E. coli} S17-1, conjugated into \textit{P. pantotrophus} GB17, and heterogenote transconjugants were selected exhibiting the Km\textsuperscript{r} and Cm\textsuperscript{r} phenotype. To obtain the homogenote GB\textit{Ω} one single colony was cultivated with shaking for 3 days in mineral medium containing 20 mM sodium succinate at a final pH of 7.0 at 30°C. About \textit{10}\textsuperscript{7} cells were plated on mineral agar containing 300 µg Km ml\textsuperscript{−1}. To verify the integration of the \textit{Ω}-Km fragment in \textit{shxV}, colonies exhibiting a Km\textsuperscript{r} and Cm\textsuperscript{r} phenotype were analyzed by Southern hybridization of \textit{EcoR}I restricted chromosomal DNA using the DIG labeled 2-kb \textit{BamHI} \textit{Ω}-Km fragment of pHP45\textit{Ω}-Km and the DIG labeled 3.4-kb \textit{SalI}-\textit{PstI} insert of pBSP3.4 as probes. One clone exhibiting a signal in the Southern blot at about 13 kb with both probes was designated GB\textit{Ω}.

3. Results

3.1. Characterization of \textit{shxVW}

\textit{shxV} predicts a channel-forming polypeptide located in the cytoplasmic membrane with six transmembrane helices and with conserved cysteine residues at helix 1 and helix 4. ShxV is 42% identical to CcdA of \textit{P. pantotrophus} essential for biogenesis of \textit{c}-type cytochromes [9]. \textit{shxW} predicts a thioredoxin-like periplasmic protein [5]. Thioredoxins are characterized by their active site including a CXXC motif and by a distinct structure consisting of four stranded β-sheets and three flanking α-helices [20]. ShxW revealed a 95% correspondence with the consensus pattern described for the active site of members of the thioredoxin family in the PROSITE database while the distribution of β-strands and α-helices of ShxW deviated concerning the proposed structure (data not shown).

3.2. Isolation and characterization of strain GB\textit{Ω}V

To determine the function of ShxV a mutant carrying an \textit{Ω}-Km interposon in \textit{shxV} was constructed (Fig. 1). This construct was conjugated from \textit{E. coli} to \textit{P. pantotrophus}, established by gene replacement, verified by Southern blot analysis (data not shown), and the resulting mutant GB\textit{Ω}V was characterized. Strain GB\textit{Ω}V was unable to grow lithoautotrophically with thiosulfate or with molecular hydrogen. Growth with formate was not affected demonstrating that autotrophic carbon dioxide fixation was functional in GB\textit{Ω}V. Also, growth with succinate or glucose was unaffected with respect to specific growth rate and cellular yields. Furthermore, anaerobic growth with nitrate or nitrite as electron acceptor was...
identical for strain GB6V and the wild-type (data not shown). This was evidence that disruption of shxV did not cause a general defect in energy metabolism and that its function differed from that of ccdA of P. pantotrophus.

3.3. Immunochemical analysis of ShxW, hydrogenase and Sox antigens

To determine whether the insertion of the Ω-Km interposon in shxV affected the formation of ShxW, of hydrogenase or of the Sox proteins, the presence of the respective antigens was examined. ShxW antigens were exclusively detected from cell free extracts of strain GB17 cultivated mixotrophically with succinate and thiosulfate while no ShxW antigens were detected from strain GB6V cultivated under identical conditions (Fig. 2). No ShxW antigens were detected from cells of strain GB17 and GB6V cultivated heterotrophically (Fig. 2). This result demonstrated that expression of ShxW was induced by thiosulfate in the wild-type and that expression of ShxW was prevented by disruption of shxV by the Ω-Km interposon.

Hydrogenase antigens were present in cell free extracts of hydrogen-induced wild-type cells while no antigens were detected in the hydrogen-induced mutant cells as determined by immunoblot analysis (data not shown). This result demonstrated a link of ShxV and ShxW to hydrogenase expression.

SoxXA, SoxB, SoxC, and SoxG antigens were detected in cell free extracts of the wild-type and in equal intensity in the mutant GB6V when cultivated with succinate plus thiosulfate, demonstrating that these sox genes were not linked with shxVW (Fig. 3).

Hydrogenase activity was not detected in strain GB6V while a specific activity of 1.7 U per mg of protein was determined from the wild-type (Table 2). After mixotrophic cultivation with succinate plus thiosulfate, strain GB6V exhibited 2% of thiosulfate-dependent oxygen uptake rate as compared to the wild-type (Table 2).

3.4. Cytochrome c analysis

Disruption of ccdA of P. pantotrophus causes complete

![Fig. 2. Western blot analysis of P. pantotrophus GB17 and strain GB6V for ShxW. ShxW was detected from cell free extracts (10 μg of protein) of strain GB17 (A) and GB6V (B) cultivated heterotrophically (I) or mixotrophically with thiosulfate (II).](image1)

![Fig. 3. Western blot analysis of P. pantotrophus GB17 and strain GB6V for Sox specific antigens. SoxXA (I), SoxB (II), SoxC (III), and SoxG (IV) antigens were detected from cell free extracts (10 μg of protein) of strain GB17 (A) and GB6V (B) cultivated mixotrophically with thiosulfate.](image2)

### Table 2

Lithoautotrophic growth of P. pantotrophus GB17 and strain GB6V with thiosulfate and molecular hydrogen* and specific thiosulfate and hydrogen oxidizing activity

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>Thiosulfate</th>
<th>H2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t4 (h)b</td>
<td>Spec. act. (μmol O2 min⁻¹ (mg protein)⁻¹)</td>
<td>t4 (h)b</td>
</tr>
<tr>
<td>GB17</td>
<td>13.9</td>
<td>0.52</td>
<td>6.5</td>
</tr>
<tr>
<td>GB6V</td>
<td>≈</td>
<td>0.01</td>
<td>≈</td>
</tr>
<tr>
<td>GB6V (pBHP6)</td>
<td>14.4</td>
<td>0.55</td>
<td>7.3</td>
</tr>
</tbody>
</table>

*Media and growth conditions are given in the text.

bDoubling time.

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**References:**

deficiency in c-type cytochromes [9], suggesting that ShxV did not compensate the CcdA function and led us to examine the role of shxVW in cytochrome c formation. When P. pantotrophus GB17 and strain GBΩV were cultivated with glucose no qualitative difference in c-type cytochromes was detected from heme staining of cell free extracts after separation of proteins by SDS–PAGE (data not shown). Moreover, quantitative analysis by pyridine hemochrome analysis revealed 1.83 nmol c-type cytochromes per mg of protein in strain GBΩV and 1.79 nmol c-type cytochromes per mg of protein in the wild-type. Almost identical concentrations of c-type cytochromes were also evident from analysis of redox difference spectra of both strains (Fig. 4). These data demonstrated that ShxVW did not play a role in cytochrome c biogenesis.

3.5. Complementation of the shxV::Ω-Km mutation

To confirm that the phenotype of strain GBΩV was caused by the disruption of shxV, plasmid pBHP6 containing shxVW was used for complementation (Fig. 1). This plasmid lacks the soxCD genes, essential for lithotrophic growth with thiosulfate [21]. Growth with hydrogen and hydrogenase activity were fully restored in strain GBΩV harboring pBHP6 (Table 2). Also, lithothrophic growth of strain GBΩV(pBHP6) with thiosulfate was fully restored (Table 2). Strain GBΩV(pBHP6) formed significant amounts of ShxW antigens (data not shown) indicating that the respective gene was well expressed from plasmid pBHP6.

4. Discussion

shxVW was identified to be essential for lithotrophic growth of P. pantotrophus. Due to the polarity of the Ω-Km interposon, inactivation of shxV resulted in the inability to form ShxW antigens, and the absence of ShxV/W resulted in the inability to grow with molecular hydrogen and thiosulfate as electron donors for autotrophic carbon dioxide fixation. The inability of strain GBΩV to grow with molecular hydrogen appeared to be based on the absence of hydrogenase antigens while the inability to grow with thiosulfate appeared to be based on an unknown mechanism leading to the activation of the multienzyme system since the proteins required for thiosulfate oxidation were expressed. Lithotrophic growth of strain GBΩV with thiosulfate or molecular hydrogen was restored upon complementation with plasmid pBHP6 carrying the shxVW genes.

The predicted ShxV was highly homologous to CcdA of P. pantotrophus, essential for cytochrome c biogenesis [9]. Both genes, shxV and ccdA, predicted channel-forming transmembrane proteins homologous to CcdA of Bacillus subtilis, Rhodobacter capsulatus and DipZ of E. coli and Pseudomonas aeruginosa which participate in cytochrome c biogenesis [8]. Disruption of shxV affected exclusively lithotrophic traits but not cytochrome c biogenesis. Also, autotrophic carbon dioxide fixation was not linked to shxV since growth with formate was not impaired in strain GBΩV. In P. pantotrophus formate is oxidized by formate dehydrogenase to carbon dioxide which is then fixed via the reductive ribulose bisphosphate cycle [22].

DipZ is proposed to be involved in transport of electrons into the periplasm via two conserved cysteines [8,23]. The high structural identity of the predicted shxV gene product with CcdA or DipZ and the neighboring putative thioredoxin ShxW also suggest a function for electron transport which direction is presently unknown.

Different periplasmic thioldisulfide oxidoreductases have been identified in E. coli (reviewed in [24]). Two of these enzymes, DsbA and DsbB, are involved in formation of disulfide bonds of periplasmic proteins. While DsbA catalyzes the oxidation of reduced periplasmic proteins, DsbB is responsible for maintaining DsbA in an oxidized, active form. In analogy ShxV may be involved in the transport of reductant to the cytoplasmic membrane as similarly proposed for DsbA/B. So far, no DsbA and DsbB homologues have been identified in P. pantotrophus.

The sulfur-oxidizing enzyme system of P. pantotrophus is located in the periplasm [5] and thiosulfate oxidation may depend on the formation of disulfide bonds between cysteine residues. The putative thioredoxin ShxW may accept electrons from disulfide formation of proteins involved in metabolism of inorganic sulfur and molecular hydrogen. Therefore, disruption of shxV may prevent disulfide bond formation in periplasmic proteins. Candidates for such oxidation reaction may be SoxY and SoxZ since
both proteins appear to be covalently linked via a disulfide bond (A. Quentmeier, unpublished data).

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References
