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Cloning and Characterization of Sulfite Dehydrogenase, Two *c*-Type Cytochromes, and a Flavoprotein of *Paracoccus denitrificans* GB17: Essential Role of Sulfite Dehydrogenase in Lithotrophic Sulfur Oxidation

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A 13-kb genomic region of *Paracoccus dentrificans* GB17 is involved in lithotrophic thiosulfate oxidation. Adjacent to the previously reported *soxB* gene (C. Wodara, S. Kostka, M. Egert, D. P. Kelly, and C. G. Friedrich, J. Bacteriol. 176:6188–6191, 1994), 3.7 kb were sequenced. Sequence analysis revealed four additional open reading frames, *soxCDEF*. *soxC* coded for a 430-amino-acid polypeptide with an M_r of 47,339 that included a putative signal peptide of 40 amino acids (M_r of 3,599) with a RR motif present in periplasmic proteins with complex redox centers. The mature *soxC* gene product exhibited high amino acid sequence similarity to the eukaryotic molybdoenzyme sulfite oxidase and to nitrate reductase. We constructed a mutant, GB*soxC*Δ, carrying an in-frame deletion in *soxC* which covered a region possibly coding for the molybdenum cofactor binding domain. GB*soxC*Δ was unable to grow lithoautotrophically with thiosulfate but grew well with nitrate as a nitrogen source or as an electron acceptor. Whole cells and cell extracts of mutant GB*soxC*Δ contained 10% of the thiosulfate-oxidizing activity of the wild type. Only a marginal rate of sulfite-dependent cytochrome *c* reduction was observed from cell extracts of mutant GB*soxC*Δ. These results demonstrated that sulfite dehydrogenase was essential for growth with thiosulfate of *P. dentrificans* GB17. *soxD* coded for a periplasmic diheme *c*-type cytochrome of 384 amino acids (M_r of 39,983) containing a putative signal peptide with an M_r of 2,363. *soxE* coded for a periplasmic monoheme *c*-type cytochrome of 236 amino acids (M_r of 25,926) containing a putative signal peptide with an M_r of 1,833. SoxD and SoxE were highly identical to *c*-type cytochromes of *P. denitrificans* and other organisms. *soxF* revealed an incomplete open reading frame coding for a peptide of 247 amino acids with a putative signal peptide (M_r of 2,629). The deduced amino acid sequence of *soxF* was 47% identical and 70% similar to the sequence of the flavoprotein of flavocytochrome *c* of *Chromatium vinosum*, suggesting the involvement of the flavoprotein in thiosulfate oxidation of *P. denitrificans* GB17.

Paracoccus denitrificans grows heterotrophically with a large variety of carbon sources and lithoautotrophically with molecular hydrogen (4, 25) or with thiosulfate as an electron donor (17). *P. dentrificans* GB17 (formerly *Thiosphaera pantotropha* GB17^T [30, 41]) exhibits most of the characteristics of the *P. denitrificans* type strain, which is well characterized on the genetic level (48), but strain GB17 is amenable to transposon Tn*5* mutagenesis (8). The 16S rRNA nucleotide sequence is the same in both strains, and their DNA-DNA homology is 86%. These criteria led to the transfer of the former *T. pantotropha* GB17^T to *P. denitrificans* GB17 (30).

The genes involved in thiosulfate oxidation of *P. denitrificans* GB17 (*sox* genes) were located by transposon Tn*5* mutagenesis, which yielded three classes of Sox$^-$ mutants unable to grow with thiosulfate as an energy source. Class I mutants are exclusively Sox$^-$; class II mutants have also lost the ability to oxidize hydrogen and to reduce nitrite anaerobically; class III mutants are also unable to oxidize formate and xanthine and to reduce nitrate (8) due to their inability to synthesize a functional molybdopterin cofactor (reviewed in reference 16).

The gene region involved in thiosulfate oxidation of *P. denitrificans* GB17 was identified and cloned with the aid of the class I Sox$^-$ mutant, yielding the hybrid plasmid pEG12 with a 13-kb insert (Fig. 1) (34). Partial sequence analysis of the 13-kb insert and comparative biochemistry suggested identity of the *soxB* gene product of *P. denitrificans* GB17 with enzyme B of the thiosulfate-oxidizing enzyme system of *Paracoccus versutus* (56) (formerly *Thiobacillus versutus* [23]). In *P. versutus*, four periplasmic proteins are required for thiosulfate oxidation in vitro: enzyme A (M_r of 16,000), enzyme B (M_r of 63,000), cytochrome $c_{552.5}$ (M_r of 56,000; subunit M_r of 29,000), and cytochrome c_{551} (M_r of 260,000; subunit M_r of 43,000). Sulfite dehydrogenase of *P. versutus* (M_r of 44,000) is tightly associated with cytochrome c_{551} but was reported not to be required for thiosulfate oxidation (24, 28, 29). Sulfite oxidase from *Thiobacillus novellus* (M_r of 40,000) was also reported to be associated with a *c*-type cytochrome (58) and to contain a heme moiety similar to mammalian sulfite oxidases which contain cytochrome b_5-type heme (49). Molybdenum or the molybdopterin cofactor was detected in sulfite dehydrogenases so far examined (5, 21, 49). Molybdate is required for lithoautotrophic growth with thiosulfate of *P. denitrificans* and of *P. versutus* (8, 18). Provided sulfite dehydrogenase of *P. denitrificans* GB17 contained molybdenum, as do the enzymes from other sources, the fact that there are Sox-negative mutants unable to form a functional molybdenum cofactor suggests that this enzyme is essential for thiosulfate oxidation in vivo in *P. denitrificans* GB17.

We here report on the identification of four open reading frames 3′ of *soxB* (56), *soxCDEF*, coding for periplasmic proteins. The protein deduced from *soxC* is highly identical to the

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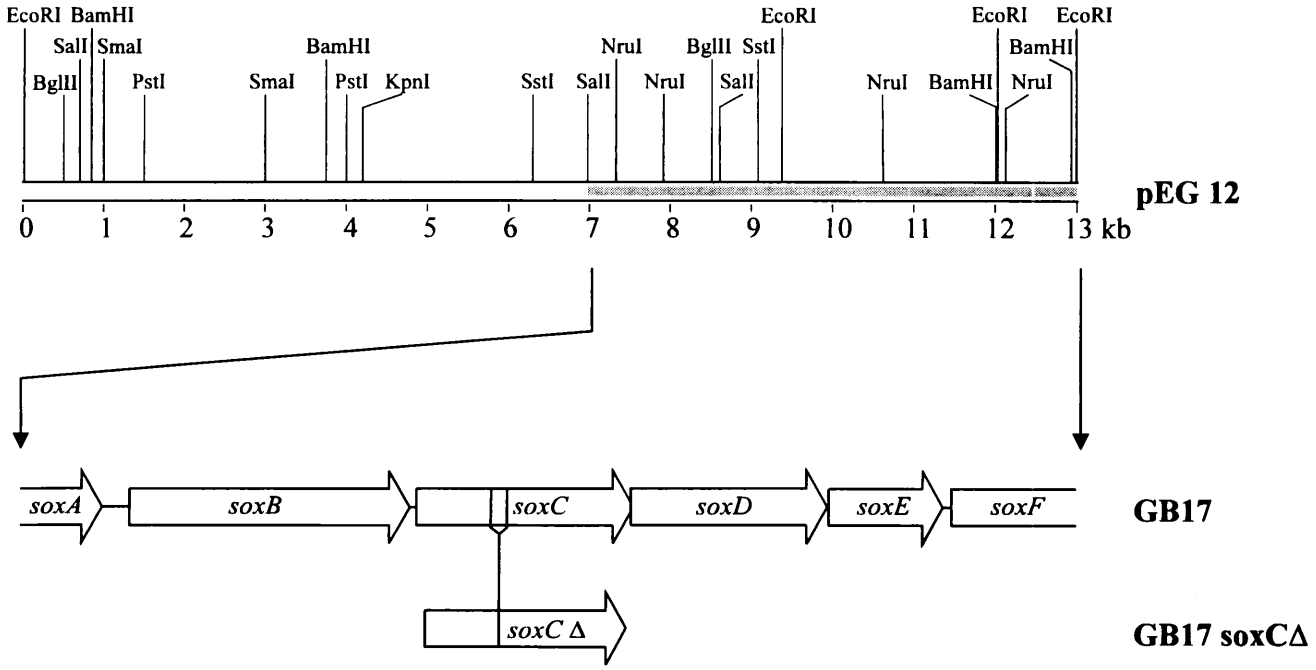

FIG. 1. Physical map of plasmid pEG12 and *sox*-relevant open reading frames.

eukaryotic type of sulfite dehydrogenase. Disruption of this gene by mutation eliminates sulfite dehydrogenase activity from crude extracts and demonstrates that *soxC* is essential for growth of *P. denitrificans* GB17 on thiosulfate. *soxD* and *soxE* code for *c*-type cytochromes, and *soxF* exhibits high identity to the flavoprotein of *Chromatium vinosum.*

MATERIALS AND METHODS

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

Media and growth conditions. The thiosulfate mineral medium for lithoautotrophic growth of *P. denitrificans* strains and of *P. versutus* and the mineral media for examination of assimilatory and dissimilatory nitrate reduction were previously described (8). To express proteins relevant for thiosulfate metabolism,

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype or phenotype[a]	Reference or source
Escherichia coli		
S17-1	*recA pro thi hsdS*, RP4-*tra* functions	47
XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac* [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10*(Tet^r)]	7, Stratagene
Paracoccus denitrificans		
GB17	Sox$^+$ Hox$^+$	30, 41, L. A. Robertson
GBJD*soxC*Δ	Sox$^+$ Km^r *lacZ*, cointegrate of pJD*soxC*Δ	This study
GB*soxC*Δ	Sox$^-$ *soxC*Δ	This study
Paracoccus versutus	Sox$^+$ Hox$^-$	DSM 582
Plasmids		
pBluescript SK$^+$	Ap^r *lacZ* f1 ori; T7 ϕ10 promoter	Stratagene
pBluescript KS$^+$	Ap^r *lacZ* f1 ori; T7 ϕ10 promoter	Stratagene
pEG12	13-kb *sox*-relevant DNA in pSUP202	34
pEG5	5.2-kb *Eco*RI fragment of pEG12 with an 8-kb *Bgl*II deletion in pSUP202	56
pJD1	Km^r *lacZ* RP4 oriT ColE1 ori	12
pSUP202	Ap^r Cm^r Tc^r Tra$^-$ Mob$^+$	47
pBKS$^+$E2.7	2.7-kb *Eco*RI fragment from pEG12 in pBluescript KS$^+$	This study
pBKS$^+$E2.7R	2.7-kb *Eco*RI fragment inserted in pBKS$^+$ in orientation opposite that in pBKS$^+$E2.7	This study
pBKS$^+$E2.7SN	pBKS$^+$E2.7 with deletion of *Sma*I/*Nru*I	This study
pBKS$^+$E2.7RSN	pBKS$^+$E2.7R with deletion of *Sma*I/*Nru*I	This study
pBSK$^+$E1	1-kb *Eco*RI fragment from pEG12 in pBluescript KS$^+$	This study
pBSK$^+$E1R	1-kb *Eco*RI fragment inserted in pBSK$^+$ in orientation opposite that in pBSK$^+$E1	This study
pBKS$^+$E4.8	4.8-kb *Eco*RI fragment from pEG5 inserted in pBluescript KS$^+$	This study
pBSK$^+$*soxC*Δ	562-bp *Xba*I-*soxC*Δ PCR fragment in pBluescript SK$^+$	This study
pJD*soxC*Δ	1,020-bp *Pvu*II fragment from pBSK$^+$ *soxC*Δ in pJD1	This study

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

Sox-negative derivatives of *P. denitrificans* GB17 were cultivated under mixotrophic growth conditions in mineral medium with 0.1% (wt/vol) sodium pyruvate and 20 mM sodium thiosulfate. Seed cultures were cultivated in glucose (0.1% [wt/vol]) mineral medium for 24 h and used as 0.1% (vol/vol) inocula. *P. denitrificans* strains harboring the *lacZ* gene were selected on mineral agar (1.5% [wt/vol]) plates containing 0.4% (wt/vol) glucose and 0.1 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Antibiotics were added where appropriate (for *P. denitrificans*, 300 μg of kanamycin/ml; for *E. coli*, 50 μg of kanamycin/ml or 100 μg of ampicillin/ml).

Conjugal transfer of plasmids and gene replacement. Spot matings were used to transfer mobilizable plasmids from *E. coli* S17-1 to *P. denitrificans* GB17. Transconjugants were generated by transfer of the suicide vector plasmid pJD1 (12). Heterogenote transconjugants exhibiting the kanamycin-resistant and LacZ-positive phenotype were selected on mineral medium agar plates containing 0.4% (wt/vol) glucose and 300 μg of kanamycin/ml. Kanamycin-resistant clones were purified, and single colonies were inoculated to mineral medium (30 ml) containing glucose without antibiotic and cultivated with shaking for 2 days at 30°C. After appropriate dilutions, about 10^3 cells were plated on mineral agar containing isopropylthiogalactopyranoside. Lac-negative and kanamycin-sensitive recombinants were selected and were candidates for homogenote mutants carrying the intended deletions.

Construction of the *soxC* deletion. Ninety base pairs of *soxC* coding for the putative molybdenum cofactor binding domain were deleted with the aid of PCR technology due to the lack of convenient restriction sites (Fig. 2). The strategy used for the in-frame deletion is summarized in Fig. 3. Primers 1 and 2 generated a fragment of 246 bp, designated left site, and primers 3 and 4 generated a fragment of 347 bp, designated right site. Both fragments were fused via the complementary ends of primers 2 and 3 and multiplied by PCR with primers 1 and 4. The resulting fragment of 572 bp contained *Xba*I restriction sites at both ends and was cloned via these sites into pBluescript SK^+ and designated $pBSK^+soxC\Delta$ (Fig. 3). The intended deletion determined by the flanking ends was verified from nucleotide sequencing. $pBSK^+soxC\Delta$ was restricted with *Pvu*II, and the 1,020-bp fragment was ligated into the *Msc*I site of pJD1, resulting in pJD*soxC*Δ. This plasmid was transferred to *P. denitrificans* GB17 via conjugation. Homogenote recombinants with the deletion in the *soxC* gene were screened by amplification of the appropriate DNA fragments by PCR essentially as described elsewhere (32), using the *soxC* primers 1 and 4 (Fig. 3). After cultivation overnight in Luria-Bertani medium, cells (1 ml) were harvested, washed once with 20 mM Tris-HCl (pH 8.0), and concentrated 10-fold. Cells were boiled for 5 min, and 10 μl of the crude extract was subjected to DNA amplification (95°C for 1 min, 60°C for 1 min, 72°C for 1 min; 20 cycles). The PCR products were extracted and analyzed by agarose-gel electrophoresis.

To verify the deletion described in Fig. 3 in the homogenote recombinants, primers 1 and 4 were used with total DNA as a template. A PCR fragment of 660 bp was obtained from the wild-type strain GB17 and the heterogenote recombinant GBJD*soxC*Δ. From the latter, a second fragment of 560 bp was obtained. This fragment resulted also from the homogenote recombinant GB*soxC*Δ and from the suicide vector plasmid pJD*soxC*Δ (Fig. 4).

DNA techniques and DNA sequencing. Standard DNA techniques (43) were used. Sequencing was performed by primer walking with a T7 sequencing kit (Pharmacia, Freiburg, Germany) and [^{35}S]dATP[αS] (Amersham-Buchler, Braunschweig, Germany) by the method of Sanger et al. (45). The 1.0- and 2.7-kb *Eco*RI fragments of pEG12 were cloned in pBluescript SK^+ in both orientations. The 2.7-kb inserts in the resulting plasmids were deleted with *Nru*I/*Sma*I to reduce the sizes of the plasmids. With PCR, the locations of the 9.5-, 2.7-, and 1.0-kb *Eco*RI fragments were determined for the chromosome of *P. denitrificans* GB17 and for pEG12. Five primers were deduced from the ends of the fragments of pEG12: RSN9 (5′-CCGGAATGGTGGCGTTC-3′), SN9 (5′-CTATATCGAGAACCCCA-3′), KS4 (5′-TGCATCGCGACCACGAA-3′), SK4 (5′-CTCAAGCAGACCAATCC-3′), and SE2 (5′-ACGATCTGATCTGTGAC-3′). Nucleotide sequence analysis was done with the HUSAR (EMBL, Heidelberg, Germany) and DNASIS and PC/GENE (IntelliGenetics Inc., Mountain View, Calif.) software packages.

Preparation of cell extracts. Crude extracts were prepared as previously described (14). To eliminate unspecific cytochrome *c* reductions from crude extracts in enzyme assays, saturated ammonium sulfate was added to crude extracts in a 1:1.8 ratio, giving a 65% saturation of ammonium sulfate. Precipitated proteins were separated by centrifugation at 4,000 × *g* for 10 min, redissolved in 50 mM Tris buffer (pH 7.0), and designated cell extracts.

Immunoblot analysis. SoxC antigens from crude extracts were detected with antibodies raised against the peptide VESREETSKYTDLM deduced from the *soxC* nucleotide sequence. This internal peptide was predicted to be highly antigenic according to the PC/GENE program. Antibodies were raised in rabbits at the facilities of Eurogentec (Seraing, Belgium). Immunosorbent assays were performed according to the semidry procedure, using the Multiphor electrophoretic system (Pharmacia). After denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (27), the proteins were electroblotted onto nitrocellulose membranes (pore size, 0.45 μm) as described by Towbin et al. (50). SoxC antigens were detected on the membranes with anti-SoxC serum (diluted 1:1,000) and anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (diluted 1:15,000; Sigma, Deisenhofen, Germany) and exposed for 30 min. The stain was developed for 30 min in 100 ml of 50 mM sodium carbonate buffer to which were added 1.0 ml of 0.5% (wt/vol) disodium 5-bromo-4-chloro-3-indolylphosphate and 10 ml of 0.1% (wt/vol) nitroblue tetrazolium (Serva, Heidelberg, Germany).

Identification of the Sox character. To identify the Sox character of transconjugants, a single colony was inoculated to thiosulfate mineral medium (2 ml, pH 7.2), supplemented with 0.1% (wt/vol) sodium pyruvate and 10 μg of phenol red/ml, and cells were aerated on a roller overnight. Thiosulfate oxidation caused a drop in pH, indicated by the color change from phenol red to yellow.

Enzyme assays. The rate of thiosulfate oxidation was determined from whole cells and cell extracts. The thiosulfate-oxidizing activity of whole cells was determined with an oxygen electrode (Rank Brothers, Bottisham, England). The assay mixture (3.0 ml) contained 150 μmol of Tris buffer (pH 8.0), about 0.5 mg (dry weight) of cells, and 6.0 μmol of thiosulfate or 0.3 μmol of sodium sulfide to start the reaction. One unit of activity was defined as 1 μmol of molecular oxygen consumed per min at 30°C. The activity of the thiosulfate-oxidizing enzyme system and of sulfite dehydrogenase from cell extracts was determined by monitoring the reduction of cytochrome *c* at 550 nm. The assay mixture (1.0 ml) contained 50 μmol of potassium phosphate buffer (pH 7.5), 35 nmol of horse heart cytochrome *c*, about 1 mg of cell extract, and 2.0 μmol of sodium thiosulfate to start the reaction. The sulfite dehydrogenase assay (1.0 ml) contained 100 μmol of Tris buffer (pH 7.5), about 1 mg of cell extract, 35 nmol of horse heart cytochrome *c*, and 20 nmol of disodium sulfite to start the reaction. One unit of enzyme activity was defined as 1 μmol of cytochrome *c* (ε = 27.8 $cm^2/\mu mol$) reduced per min at 30°C. Protein was determined by the method of Bradford (6), with bovine serum albumin as a reference.

Nucleotide sequence accession number. The nucleotide sequence of *soxCDEF* was submitted to the EMBL data library under accession no. X79242 PDSOXL.

RESULTS

Sequence analysis. The three *Eco*RI fragments of pEG12 were found by PCR to be in the order 9.5, 2.7, and 1.0 kb. Using chromosomal DNA as the template, the primer pair SE2-RSN9 and SN9-KS4 generated fragments of 900 and 350 bp, respectively, while primer pairs SE2-SN9 and SN9-SK4 did not generate fragments. This result was consistent with the orientation of the fragments shown in Fig. 1. To characterize the 3.7-kb DNA region downstream of the 2.5-kb *Sal*I/*Eco*RI fragment coding for *soxB*, the nucleotide sequence was determined from both strands to the end of the cloned DNA (Fig. 1). Analysis of this sequence revealed four open reading frames, designated *soxCDEF*.

soxC. *soxC* was located 23 nucleotides downstream of *soxB* (56) and had the potential to encode a protein of 430 amino acids (M_r of 47,339). A putative ribosome binding site was located nine nucleotides 5′ of the start codon (underlined in Fig. 2). A closer analysis of the amino-terminal amino acid sequence revealed a signal peptide of 40 amino acids (M_r of 3,617), relatively large for prokaryotes. The putative cleavage site was in accordance with the −1,−3 rule (54, 55). The total charge of amino acids 1 to 18 was +1; amino acids 19 to 36 were highly hydrophobic, with the potential to form a transmembrane helix (data not shown) (38). The putative signal peptide exhibited an RR motif present in signal peptides of periplasmic or membrane-bound proteins with complex redox centers (5, 17, 31, 59). The hydropathic index of the mature *soxC* gene product of −0.34 indicated a soluble protein. These properties suggested a periplasmic location of the deduced mature SoxC protein with a predicted M_r of 43,740 and a calculated pI of 5.7.

To determine the function of the *soxC* gene product, the deduced amino acid sequence was subjected to a homology search of the SWISSPROT database, using the TFASTA algorithm (38). The best alignments of SoxC were given to proteins from eukaryotic sources. The top eight alignments showed identities of 32.1 to 25.8% to sulfite oxidases and to nitrate reductases (Table 2).

Sulfite oxidases and nitrate reductases examined so far contain the molybdopterin cofactor. This cofactor was proposed to bind at a conserved cysteine residue of sulfite oxidase from rat or chicken liver and from nitrate reductases of fungi, algae, and

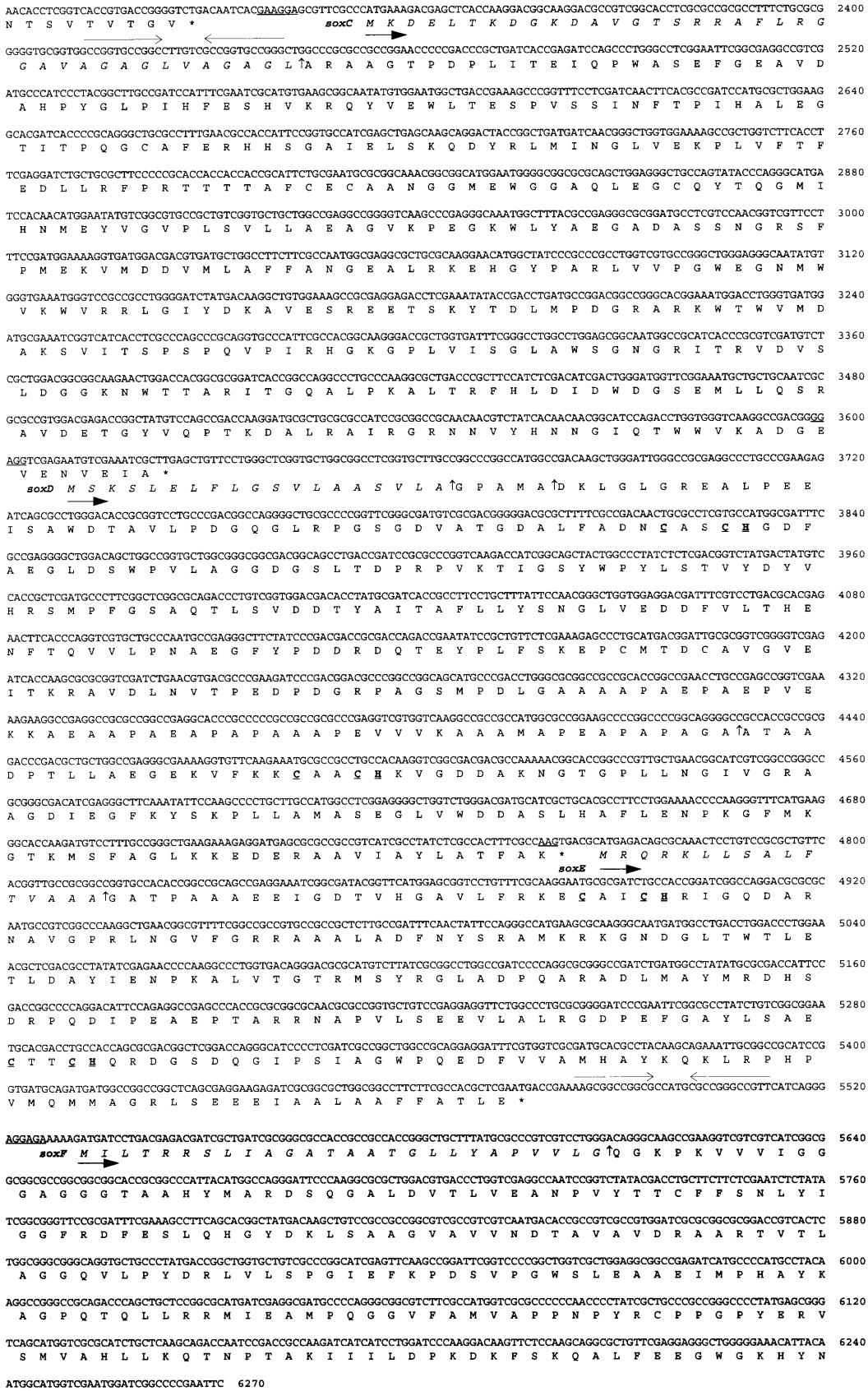

FIG. 2. Nucleotide and deduced amino acid sequences of *soxCDEF* of *P. denitrificans* GB17.

FIG. 3. Strategy for the deletion in *soxC*. The *Xba*I restriction sites of primers *soxC*Δ1 and *soxC*Δ4 are shown in boldface; the complementary ends of primers *soxC*Δ2 and *soxC*Δ3 and of the left-site and right-site fragments are underlined.

higher plants (3). A cysteine residue was present in the SoxC sequence about at a position characteristic for molybdoenzymes from eukaryotic sources but with a low identity to these enzymes. A region with higher identity to the above-mentioned enzymes was observed from amino acids 330 to 348 (Fig. 5). A second cysteine residue conserved in chicken liver sulfite oxidase and in nitrate reductase of *Arabidopsis thaliana* (10) was not present in SoxC. Sulfite oxidases from eukaryotes contain a nonconserved cytochrome b_5 binding domain at the amino-terminal end of the protein which is not evident from the amino acid sequences and was not evident from the amino acid sequence of SoxC.

soxD. The *soxD* gene overlapped with the last six codons of *soxC*. Six nucleotides upstream of the *soxD* start codon, a putative ribosome binding site was located (underlined in Fig. 2). *soxD* coded for a protein of 384 amino acids (M_r of 39,982) with two conserved heme binding sites (CxxCH). The amino-terminal sequence of the protein exhibited characteristics of a signal peptide with two possible cleavage sites consistent with the −3,−1 rule (54, 55), yielding possible signal peptides of 19 and 24 amino acids with M_rs of 1,936 and 2,363, respectively. Thus, *soxD* coded for a periplasmic *c*-type cytochrome. Both heme binding sites were separated by a hydrophobic alanine- and proline-rich intervening sequence of about 50 amino acids. The mature protein was slightly hydrophilic, with an overall hydropathy index of −0.14 (26) and a pI of 5.67. However, the carboxy-terminal end of the protein was highly hydrophobic (Fig. 2). The region with the first heme binding site exhibited 50% identity in a 34-amino-acid overlap to cytochrome $c_{554(547)}$ of the obligate lithoautotroph *Thiobacillus neapolitanus* (Table 2; reference 1) and was 17.5% identical in a 165-amino-acid overlap to the *moxG* gene product, cytochrome c_{551}, the electron acceptor of methanol dehydrogenase of *Methylobacterium extorquens* (37). The intervening sequence contained 26 alanine, 11 proline, 7 glutamate, and 4 valine residues with the potential to form a helix suitable for integration into the cytoplasmic membrane (40). A similar amino acid composition is present in the amino-terminal region of cytochrome c_1 of *P. denitrificans* and of cytochrome c_Y of *Rhodobacter capsulatus*, being 39.0 and 44.7% identical in 41- and 38-amino-acid overlaps, respectively (data not shown). The region with the second heme binding site was 54.0% identical in a 100-amino-acid overlap with cytochrome c_{550} of *Thiobacillus novellus* (Table 2). This region was also highly identical to membrane-bound *c*-type cytochromes of the respiratory chain of different strains of *P. denitrificans* and of other lithoautotrophic bacteria as well as to *c*-type cytochromes involved in anoxygenic photosynthesis of some phototrophic bacteria (Table 2).

soxE. Three nucleotides 3′ of the *soxD* termination codon was located *soxE*, coding for a protein of 236 amino acids (M_r of 25,926) with two conserved heme binding sites (CxxCH). Sequence analysis predicted a signal peptide of 17 amino acids (M_r of 1,833) with a cleavage site consistent with the −3,−1

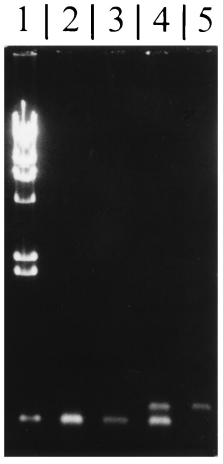

FIG. 4. Analysis of the deletion in *soxC* of GB*soxC*Δ. Analysis was done from DNA preparations of the strains indicated by PCR technology using primers 1 and 4 (Fig. 3) as described in Materials and Methods. Lanes 1, *Hin*dIII-digested λ DNA; 2, pJD*soxC*Δ; 3, GB*soxC*Δ; 4, GBJD*soxC*Δ; 5, GB17.

TABLE 2. Amino acid sequence identities of SoxB, -C, -D, -E, and -F to other proteins

Sox protein and organism	Gene	Function	% Identity (no. of amino acids)	Accession no.[a] (reference)
SoxB (554 amino acids)				
Bos taurus		5′-Nucleotidase	20.7 (522)	Q05927
SoxC (430 amino acids)				
Hordeum vulgare	*nar-7*	Nitrate reductase	29.9 (184)	P27968 (35)
Gallus gallus		Sulfite oxidase	30.7 (326)	P07850 (3)
Phytophthora infestans	*niaA*	Nitrate reductase	25.8 (291)	P39864 (39)
Lycopersicon esculentum	*nia*	Nitrate reductase	30.2 (192)	P17570 (11)
Rattus norvegicus		Sulfite oxidase	26.9 (264)	Q07116 (21)
Homo sapiens		Sulfite oxidase	32.1 (277)	P51687 (20)
SoxD (384 amino acids)				
Heme region 1 (amino acids 27–224)				
Thiobacillus neapolitanus		Cytochrome $c_{554(547)}$	50.0 (34)	P25938 (1)
Methylobacterium extorquens	*moxG*	Cytochrome c_{551}	17.5 (165)	X07856[b]
Heme region 2 (amino acids 279–384)				
Thiobacillus novellus		Cytochrome c_{550}	54.0 (100)	P23021 (57)
Agrobacterium tumefaciens		Cytochrome c_2	46.9 (98)	P00081 (52)
Paracoccus denitrificans GB17[c]	*soxE*	Cytochrome *c*	39.2 (120)	This study
Rhodomicrobium vannielii		Cytochrome c_2	52.5 (99)	P00082 (2)
Rhodopseudomonas acidophila		Cytochrome c_2	50.0 (98)	P00084
Paracoccus denitrificans	*cycM*	Cytochrome c_{552}	42.4 (144)	P54820 (51)
Paracoccus denitrificans LMD82.5[d]		Cytochrome c_{550}	43.9 (82)	P80288 (44)
SoxE (236 amino acids)				
Paracoccus denitrificans GB17	*soxD*	Cytochrome *c*	39.2 (120)	This study
Thermomyces lanuginosus		Cytochrome *c*	40.0 (105)	P00047 (36)
Schizosaccharomyces pombe	*cyc1*	Cytochrome *c*	42.2 (102)	P00046 (42)
Rhodomicrobium vannielii		Cytochrome c_2	52.3 (107)	P00082
Rhodopseudomonas viridis	*cycA*	Cytochrome c_2 precursor	44.4 (126)	P00083 (22)
SoxF (247 amino acids)				
Chromatium vinosum	*fccB*	Flavoprotein	47.4 (251)	Q06530 (9)

[a] All are accession numbers of the SWISSPROT databank except as noted otherwise.
[b] Accession number of the EMBL databank.
[c] Filed as *T. pantotropha* LMD 82.5 in the Delft culture collection.
[d] Identical to *T. pantotropha* LMD 82.5 (41).

rule (54). These characteristics identified also the *soxE* gene product as a periplasmic *c*-type cytochrome. The carboxy-terminal end of the protein was highly hydrophobic. Amino acid sequence comparison revealed high identities to the second heme region of SoxD of *P. denitrificans* GB17. Consequently, like SoxD, SoxE was highly identical to *c*-type cytochromes involved in anoxygenic photosynthesis of, e.g., *Rhodomicrobium vannielii* and *Rhodopseudomonas viridis* (Table 2).

soxF. Fifty-three nucleotides downstream of *soxE*, we identified an incomplete open reading frame, designated *soxF*, interrupted by the end of the cloned DNA. The intervening sequence contained a short inverted repeat with a potential formation of a hairpin structure with a free energy of formation of −72 kJ/mol. A typical ribosome binding site was located six nucleotides 5′ of the start codon (underlined in Fig. 2). *soxF* had the potential to encode a peptide of 247 amino acids (M_r of 26,444) with a putative signal peptide of 26 amino acids (M_r of 2,629) and a cleavage site which was in accordance to the −1,−3 rule (54, 55). Within the putative signal peptide was detected the RR motif, indicating a periplasmic protein with a redox center (5, 13, 31, 59). Sequence analysis of the deduced amino acid sequence of *soxF* revealed a relationship to only one other protein, the *fccB* gene product of *C. vinosum*. fccB codes for the 431 amino acids containing flavoprotein of flavocytochrome *c* of *C. vinosum* (9, 12). SoxF was 47.4% identical to FccB in a 251-amino-acid overlap (Fig. 6). SoxF contained a conserved cysteine equivalent to Cys^{42} proposed to bind flavin in FccB in *C. vinosum* (Fig. 6; reference 9).

Construction of a deletion in *soxC*. The significance of sulfite dehydrogenase in lithotrophic thiosulfate oxidation of *P. denitrificans* GB17 was determined from a mutant carrying an in-frame deletion in the *soxC* gene. This deletion was designed to inactivate the function of sulfite dehydrogenase. Therefore, bp 2781 to 2870, coding for 30 amino acids including the cysteine of the putative molybdenum cofactor binding site, were deleted from *soxC* with the aid of PCR technology (Fig. 3). Homogenote recombinants carrying this deletion were isolated as described in Materials and Methods. The deletion was verified from *P. denitrificans* GB*soxC*Δ from the reduction in the size of the PCR fragment (Fig. 4).

Immunochemical analysis of SoxC. The effect of the deletion in *soxC* was first investigated by Western (immunoblot) analysis using antibodies raised against a highly immunogenic synthetic peptide deduced from the SoxC amino acid sequence (see Materials and Methods). Wild-type extracts prepared from cells grown mixotrophically with pyruvate plus thiosulfate revealed an immunogenic band corresponding to an M_r of 44,000 (Fig. 7, lane 2). Extracts of mutant GB*soxC*Δ cultivated under identical conditions showed an immunogenic band reduced to an M_r of 42,000, in good agreement with the molecular weight of the product expected to be formed by the deletion strain (Fig. 7, lane 4). No specific cross-reacting material

```
                                                  ↓
SoxC     1   MKDELTKDGKDAVGTSRRAFLRGGAVAGAGLVAGAGLARAAGTPDPLITE
ggsuo   25   THGTDVFDVTDFVELHPGGPDKILLAAGGALEPFWALYAVHGEPHVLELL

         51   IQPWASEFGEAVDAHPYGLPIHFESHVKRQYVEWLTESPVSSINFTPIHA

         75   QQYKVGELSPDEAPAAPDAQDPFAGDPPRHPGLRVNSQKPFNAEPPAELL

        101   LEGTITPQGCAFERHHSGAIELSKQDY-RLMINGLVEKPLVFTFEDL-LR
              :||::  |:|:|  : :::  :| ||:::|  :::| :::::|  |
        125   AERFLTPNELFFTRNHLPVPAVEPSSYRRLRVDGPGGRTLSLSLAELRSR

        149   FPRTTTTAFCECAANGGMEWGGAQ-LEGCQYTQGMIHNMEYVGVPLSVLL
              ||: ::|| :||:|  | : :: ::| ::: | | :  : |::|: :|
        175   FPKHEVTATLQCAGNRRSEMSRVRPVKGLPWDIGAISTARWGGASLRDVL

        198   AEAGVKPE---GKWLYAEGADASSNGRSF----PMEKVM---DDVMLAFF
               :||  |  |: :: || ||:::| ::  | ::::  :||:||:
        225   LHAGFPEELQGGEHVCFEGLDADPGGAPYGASIPYGRALSPAADVLLAYE

        238   ANGEALRKEHGYPARLVVPGWEGNMWVKWVRRLGIYDKAVESREETSKYT
               ||::|:::| :|:|:|||| |:: |||:||::: :: || : ::|:
        275   MNGTELPRDHRFPVRVVVPGVVGARSVKWLRRVAVSPDESPSRWQQNDYK

        288   DLMP-------DGRARKWTWVMDAKSVITSPSPQVPIRHGKGPLVISGLA
              :: |       | |:  :  : ::|::| |:| ::::: | |:::| |
        325   GFSPCVDWDTVDYRTAPAIQELPVQSAVTQPRPGAAVPP--GELTVKGYA

        331   WSGNGR-ITRVDVSLDGGKNWTTARITGQALPKA----LTRFHLDIDWD-
              |||:|| ::|||||||||::|::||: |:: | :    : ::|::  :
        373   WSGGGREVVRVDVSLDGGRTWKVARLMGDKAPPGRAWAWALWELTVPVEA

        375   GSEMLLQSRAVDETGYVQPTK-DALRAIRGRNNVYHNN
              |:|: : ::|||::  |||:: ::::::||
        423   GTELEIVCKAVDSSYNVQPDSVAPIWNLRGVLSTAWHR
```

FIG. 5. Alignment of the deduced amino acid sequence of *soxC* with that of chicken liver sulfite oxidase (ggsuo).

```
                                                  ↓
SoxF     1   MILTRRSLI---AGATAATGLL-YAPVVLGQGKPKVVVIGGGAGGGTAAH
             |:|:|::|   : |:||:|:| ::::::|:|: ||||:|||:||:|||:
cvfccb    1   MTLNRRDFIKTSGAAVAAVGILGFPHLAFGAGR-KVVVVGGGTGGATAAK

         48   YMARDSQGALDVTLVEANPVYTTCFFSNLYIGGFRDFESLQHGYDKLSAA
              |: : :: :::|||:|:|: | ||::||  ||| |::||::|||| |:|
         50   YI-KLADPSIEVTLIEPNTDYYTCYLSNEVIGGDRKLESIKHGYDGLRAH
                                   •
         98   GVAVVNDTAVAVDRAARTVTLAGGQVLPYDRLVLSPGIEFKPDSVPGWSL
              |::||:|:|:::|:: : |: |||: : ||| |::||||:  |:: |:|
         99   GIQVVHDSATGIDPDKKLVKTAGGAEFGYDRCVVAPGIELIYDKIEGYSE

        148   EAAEIMPHAYKAGPQTQLLRRMIEAMPQGGVFAMVAPPNPYRCPPGPYER
              |||: :|||:||| ||::||: :|:|::||: ::::|::|:|||||||||
        149   EAAAKLPHAWKAGEQTAILRKQLEDMADGGTVVIAPPAAPFRCPPGPYER

        198   VSMVAHLLKQTNPTAKIIILDPKDKFSKQALFEEGWGKHY-----NGMVE
              :| ||: ||: :| :|:||||::::||||: |::||::       |:|:|
        199   ASQVAYYLKAHKPMSKVIILDSSQTFSKQSQFSKGWERLYGFGTENAMIE

        243   WIGPEF
              |
        249   WHPGPD
```

FIG. 6. Alignment of the deduced amino acid sequence of *soxF* with that of the partial sequence available of the flavoprotein of flavocytochrome *c* of *C. vinosum* (cvfccb). The dot indicates the cysteine proposed to bind flavin in *C. vinosum.*

was observed in the wild type after growth on pyruvate alone (Fig. 7, lane 1), indicating that SoxC is specifically induced by thiosulfate.

Physiological characterization of strain GB*soxC*Δ. Strain GB*soxC*Δ was unable to grow lithoautotrophically with thiosulfate (data not shown). Under mixotrophic growth conditions, the optical density of the wild-type GB17 was significantly increased upon addition of thiosulfate to pyruvate mineral medium. However, under identical growth conditions, the optical density of mutant GB*soxC*Δ was not increased (Table 3). On the other hand, the mutant was still capable of thiosulfate-dependent oxygen uptake, albeit at a rate 10% of the wild-type rate (Table 3).

Anaerobic growth in glucose mineral medium with nitrate as the electron acceptor or aerobic growth with nitrate as the nitrogen source was the same for *P. denitrificans* GB17 and its GB*soxC*Δ derivative (data not shown), demonstrating that the deletion in *soxC* affected neither assimilatory nor dissimilatory nitrate reduction.

Sulfur oxidation in cell extracts. Sulfite is hardly oxidized by whole cells of *P. denitrificans* (17). To examine if the low thiosulfate-oxidizing activity of whole cells of strain GB*soxC*Δ was also present in cell extracts, activities of the cytochrome *c*-dependent thiosulfate-oxidizing system, of sulfite dehydrogenase, and of sulfide dehydrogenase were determined. The respective specific activities from extracts of the wild type grown mixotrophically with pyruvate plus thiosulfate were 8.90, 7.98, and 11.1 mU/mg of protein (Table 4). From GB*soxC*Δ, a low but distinct activity of thiosulfate oxidation of 0.88 mU/mg of protein was observed, while sulfite dehydrogenase exhibited a trace activity of 0.06 mU/mg of protein. In cell extracts of strain GB*soxC*Δ, an initial minor hydrogen sulfide-dependent reduction of 3.57 nmol of cytochrome *c* was observed, which ceased completely after 30 s (Table 4). These data demonstrated that sulfite dehydrogenase of *P. denitrificans* GB17 was involved in hydrogen sulfide and in thiosulfate oxidation.

DISCUSSION

Four genes, *soxCDEF*, have been identified in addition to the previously reported *soxAB* genes within a region coding for lithotrophic thiosulfate oxidation in *P. denitrificans* GB17 (56). From *P. versutus*, a close relative of *P. denitrificans*, sulfite dehydrogenase was reported not to be required for the oxidation of thiosulfate in vitro (29). Sequence analysis of the *soxC* gene of *P. denitrificans* GB17 suggests that *soxC* codes for sulfite dehydrogenase. It is essential for lithotrophic growth with thiosulfate in *P. denitrificans* GB17, as evident from (i) the inability of a mutant carrying an in-frame deletion in *soxC* eliminating sulfite dehydrogenase activity and to grow with thiosulfate as an energy source and (ii) the inducibility of sulfite dehydrogenase activity and of SoxC antigens by thiosulfate.

The *soxBCDEF* genes overlap or are separated by short intergenic sequences insufficient to code for a promoter region. The observed ribosome binding sites and the thiosulfate-de-

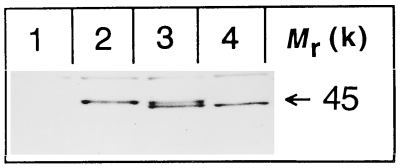

FIG. 7. Immunoblot analysis of the *soxC* and *soxC*Δ gene products. Western blot analyses of crude extracts (about 10 μg of protein per slot) of cells cultivated as indicated were performed as described in Materials and Methods. Lanes: 1, GB17 cultivated with pyruvate; 2, GB17 cultivated with pyruvate plus thiosulfate; 3, mixed extracts (10 μg of protein each) of induced strains GB17 and GB*soxC*Δ shown in lanes 3 and 4; 4, GB*soxC*Δ cultivated with pyruvate plus thiosulfate.

TABLE 3. Physiological characteristics of *P. denitrificans* GB17 and GB*soxC*Δ

Strain	Growth condition[a]	Final OD_{436}[b]	Final pH	Specific thiosulfate oxidation rate (μmol of oxygen/min · mg of protein)
GB17	Pyruvate	1.83	7.45	0.011
	Pyruvate, thiosulfate	2.41	6.61	0.840
GB*soxC*Δ	Pyruvate	1.73	7.47	0.022
	Pyruvate, thiosulfate	1.76	7.35	0.081

[a] Mineral medium (pH 7.2) with 0.1% (wt/vol) sodium pyruvate and 20 mM sodium thiosulfate, if indicated.
[b] OD_{436}, optical density at 436 nm.

pendent expression of sulfite dehydrogenase by *P. denitrificans* GB17 suggest a common regulation of at least these genes and indicate the presence of an operon. Amino acid sequence analysis suggests that the *soxBCDEF* gene products are periplasmic due to the characteristics for a signal peptide (54, 55). This analysis is in accordance with biochemical data suggesting that the thiosulfate-oxidizing enzyme system of the closely related *P. versutus* is periplasmic (28). The predicted signal peptide of sulfite dehydrogenase of *P. denitrificans* GB17 contains 40 amino acids and an RR motif. Unusually long signal peptides of 32 to 50 amino acids with an RR motif are observed in metal-containing proteins like periplasmic molybdoenzyme formate dehydrogenases (5), membrane-bound [NiFe] hydrogenases (15, 31), or respiratory copper nitrous oxide reductases (59). Although the deduced amino acid sequence of *soxC* is also highly similar to sequences of eukaryotic nitrate reductases, its essential role is in thiosulfate metabolism. This was evident from the ability of the mutant GB*soxC*Δ to grow with nitrate but not with thiosulfate. Also, sulfite-dependent cytochrome *c* reduction was observed in extracts of wild-type cells induced with thiosulfate, while only a marginal rate was detected from the mutant GB*soxC*Δ. Therefore, *soxC* coded for sulfite dehydrogenase.

Chicken liver sulfite oxidase is composed of 460 amino acids and is 30.7% identical to the enzyme of *P. denitrificans* GB17 in a 326-amino-acid overlap (Fig. 5). Chicken liver sulfite oxidase exhibits at the amino-terminal region a binding domain for heme b_5 as an internal electron acceptor which is not evident from the primary structure (1, 16). Binding of a *b*-type heme to sulfite dehydrogenase of *P. denitrificans* GB17 awaits biochemical proof. According to the biochemical characterization, sulfite dehydrogenase of *P. versutus* (M_r of 44,000) requires cytochrome c_{551} (M_r of 43,000) for activity (29), as reported for the *T. novellus* enzyme (49, 57). In analogy, we conclude that sulfite dehydrogenase of *P. denitrificans* also requires a cytochrome for activity. Such an electron mediator may be the *soxD* gene product, which is a diheme *c*-type cytochrome with an M_r of 39.982, comparable to that of *P. versutus.*

Eukaryotic sulfite oxidases and nitrate reductases contain a region with an apparently conserved cysteine which was proposed to bind the molybdopterin cofactor (3). Although the similarity of sulfite dehydrogenase of *P. denitrificans* GB17 around this cysteine to eukaryotic molybdoenzymes is not pronounced, it is proposed that sulfite dehydrogenase of *P. denitrificans* GB17 also contains the molybdenum cofactor. This proposal is based on the requirement of molybdenum for lithotrophic growth with thiosulfate and the Sox^- phenotype of the class III mutants unable to synthesize a functional molybdenum cofactor (18). It is noteworthy that no similarity was detectable to prokaryotic molybdoenzymes. However, the RR motif within the signal peptide characteristic for prokaryotic metal enzymes (5, 31, 59) may indicate a linkage of the molybdenum cofactor to the nascent enzyme prior to the export of the protein to the periplasm.

Sulfite is hardly oxidized by thiosulfate-induced cells of *P. denitrificans* (17). Thus, sulfite dehydrogenase is not likely to utilize sulfite from the environment but may be required within the reaction sequence for thiosulfate metabolism. No indication that free sulfite is an intermediate of thiosulfate oxidation of *P. denitrificans* GB17 or of *P. versutus* has been obtained (8, 18, 24). Sulfite dehydrogenase of *P. versutus* enhances the activity of the reconstituted thiosulfate-oxidizing enzyme system by 20 to 25%. The enzyme was considered to exhibit a gratuitous function which in the complex in vivo could oxidize enzyme-bound intermediates analogous to sulfite or sulfonate residues (29). Since a trace activity of thiosulfate and an initial hydrogen sulfide oxidation were still detectable in whole cells and cell extracts of strain GB*soxC*Δ, sulfite dehydrogenase may either act on an enzyme-bound intermediate, eliminate sulfite as a possible toxic by-product of the reaction, or enhance the overall reaction rate of thiosulfate and of hydrogen sulfide oxidation. The inability of GB*soxC*Δ to grow lithoautotrophically with thiosulfate demonstrates the essential role of sulfite dehydrogenase in thiosulfate oxidation by *P. denitrificans* GB17.

The *soxD* gene product may function as electron mediator for sulfite dehydrogenase of *P. denitrificans* GB17. This proposal is based on its similarity in amino acid sequence and molecular mass to *c*-type cytochromes of comparable function of *P. versutus*. A cytochrome *c* equivalent to the size of the *soxE* gene product (M_r of 25,926) was not reported to be involved in thiosulfate oxidation of *P. versutus* in vitro (24). SoxE may be integrated in the cytoplasmic membrane by the hydrophobic carboxy-terminal end and transfer electrons from thiosulfate oxidation to the respiratory chain, or it may be associated with SoxF.

The product of the incomplete *soxF* gene was highly identical to the flavoprotein of flavocytochrome *c* of the phototrophic sulfur-oxidizing bacterium *C. vinosum*, which suggests an equivalent function in *P. denitrificans* GB17. The structure of flavocytochrome *c* has been determined at a resolution of 2.53 Å (9). The protein is probably located in the periplasm of *C. vinosum*, heterodimeric, and composed of a flavoprotein (M_r of 46,000) and a diheme cytochrome *c* (M_r of 21,000). Similar proteins have been detected in other phototrophic bacteria (16) and in a *Thiobacillus* species (53). Flavocytochrome *c* exhibits in vitro hydrogen sulfide-oxidizing activity to elemental sulfur or polysulfide, with concomitant reduction of horse heart cytochrome *c* (19). The physiological function of flavocytochrome *c* is, however, a matter of debate. Flavocytochrome *c* forms stable adducts at a disulfide bond with sulfite,

TABLE 4. Thiosulfate-, sulfite-, and hydrogen sulfide-dependent cytochrome *c* reduction in cell extracts of *P. denitrificans* GB17 and GB*soxC*Δ[a]

Strain	Specific cytochrome *c* reduction rate (nmol of cytochrome *c*/min · mg of protein)		
	Thiosulfate	Sulfite	Sulfide
GB17	8.90	7.98	11.1
GB*soxC*Δ	0.88	0.06	0.0[b]

[a] Cultivated mixotrophically with pyruvate plus thiosulfate as described in Materials and Methods.
[b] Activity after 30 s; total reduction of 3.57 nmol of cytochrome *c*.

thiosulfate, and some mercaptans without reduction of flavin or heme. Sulfite forms adducts at the flavin moiety and opens cysteinyl disulfide bonds (9, 33). Flavocytochrome *c* appears to be correlated with the ability to oxidize thiosulfate to sulfate by phototrophic or lithotrophic thiobacteria but not to be linked to hydrogen sulfide oxidation (reviewed in reference 16). Mutational inactivation of *soxF* will determine its role in lithotrophic sulfur metabolism. Hydrogen sulfide oxidation has previously been found in *P. denitrificans* (8, 17). The in vitro activity of strain GB17 was assigned to a protein with an M_r of 34,000 copurified with the SoxB protein (46). The *soxB* gene, however, codes for a mature protein with an M_r of 59,063 and is essential for hydrogen sulfide and for thiosulfate oxidation (8, 34). Amino acid sequence analysis of SoxB exhibits an identity of 20.7% in a 522-amino-acid overlap to bovine 5′-nucleotidase (Table 2). This relationship may point to the covalent linkage of sulfur to a protein and the release of the oxidized sulfur by sulfonate hydrolysis by SoxB.

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