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Identification and Sequence Analysis of the *soxB* Gene Essential for Sulfur Oxidation of *Paracoccus denitrificans* GB17

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The coding region for lithotrophic sulfur oxidation (Sox) in *Paracoccus denitrificans* GB17 was identified by isolation of a transposon Tn*5-mob* mutant with a Sox⁻ phenotype (strain TP19). The corresponding wild-type region was cloned previously (G. Mittenhuber, K. Sonomoto, M. Egert, and C. G. Friedrich, J. Bacteriol. 173:7340–7344, 1991). Sequence analysis of a 2.5-kb subclone that complemented strain TP19 revealed that Tn*5-mob* was inserted into a coding region for a 553-amino-acid polypeptide named SoxB. This polypeptide had an M_r of 60.573, including a possible signal peptide. The function of the SoxB protein of *P. denitrificans* GB17 appeared to be identical to that of enzyme B of the thiosulfate-oxidizing enzyme system of *Thiobacillus versutus*. The amino acid compositions of the two proteins were identical, and the amino acid sequences of three internal peptides of enzyme B as determined by Edman degradation were identical to corresponding sequences of the deduced SoxB protein of *P. denitrificans* GB17.

Paracoccus denitrificans is a gram-negative, neutrophilic, facultatively chemolithoautotrophic eubacterium that grows heterotrophically with a large variety of carbon sources. It also grows lithoautotrophically with molecular hydrogen as an electron donor (9) or with reduced inorganic sulfur compounds, such as thiosulfate (5). Attempts to analyze genetically the thiosulfate-oxidizing enzyme system (Sox system) of *T. versutus* or *P. denitrificans* DSM65^T have been unsuccessful. However, *P. denitrificans* GB17 is accessible to transposon mutagenesis (3). Three classes of mutants unable to grow lithotrophically with thiosulfate as the energy source were isolated. These mutants were unable to oxidize thiosulfate or sulfide to sulfuric acid (Sox⁻). Class I mutants were exclusively Sox⁻. Class II Sox⁻ mutants were also unable to oxidize hydrogen or to reduce nitrite. These mutants are defective in the formation of a common cytochrome *c*. Class III Sox⁻ mutants were unable to synthesize a functional molybdopterin cofactor involved in formate oxidation or in nitrate reduction (3, 4).

From the class I mutant strain TP19, the Tn*5-mob*-containing fragment with the adjacent *sox* DNA was cloned, resulting in the hybrid plasmid pKS3-13. This fragment was used to identify and clone the corresponding wild-type *sox* DNA region, resulting in the hybrid plasmid pEG12. The 13-kb insert of pEG12 contained three *Eco*RI fragments of 0.8, 2.7, and 9.5 kb and was used to identify DNA homologies of other thiobacteria. Strong hybridization signals were obtained with *T. versutus* DNA (14). For *T. versutus*, the biochemistry of the thiosulfate-oxidizing enzyme system has been described. It is composed of four periplasmic proteins: enzyme A (M_r, 16,000), enzyme B (M_r, 63,000), cytochrome $c_{552.5}$ (M_r, 56,000), and cytochrome c_{551} (M_r, 260,000), as reviewed by Kelly (7).

Here, we report on the identification of the *soxB* gene of *P. denitrificans* GB17 and its sequence analysis. The function of the *soxB* gene product was identified as equivalent to that of enzyme B of the thiosulfate-oxidizing enzyme system of *T. versutus*.

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MATERIALS AND METHODS

Strains, phages, and plasmids. Bacteria, phages, and plasmids used are listed in Table 1.

Media and growth conditions. *Escherichia coli* was cultivated in Luria-Bertani medium. Solid media contained 1.5% (wt/vol) agar. Selective media contained 100 μg of ampicillin per ml.

Plasmid analysis, cloning, and sequencing. Standard DNA techniques were employed (15). Plasmids were analyzed by the method of Kado and Liu (6). For single-stranded DNA sequencing, appropriate restriction fragments of the 9.5-kb *sox* DNA region of pEG9 were subcloned into M13 vectors (Table 1). For sequencing of the insertion site of transposon Tn*5-mob*, pBluescript vectors were used with *E. coli* XL1-Blue as the host strain (Stratagene Cloning Systems, Inc.). For DNA sequencing, the dideoxy-chain termination method (16) was used with a T7 sequencing kit (U.S. Biochemicals) and ^{35}S-dATP (Amersham-Buchler, Braunschweig, Germany). The nucleotide sequence analysis software included HUSAR (European Molecular Biology Laboratory, Heidelberg, Germany), DNASIS, and PC/GENE software packages (IntelliGenetics Inc., Mountain View, Calif.). The precise insertion locus of Tn*5-mob* was determined from a 3.0-kb *Sal*I fragment of plasmid pKS3-13. This fragment carried one transposon-chromosome junction and was subcloned in pBluescript KS$^+$ (Table 1). The junction was sequenced as described elsewhere (16) with the oligonucleotide GTTAGGAGGTCACATGG as the primer for the terminal Tn*5* inverted-repeat sequence.

DNA-DNA hybridization. DNA-DNA hybridizations were performed at the facilities of the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany, by a procedure described previously (13).

Protein sequencing and oligonucleotide probe. A partially purified sample of enzyme B of the thiosulfate-oxidizing enzyme system of *T. versutus* was further purified by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TABLE 1. Bacterial strains, phages, and plasmids

Strain, phage, or plasmid	Relevant characteristics	Reference or source
Strains		
E. coli		
XL1-Blue	*recA recA1 lac endA1 gyrA96 thi hsdR17 supE44 relA1* [F' *proAB lacI*^{q} *lacZ*Δ(M15 Tn*10*)]	Stratagene
SMH50	*endA gyrA96 thi hsdR17 supE44 relA1* Y$^-$ Δ(*lac proAB*) (F' *traD36 proAB lacI*^{q}ZM15)	21
P. denitrificans		
DSM65	Type strain; Sox$^+$ Km^s	DSM[a]
GB17	Wild type; Sox$^+$ Km^s	10, 13
TP19	Sox$^-$ Km^r *sox*::Tn*5-mob*	3, 14
TPX10	Sox$^-$ Ap^r Tc^r Km^r; integration of pEG10	This study
TPX9	Sox$^+$ Ap^r Tc^r Km^r; integration of pEG9	This study
TPX7	Sox$^+$ Ap^r Tc^r Km^r; integration of pEG7	This study
TPX5	Sox$^-$ Ap^r Tc^r Km^r; integration of pEG5	This study
TPX4	Sox$^-$ Ap^r Tc^r Km^r; integration of pEG4	This study
T. versutus DSM700	Type strain	DSM
Bacteriophages		
M13BM-20	M13 derivative	Boehringer
M13BM-21	M13 derivative	Boehringer
Plasmids		
pSUP202	Ap^r Cm^r Tc^r Tra^{-b} Mob^{+c}	18
pVK101	Tc^r Km^r Mob$^+$	8
pBluescript KS$^+$	Ap^r *lacZ'*; T7 Phil10 promoter; f1 *ori*	Stratagene
pKS3-13	9-kb *sox*::Tn*5-mob* insert of strain TP19 in pSUP202	14
M1321-EB	1.2-kb *Eco*RI-*Bgl*II fragment in M13BM-21	This study
M1320-EB	1.2-kb *Eco*RI-*Bgl*II fragment in M13BM-20	This study
M1321-S	1.4-kb *Sal*I fragment in M13BM-21	This study
M1321-SR	1.4-kb *Sal*I fragment in M13BM-21	This study
pBKS-Tn51	3.0-kb *Sal*I fragment of the 9-kb *sox*::Tn*5-mob* insert of pKS3-13 subcloned in pBluescript KS$^+$	This study
pEG4	3.7-kb *Eco*RI fragment of pEG12 in pSUP202	This study
pEG5	5.2-kb *Eco*RI fragment of pEG12 with an 8-kb *Bgl*II deletion in pSUP202	This study
pEG7	7.0-kb *Eco*RI fragment of pEG12 with a 2.5-kb *Pst*I deletion in pSUP202	This study
pEG9	9.5-kb *Eco*RI fragment of pEG12 in pSUP202	This study
pEG10	9.7-kb *Eco*RI fragment of pEG12 with a 3.1-kb *Sst*I deletion in pSUP202	This study
pEG12	13-kb wild-type *sox* insert in pSUP202	14
pEV3	2.7-kb *Eco*RI fragment of pEG12 in pVK101	This study
pEV7	7.0-kb *Eco*RI fragment of pEG9 with a 2.5-kb *Pst*I deletion in pVK101	This study
pEV9	9.5-kb *Eco*RI fragment of pEG12 in pVK101	This study

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.
[b] Tra, transfer of mobilizable plasmids.
[c] Mob, mobilizability.

(SDS-PAGE) (17). The amino acid sequence was determined from 300 μg of protein with a pulsed gas-liquid-phase protein sequenator (model 477A; Applied Biosystems). Since the amino-terminal end of enzyme B was blocked, a sample was treated with ethanol. The precipitated protein (1 mg) was cleaved with cyanogen bromide, and the resulting peptides were separated by SDS-PAGE and transferred to ProBlott membranes and sequenced.

The amino acid composition of enzyme B of *T. versutus* was determined from 100 μg purified by SDS-PAGE, blotted on ProBlott membranes, and hydrolyzed for 24 h in 6 N hydrochloric acid at 100°C.

Nucleotide sequence accession number. The nucleotide sequence was submitted to the EMBL data library under accession number X79242 PDSOXB.

RESULTS AND DISCUSSION

Complementation of the *sox*::Tn*5-mob* mutant. Strain TP19 is unable to oxidize thiosulfate or sulfide to sulfate (Sox$^-$) (3). The Sox$^-$ phenotype of strain TP19 could be due to an insertion of Tn*5-mob* into a structural or regulatory gene or due to polarity. To determine the nature of the mutation, the corresponding 13-kb wild-type DNA region relevant to Sox was subcloned. Fragments of about 9.7, 9.5, 7.0, 5.2, and 3.7 kb were physically mapped and inserted into pSUP202, which cannot be maintained in strains other than *E. coli*. The resulting hybrid plasmids were designated pEG10, pEG9, pEG7, pEG5, and pEG4, respectively (Fig. 1; Table 1). Each of the plasmids was introduced into the Sox$^-$ mutant TP19 from *E. coli* via conjugation and rescued by general recombination. Transconjugants were selected for tetracycline resistance (Tc^r). From each transfer, 20 kanamycin-resistant (Km^r), Tc^r strains were examined for the Sox phenotype. All transconjugants having integrated pEG9 or pEG7 were Sox$^+$. Those resulting from integration of pEG10 or pEG4 remained Sox$^-$ (Table 1). This result provided evidence that a 5.5-kb region located between the *Eco*RI and *Pst*I restriction sites was necessary for sulfur oxidation.

To determine if the 5.5-kb DNA region could complement the Tn*5-mob* insertion in *trans*, the 9.7-, 7.0-, and 2.7-kb fragments were cloned into the broad-host-range vector pVK101. The resulting hybrid plasmids, pEV9, pEV7, and pEV3 (Fig. 1), were each introduced into the Sox$^-$ mutant TP19. From each cross, 20 Tc^r, Km^r transconjugants of strain

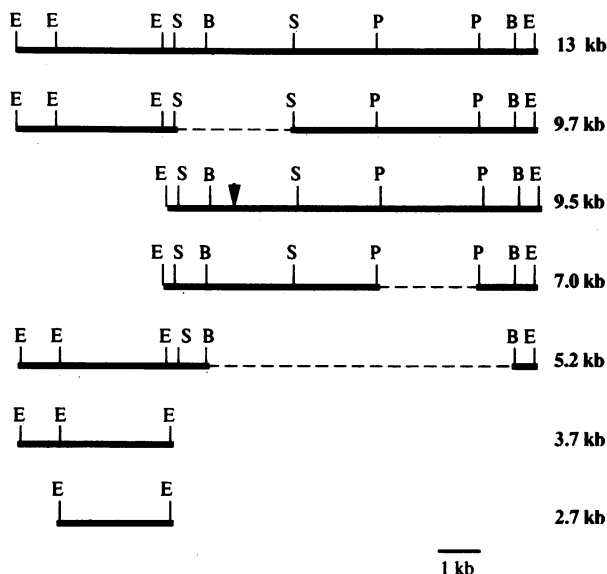

FIG. 1. Physical and restriction maps of subclones of the 13-kb *sox* region of *P. denitrificans* GB17. *Eco*RI fragments were cloned into pSUP202 or pVK101 as listed in Table 1. Dashed lines indicate deletions between identical restriction enzyme sites (B, *Bgl*II; E, *Eco*RI; P, *Pst*I; S, *Sst*I). The arrowhead at the 9.5-kb insert indicates the position of Tn*5*-*mob* in the insert of pKS3-13.

TP19 were examined for the presence of the respective plasmid. Transconjugants harboring pEV9 or pEV7 were Sox$^+$ while those harboring pEV3 remained Sox$^-$. This result demonstrated *trans* complementation of the region between the *Eco*RI and *Pst*I restriction sites of pEV9 or pEV7 (Fig. 1).

Nucleotide sequence analysis of a *sox* locus. The *sox*::Tn*5*-*mob* insertion of the Sox$^-$ mutant TP19 mapped between the *Bgl*I and *Sst*I restriction sites of the 18-kb *Eco*RI insert of pKS3-13 (14) (Fig. 1). The precise Tn*5*-*mob* insertion was determined from a 3-kb *Sal*I subclone of the 9-kb insert of pKS3-13 carrying the Tn*5* inverted-repeat–chromosome junction. The adjacent chromosomal nucleotide sequence revealed coding characteristics according to codon preference analysis (19). Thus, Tn*5*-*mob* was possibly inserted into a *sox* structural gene.

From the corresponding wild-type *sox* region of pEG9 (14) (Fig. 1), a region of about 2.5 kb was subcloned (Fig. 2) and sequenced (data are accessible under no. X79242 PDSOXB at the EMBL data library). The *Sal*I-*Eco*RI fragment contained 2,502 bp and exhibited one open reading frame with coding characteristics and a GC content of 64.6%. The open reading frame was separated from upstream sequences with noncoding characteristics and a GC content of 61.8%. The open reading frame contained 554 codons, and it was designated *soxB* and its product was designated the SoxB protein. The deduced protein contained 553 amino acids, with a calculated M_r of 60,573. Sequence analysis revealed a possible signal peptide of 16 amino acids, suggesting that the SoxB protein was located in the periplasm. The M_r of the mature protein was determined to be 59,063, with a calculated isoelectric point (pI) of 4.76.

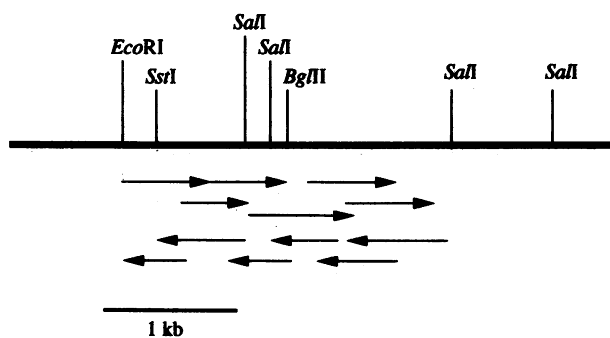

FIG. 2. Strategy for sequencing the *sox* region of *P. denitrificans* GB17.

The *soxB* gene was separated from another start codon by 23 intervening nucleotides with a well-conserved putative ribosome binding site. The adjacent sequence exhibited peptide coding characteristics similar to those observed for *soxB* and extended to the *Eco*RI restriction site at the end of the *sox* DNA region in pEG9. Since the intervening sequence exhibited no signal for termination, the adjacent coding region may be part of another *sox* gene.

Nucleotide sequence comparison of the Tn*5*-*mob*–chromosome junction revealed an insertion between bp 720 and 721 within the *soxB* gene and within the codon of the 24th amino acid of the protein.

Identification of the *soxB* gene of *P. denitrificans* GB17. To identify the function of the SoxB protein of *P. denitrificans* GB17, it was compared with enzyme B (M_r, 63,000) of the thiosulfate-oxidizing enzyme system of *T. versutus* (12). *T. versutus* is the closest relative of *P. denitrificans* DSM65^T (13). DNA-DNA hybridization of both type strains revealed (56 ± 1)% homology (data not shown), confirming that these strains were closely related.

The close relationship of the two strains and the sizes of the two proteins and their involvement in sulfur oxidation led us to analyze enzyme B of *T. versutus* to identify the function of the SoxB protein of *P. denitrificans*. Enzyme B of *T. versutus* was purified and characterized previously (12). The M_r of enzyme B was redetermined by SDS-PAGE to be 60,000 (data not shown). The amino acid composition of this protein was determined chemically and compared with the deduced amino acid composition of the SoxB protein (without the signal peptide). The two proteins were almost identical, with minor differences attributed to the chemical detection method (data not shown).

To obtain further evidence for a possible identity of the two proteins, the amino acid sequences from three internal peptides of enzyme B of *T. versutus* were analyzed. The sequence of peptide B1 was GGRVKGIDVILSGHTHD, that of peptide B2 was AALIDAERAPFQAQLEEXIG, and that of peptide B3 was ALADTGEAIEPAKSYTVAGXASVNEGXEGP. The amino acid sequences of these peptides were compared with the deduced sequence of the SoxB protein. The nucleotides corresponding to the amino acid sequence for the peptides were as follows: B1, bp 1477 to 1528; B2, bp 1696 to 1766; and B3, bp 2126 to 2215. These amino acid sequences were identical for the proteins of *P. denitrificans* GB17 and *T. versutus*. Also, the molecular mass was 60 kDa for both proteins, and the pI of 4.76 for SoxB was similar to the pI of 4.25 for enzyme B (12). Moreover, the signal peptide indicated a periplasmic location of the deduced protein of *P. denitrificans*. The periplasmic location of enzyme B of *T. versutus* has been demonstrated by biochemical methods (11). These data were evidence for identical functions of enzyme B of *T. versutus* and the SoxB protein of *P. denitrificans* GB17.

Amino acid sequence analysis of SoxB. Enzyme B of *T. versutus* contains a binuclear manganese cluster (2). The coordination of manganese is known for several Mn-containing

enzymes, such as Mn-superoxide dismutase (20). However, no known conserved amino acid motifs of the deduced SoxB protein for the coordination of manganese could be detected.

Molybdenum is required for thiosulfate oxidation of *P. denitrificans* and *T. versutus* (4). Molybdenum oxotransferases contain a conserved cysteine which may play a role in the binding of molybdopterin to the protein (1). However, comparison with this region revealed only marginal homology of the amino acid sequence of the deduced SoxB protein of *P. denitrificans* GB17 with those of molybdoenzymes from eucaryotic sources and no homology with enzymes from procaryotic sources. Therefore, it is doubtful whether molybdopterin is part of the SoxB protein, and it may play a role in a different protein.

The M_r of the mature SoxB protein is 60,000. Enzyme B of *T. versutus* was reported to appear in two forms, with M_rs of 63,000 and 32,000 (12). Recently, the M_r of the SoxB protein of *P. denitrificans* GB17 was determined to be 32,000 (17a), possibly indicating a processing of this protein.

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