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Transposon Mutagenesis Affecting Thiosulfate Oxidation in *Bosea thiooxidans*, a New Chemolithoheterotrophic Bacterium

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Transposon insertion mutagenesis was used to isolate mutants of *Bosea thiooxidans* which are impaired in thiosulfate oxidation. Suicide plasmid pSUP5011 was used to introduce the transposon Tn*5* into *B. thiooxidans* via *Escherichia coli* S17.1-mediated conjugation. Neomycin-resistant transconjugants occurred at a frequency of 2.2×10^{-4} per donor. Transconjugants defective in thiosulfate oxidation were categorized into three classes on the basis of growth response, enzyme activities, and cytochrome patterns. Class I mutants were deficient in cytochrome *c*, and no thiosulfate oxidase activity was detected. Class II mutants retained the activities of key enzymes of thiosulfate metabolism, although at reduced levels. Mutants of this class grown on mixed-substrate agar plates deposited elemental sulfur on the colony surfaces. Class III mutants were unable to utilize thiosulfate, though they had normal levels of cytochrome *c*. The transposon insertions occurred at different chromosomal positions, as confirmed by Southern blotting of chromosomal DNA of mutants deficient in thiosulfate oxidation, a deficiency which resulted from single insertions of Tn*5*.

Little is known about the structure, expression, and regulation of the genes associated with the oxidation of inorganic sulfur compounds of the obligately (17, 44) or facultatively (13, 31, 43) chemolithotrophic sulfur bacteria. Oxidation of inorganic sulfur is performed by a number of heterotrophic bacteria, including *Pseudomonas* spp. (33), *Paracoccus* spp. (11), *Mycobacterium* spp. (49), and *Xanthobacter* spp. (39). These heterotrophs may benefit from sulfur oxidation in the detoxification of hydrogen peroxide (18, 29). To date, preliminary work has been done to understand the genetical basis of sulfur metabolism by obligate chemolithoautotrophic (*Thiobacillus ferrooxidans*) (30) and facultatively chemolithoautotrophic (*Thiobacillus versutus* and *Thiobacillus novellus*) bacteria (9). However, little is known at the genetic level, and no genes involved in oxidation of sulfur have been identified or cloned (47–49). Until recently, no DNA probes for reduced inorganic sulfur oxidation genes existed (30). In addition, there is no defined mutant deficient in sulfur oxidation and an only rudimentary genetic system has been discussed for *Thiobacillus novellus*, *Thiobacillus versutus* (9), *Paracoccus denitrificans* GB17 (51), *Thiosphaera pantotropha* (5, 28), and a bacterial endosymbiont of the hydrothermal vent tubeworm *Riftia pachyptila* (19). Genetic systems to study bacterial species capable of oxidizing inorganic sulfur compounds are only now being developed. Transposon mutagenesis of *Bosea thiooxidans* (8) was used to generate mutants impaired in thiosulfate oxidation.

Transposon insertion mutagenesis has been applied to a wide range of gram-negative bacteria (5, 16, 34, 35, 45) for both strain construction and the analysis of mutant phenotypes, but this approach has not been exploited to study the mechanism of inorganic sulfur oxidation for chemolithoheterotrophs.

B. thiooxidans BI-50 was selected for genetic studies of thiosulfate-oxidizing activity by transposon Tn*5* insertion mutagenesis because (i) its level of spontaneous neomycin resistance above its base sensitivity level is very low; (ii) its generation time in rich medium (Luria broth and nutrient broth) is short; (iii) the nutrition of this strain is chemolithoheterotrophic; (iv) broad-host-range IncP plasmids like RP4 and RP4.4 can be transferred from other donors, such as *Escherichia coli*, *Thiobacillus versutus*, or *Pseudomonas* spp., into this bacterium at high frequencies (data not shown); (v) the strain is accessible to the introduction of transposon Tn*5*-*mob* by the use of a suicide vector; and (vi) mutants generated by Tn*5* insertion are highly stable and have very low reversion frequencies.

Thus, the objectives of this investigation were to develop transposon mutagenesis techniques for use with *B. thiooxidans* and to isolate mutants defective in thiosulfate oxidation.

MATERIALS AND METHODS

Chemicals. The following analytical-grade chemicals were used (their makers are mentioned in parentheses): Sephadex G-50 (fine) DNA grade (Pharmacia Chemicals, Uppsala, Sweden); restriction endonucleases (Bethesda Research Laboratory, Gaithersburg, Md., and New England Biolabs); [α-^{32}P]dATP (Bhabha Atomic Research Centre); yeast extract (Difco Laboratories, Detroit, Mich.); potassium tetrathionate (Sigma Chemical Co., St. Louis, Mo.); and other sulfur compounds (Sisco Research Laboratory Pvt. Ltd.).

Bacterial strains, plasmid, and transposons. Bacterial strains used are listed in Table 1. BI-50 is the spontaneous rifampin-resistant (Rif^r) mutant of *B. thiooxidans* (BI-42) on which transposon mutagenesis was done (7, 8). *E. coli* S17.1 [Pro res$^-$ mod$^+$ RP4.2 (Tc::Mu) (Km::Tn*7*) with trimethoprim resistance (Tp^r) and streptomycin resistance (Sm^r)], containing the suicide plasmid pSUP5011 (pBR325::Tn*5*-*mob* with ampicillin resistance [Ap^r], chloramphenicol resistance [Cam^r], and kanamycin resistance [Km^r]) (35) with a P-type-specific recognition site for mobilization (mob), was used for transposon Tn*5* mutagenesis as described by Simon et al. (36).

Media. The GYM (glutamate-yeast extract-mineral salt) medium was as described by Das et al. (8) and contained (per liter) 4.0 g of Na_2HPO_4, 1.5 g of KH_2PO_4, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.5 g of sodium glutamate, and 0.1 g of yeast extract powder. For MS (mixed-substrate) medium, sodium thiosulfate (5 g/liter) and sodium succinate (5 g/liter) were added to the GYM medium. Heterotrophic growth was checked in GYM medium with succinate (5 g/liter). For solid medium, Bacto agar (20 g/liter; Difco Laboratories) was used.

Transpositional mutagenesis. For mutagenesis, the transposon Tn*5* was introduced into strain BI-50 on the suicide plasmid vehicle pSUP5011 by conjugation with *E. coli* S17.1. The recipient BI-50 (Rif^r) and the donor *E. coli* S17.1 were grown in Luria broth for 20 h and 6 h, respectively. Two milliliters of each freshly grown culture was mixed aseptically and centrifuged at 4,000 $\times$ *g* for 10 min. The resultant pellet was suspended in 0.2 ml of sterile normal saline and plated on Luria agar medium for conjugation. The plate was incubated at 30°C for 18 h. The mated cultures were washed off from the plate with 6 ml of normal saline. Two-tenths milliliter of the dilutions was plated on Luria agar with rifampin (100 μg/ml) for the recipient count, with neomycin (70 μg/ml) for the donor count,

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TABLE 1. Bacterial strains used in this study

Bosea thiooxidans strain	Relevant phenotype[a]	Reference or source
BI-42	Sm^r Ap^r	8
BI-50	Spontaneous Rif^r mutant of strain BI-42	This study
DNK-10	Thi^- Suc^+ Neo^r	This study
DNK-11	Thi^- Suc^+ Neo^r	This study
DNK-12	Thi^- Suc^+ Neo^r	This study
DNK-13	Thi^- Suc^+ Neo^r	This study
P-3	Thi^+ Cys^- Neo^r	This study
P-5	Thi^+ Lys^- Neo^r	This study
P-6	Thi^+ His^- Neo^r	This study
P-7	Thi^+ Cys^- Neo^r	This study
P-9	Thi^+ His^- Neo^r	This study
P-15	Thi^+ Pro^- Neo^r	This study
P-18	Thi^+ Ade^- Neo^r	This study

[a] Abbreviations: Neo, neomycin; Suc, succinate; Cys, cysteine; Lys, lysine; His, histidine; Pro, proline; Ade, adenine.

and with both of the above-listed antibiotics for the selection of transconjugants. After 4 days of incubation, transconjugants appeared as neomycin- and rifampin-resistant single colonies and were picked onto master plates for the postmating selection.

Selection of mutants. Each master plate was replica plated onto the following agar media: (i) GYM medium containing thiosulfate plus succinate and phenol red (to detect acid production) and (ii) GYM medium with succinate (to detect the ability to grow as a succinate prototroph). Colonies defective in thiosulfate metabolism exhibited no color change even after 7 days of incubation. These colonies were purified from the master plate and maintained on Luria agar slants. Additionally, mutants defective in amino acid and vitamin biosynthetic pathways were identified by crossed-pool auxanography as described by Holliday (14).

Reversion frequency of Tn*5*-induced mutants. Each mutant was grown in freshly prepared Luria broth in the absence of neomycin for four to five successive transfers. Two-tenths milliliter of diluted overnight culture (up to 10^{-6} organisms) was plated on MS agar medium containing phenol red (to detect acid-producing revertant colonies) and on GYM medium with succinate (to detect the prototrophs among the auxotrophic mutants). Phenotypes of the revertant colonies were checked by replica plating.

Substrate respiration in whole cells. Oxygen consumption was measured polarographically with an oxygen monitor (model D.W2/2; Hansatech Instrument Ltd.) at 25°C. Cells grown in liquid MS medium were harvested by centrifugation, washed, and suspended in the desired volume of 100 mM KPO_4 buffer (pH 7.4) for thiosulfate- and tetrathionate-dependent oxygen uptake assay and in 100 mM Tris-HCl buffer (pH 8.0) for sulfite-dependent respiration. The thiosulfate assay mixture contained 150 μmol of KPO_4 buffer (pH 7.4), 10 μmol of $Na_2S_2O_3$, whole cells, and water to a final volume of 1.5 ml. A similar assay mixture procedure was employed to measure tetrathionate-dependent oxygen uptake except that 4 μmol of tetrathionate was used instead of thiosulfate. The sulfite-dependent oxygen uptake assay contained 150 μmol of Tris-HCl buffer (pH 8.0), 5 μmol of Na_2SO_3, whole cells, and water to a final volume of 1.5 ml. Whole cells equivalent to 525 μg of protein were used in all cases.

Oxygen consumption was calculated on the basis of 253 nmol of O_2 per ml in air-saturated water at 25°C and expressed as nanomoles of O_2 consumed per minute per milligram of protein.

Substrate induction. Growth, thiosulfate utilization, and sulfate production by the wild-type strain (BI-50) and thiosulfate-negative (Thi^-) mutants were tested by growing cultures in liquid MS medium. For measurements of cytochromes, cells were grown initially in the GYM medium supplemented with sodium succinate (5 g/liter) for 20 h. The cultures (with optical densities at 660 nm of 0.5 to 0.6) were divided into equal parts and harvested aseptically by centrifugation. The resultant pellets were resuspended in sterile water and reinoculated into equal volumes of freshly prepared medium, one part of which was supplemented with sodium thiosulfate (5 g/liter) for induction. The incubation was carried out at 30°C for 5 h.

Preparation of cell extracts. Cells grown in MS medium with shaking to an optical density at 660 nm of 1.0 to 1.2 were harvested by centrifugation at 4,000 × *g* for 10 min. The cells were washed and suspended in 100 mM KPO_4 buffer (pH 7.4) for thiosulfate oxidase, in 100 mM Tris-HCl buffer (pH 8.0) for sulfite oxidase, and in rhodanese and 100 mM KPO_4 (pH 7.0) containing 1 mM $MgCl_2$ for the assay of *c*-type cytochromes. Cells were disrupted by sonication in a Braunsonic 1510 sonicator (for 6 × 30 s at 150 W) in ice. The cell debris was removed by centrifugation at 18,000 × *g* for 30 min at 4°C, and the clear supernatant fluid was used for the assay of enzymes.

The crude extracts were further separated into soluble and membrane fractions by centrifugation at 105,000 × *g* for 60 min (Sorvall OTD 65), and the patterns of resuspended cytochrome pellets were examined.

Assay methods. Thiosulfate oxidase was assayed by the method of Trudinger (46). The assay mixture contained 250 μmol of KPO_4 buffer (pH 7.4), 20 μmol of $Na_2S_2O_3$, 3 μmol of $K_3Fe(CN)_6$, crude enzyme preparation, and water to a final volume of 2.5 ml.

Sulfite oxidase was assayed by the method of Charles and Suzuki (6). The assay mixture contained 300 μmol of Tris-HCl (pH 8.0), 10 μmol of Na_2SO_3 in 5 mM EDTA, 3 μmol of $K_3Fe(CN)_6$, crude enzyme preparation, and water to a total volume of 3.0 ml.

The reaction was started by the addition of substrate, and appropriate corrections were made for substrate and enzyme blanks. Enzyme activities were determined spectrophotometrically at room temperature by measuring the rates of reduction of ferricyanide at 420 nm with an extinction coefficient of 0.9×10^3 M^{-1} cm^{-1} (27) and were expressed as nanomoles of ferricyanide reduced per minute per milligram of protein.

Rhodanese was assayed by the method of Tabita et al. (42), and the enzyme activity was expressed as nanomoles of thiocyanate formed per minute per milligram of protein.

Measurements of cytochromes. All difference spectra were monitored with a Shimadzu UV-240 spectrophotometer with 1-ml cuvettes with a 1-cm path at 25°C. For determining difference spectra, cytochromes of the test samples were reduced with a few crystals of sodium dithionite and the spectra were measured against a reference cuvette. Difference spectra were recorded 2 min after addition of dithionite. Amounts of cytochrome *c* present were quantified from the reduced minus air-oxidized difference spectra by using the extinction coefficient of cytochrome *c*, $\varepsilon = 19$ mM^{-1} cm^{-1} at 552 and 550 nm, respectively, as described by Sweet and Peterson (41).

Analysis of particles deposited on the cell surface of mutants DNK-11 and DNK-12. Cells grown on MS agar plates were scraped into distilled water and then rigorously stirred. Suspended particles were pelleted by centrifugation at 500 × *g* for 10 min in order to avoid the sedimentation of cells. The pellet was dissolved in chloroform and dried over anhydrous sodium sulfate. A yellow solid was obtained after removal of the solvent under reduced pressure at room temperature. The infrared spectrum and melting point were determined for the yellow material with authentic elemental sulfur as the standard.

Detection of Tn*5* DNA homology. Genomic DNA was isolated by following the method of Marmur (25). DNA digested with restriction enzyme *Eco*RI was electrophoretically separated on 0.8% agarose gel. The DNA was transferred to a nitrocellulose filter (Schleicher & Schuell, Inc., Dassel, Germany) as described by Sambrook et al. (32). The probe DNA consisted of Tn*5-mob* (~5.4 kb) labelled by nick translation with [α-^{32}P]dATP. The hybridization was performed by following the method of Southern (38). Prehybridization was performed at 37°C for 4 h. After the addition of labelled probe DNA, the reaction was continued for 16 h at 37°C. Prior to autoradiography, the blot was washed three times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (for 20 min each time) and 0.1% sodium dodecyl sulfate (SDS) (for 20 min each time), after which an identical treatment with 0.2× SSC and 0.1% SDS was performed at room temperature.

Analytical techniques. Protein was estimated as described by Lowry et al. (20), with bovine serum albumin as the standard. Concentrations of thiosulfate in the culture filtrates were measured according to Kelly et al. (15). Sulfate was measured by the method of Gleen and Quastel (12).

RESULTS

Tn*5* transfer frequency and isolation of mutants. Tn*5*-containing plasmid pSUP5011 was transferred from *E. coli* S17.1 to BI-50, a spontaneous rifampin-resistant derivative of *B. thiooxidans* BI-42. Transconjugants occurred at a frequency of 2.2×10^{-4} per donor. Spontaneous neomycin (70 μg/ml)-resistant colonies of *B. thiooxidans* BI-42 did not occur ($<10^{-10}$).

A total of 10,000 neomycin-resistant transconjugants were scored by replica plating to screen for colonies unable to oxidize thiosulfate and for auxotrophy. Colonies defective in thiosulfate oxidation appeared at a very low frequency (2.5×10^{-9}) and were identified by their inability to produce acid by the oxidation of thiosulfate in MS growth medium. Among the colonies screened, only four transconjugants were identified as defective for thiosulfate oxidation (Table 1). These four transconjugants were categorized into three different phenotypic classes. Class I mutant DNK-10 was unable to utilize thiosulfate and grew slowly on MS agar medium. Class II mutants DNK-11 and DNK-12 grew poorly by thiosulfate oxidation, and sulfur was deposited on the surface of colonies (Fig. 1). Class III

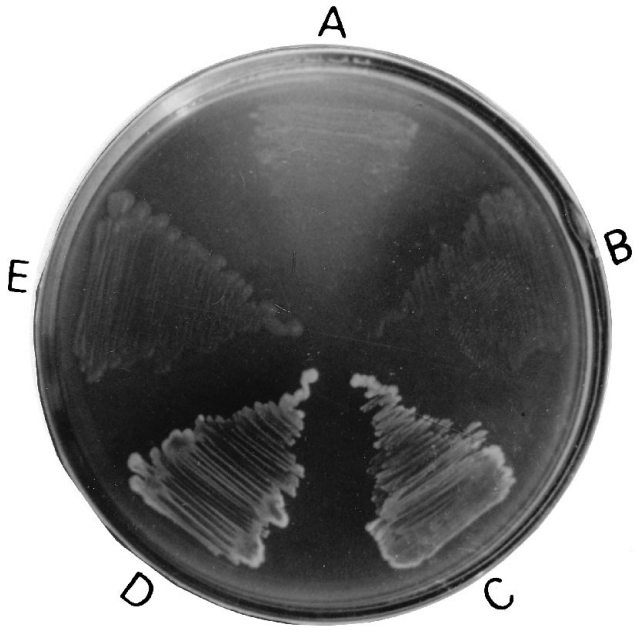

FIG. 1. Growth of wild-type and thiosulfate-negative mutants on GYM medium containing thiosulfate plus succinate. (A) Wild type; (B) mutant DNK-10; (C) mutant DNK-11; (D) mutant DNK-12; (E) mutant DNK-13.

mutant DNK-13 was unable to utilize thiosulfate but could grow well under heterotrophic growth conditions.

The auxotrophic mutants occurred at a frequency of 1%. Some auxotrophic mutants were characterized with respect to their specific nutritional requirements and could be assigned to the following phenotypes: Cys^-, Lys^-, Pro^-, His^-, and Ade^- (Table 1). All spontaneous prototrophic revertants of the auxotrophs and the revertants of thiosulfate-negative mutants were neomycin sensitive. Frequencies of reversion ranged from 10^{-8} to less then 10^{-10} (data not shown).

Growth and thiosulfate oxidation. The effects of thiosulfate on bacterial growth, consumption, and sulfate production in wild-type strain BI-50 and in mutants deficient in thiosulfate oxidation are presented in Table 2. The wild-type strain exhibited better growth when grown in MS medium than when grown under heterotrophic conditions according to results recorded after 48 h of incubation. The thiosulfate added was quantitatively converted to sulfate. However, mutants DNK-10 and DNK-13 showed neither enhanced growth nor the consumption of thiosulfate. On the other hand, mutants DNK-11 and DNK-12 showed low amounts of thiosulfate consumption (Table 2). Furthermore, the oxygen uptake study revealed that oxidation of reduced-sulfur compounds tested for the mutants DNK-11 and DNK-12 was at very low levels, though significant compared with that of the wild-type strain. However, such activities were not observed in the mutants DNK-10 and DNK-13 (Table 3).

TABLE 2. Growth, thiosulfate consumption, and sulfate production of the wild-type strain and thiosulfate-negative mutants

Strain	Growth (OD_{660})[a]		Amt (mg of atoms of S/1) of:	
	Without thiosulfate	With thiosulfate	Thiosulfate consumed	Sulfate released
Wild type				
BI-50	0.56	1.14	40.3	38.6
Mutants				
DNK-10	0.43	0.40	<0.1	<0.1
DNK-11	0.54	0.69	1.1	0.38
DNK-12	0.58	0.67	0.5	0.19
DNK-13	0.56	0.53	<0.1	<0.1

[a] Cells were grown in GYM medium with thiosulfate plus succinate for 48 h, and then amounts of bacterial growth, thiosulfate, and sulfate in the medium were determined. OD_{660}, optical density at 660 nm.

TABLE 3. Oxidation of reduced sulfur compounds by cells of wild-type and mutant strains

Strain	Oxygen uptake[a] (μmol of O_2/min/g of protein) in presence of:		
	Thiosulfate	Tetrathionate	Sulfite
Wild type			
BI-50	95	58	275
Mutants			
DNK-10	<1	<1	<1
DNK-11	27	10	68
DNK-12	22	7	74
DNK-13	<1	<1	<1

[a] Cells were grown in GYM medium with thiosulfate plus succinate, and thiosulfate-, tetrathionate-, and sulfite-dependent oxygen consumption rates were determined with a biological oxygen monitor.

Enzymatic analysis of the thiosulfate-deficient mutants. Key enzymes, such as thiosulfate oxidase, sulfite oxidase, and rhodanese, and cytochrome patterns were studied in the thiosulfate-defective mutants (Table 4).

The class I mutants represented by the strain DNK-10 were defective in thiosulfate oxidation. Significantly, sulfite oxidase activity was at levels similar to that of the wild-type BI-50 in cell extract of DNK-10 grown under the mixed-substrate condition. Both thiosulfate oxidase and sulfite oxidase are inducible in the wild-type strain BI-50. Enzyme levels in DNK-10

TABLE 4. Enzyme activities and specific cytochrome $c_{550\text{-}552}$ contents of the wild type and mutants deficient in thiosulfate oxidation

Strain[a]	Specific activity of enzyme:			Cytochrome content[d]	
	Thiosulfate oxidase[b]	Sulfite oxidase[b]	Rhodanese[c]	Soluble fraction	Particulate fraction
BI-50	55	396	132	198	461
DNK-10	<0.1	433	21	<0.1	<0.1
DNK-11	16	102	119	95	277
DNK-12	11	114	98	74	259
DNK-13	<0.1	<0.1	66	33	233
BI-50	<0.1	<0.1	52	55	197
DNK-10	<0.1	424	20	<0.1	<0.1
DNK-11	<0.1	<0.1	44	63	207
DNK-12	<0.1	<0.1	43	47	198
DNK-13	<0.1	<0.1	56	34	222

[a] Cells were grown in GYM medium with thiosulfate plus succinate (top five lines) or with succinate alone (bottom five lines).

[b] Specific activities are given as nanomoles of ferricyanide reduced per minute per milligram of protein.

[c] Enzyme activities are expressed in nanomoles of SCN^- formed per minute per milligram of protein.

[d] Expressed as nanomoles of cytochrome *c* per milligram of protein.

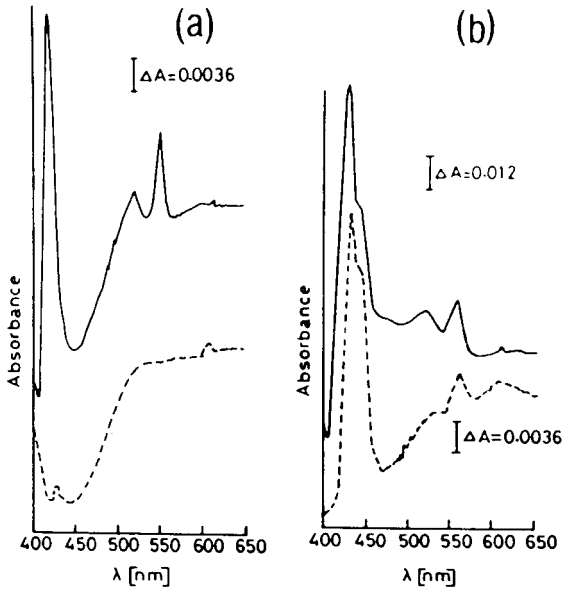

FIG. 2. Cytochrome spectra of soluble (a) and particulate (b) fractions prepared from wild-type (BI-50) and mutant (DNK-10) cells grown on thiosulfate plus succinate. Dithionite-reduced minus air-oxidized difference spectra are shown. Lines: ——, wild type cells; – – –, mutant cells.

grown heterotrophically were as high as those in induced wild-type cells. Rhodanese activity was present at reduced levels compared with that of the wild-type strain.

Cytochrome *c* was virtually absent in DNK-10, as shown by dithionite-reduced minus air-oxidized difference spectra. In comparison with the wild-type strain, the alpha absorption maxima, characteristic of cytochrome *c* (550 to 552 nm), were not detectable, whereas the presence of cytochrome aa_3 was observed, as indicated by the characteristic A_{606} (Fig. 2).

Class II mutants DNK-11 and DNK-12 utilized thiosulfate at significant but reduced levels (Table 2). Thiosulfate oxidase and sulfite oxidase were detected at only low levels of activity in both mutants compared with those of the wild type. These mutants showed rhodanese activity at levels similar to that of the wild type, but low levels of cytochrome *c* were synthesized.

Class III mutant DNK-13 failed to oxidize thiosulfate. No thiosulfate oxidase and sulfite oxidase activities were observed. Rhodanese activities were found at a level similar to that obtained with the wild-type strain when grown under heterotrophic growth conditions.

Analysis of the cell surface-deposited particles. The particles deposited on the cell surfaces of mutants DNK-11 and DNK-12 appeared to contain sulfur. Thus, infrared spectra and melting points were determined. Infrared analysis showed superimposed spectra with authentic elemental sulfur (data not shown). The melting points of the particles were found to be 114 to 116°C, which are similar to that of standard elemental sulfur.

Physical evidence for Tn*5* insertion. To examine the stability and randomness of transposon insertion, as well as to locate the transposon on the genomic DNA, the four mutants deficient in thiosulfate oxidation were analyzed by Southern hybridization. Hybridization results indicated that signals were obtained for all the mutants, and the Tn*5-mob* was identified on 15-, 9.0-, and 8.0-kb *Eco*RI fragments (Fig. 3).

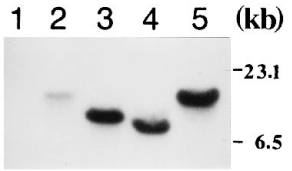

FIG. 3. Southern hybridization of chromosomal DNAs from four thiosulfate-negative mutants and the wild-type BI-50. Chromosomal DNAs were completely digested with *Eco*RI, separated by 0.8% agarose gel electrophoresis, and hybridized with the ^{32}P-labelled 5.4-kb Tn*5-mob* fragment isolated from pSUP5011 after *Hin*dIII digestion. Shown is an autoradiogram of the Southern blot. *Hin*dIII-digested lambda phage DNA size markers are shown on the right. Lanes: 1, strain BI-50; 2, DNK-10; 3, DNK-11; 4, DNK-12; 5, DNK-13.

DISCUSSION

Transposon mutagenesis techniques have been applied to many organisms, such as *E. coli* (2, 34), *Rhizobium* spp. (3), *Salmonella typhimurium* (4), and *Klebsiella pneumoniae* (26), but application of this technique to colorless sulfur bacteria has not progressed sufficiently. Several factors could be attributed to this lack of progress (9, 30). Transposon insertion mutagenesis in true sulfur bacteria, such as *Thiobacillus* spp., *Thiomicrospira* spp., *Beggiatoa* spp., and *Thiothrix* spp., has lagged (1, 16, 37), because suitable delivery vectors for the transposon have not been available. Initial studies were made with *Thiobacillus novellus* and *Thiobacillus versutus* (9). Furthermore, these species are spontaneously resistant to high concentrations of several antibiotics so that discrimination between the transposon-induced antibiotic marker and spontaneous resistance becomes difficult (9). Ignoring such difficulties inherent in obligately or facultatively chemolithoautotrophic sulfur bacteria, to explore the possibility of understanding the sulfur metabolism, we generated Tn*5* insertion mutants of a rifampin-resistant strain (BI-50) obtained spontaneously from a newly isolated chemolithoheterotrophic sulfur bacterium, *B. thiooxidans* BI-42. Mutants which were identified as (i) unable to oxidize thiosulfate and (ii) auxotrophic by their nutritional requirements were isolated.

The high frequency of auxotrophic mutants among the neomycin-resistant transconjugants of strain BI-50 suggests that the mutations are the result of Tn*5* insertion. The frequency of auxotrophic mutants produced is similar to those observed for other bacteria (3, 34) and indicates that insertion of Tn*5* was random, resulting in a range of altered bacterial phenotypes (Thi^-, Cys^-, Lys^-, Pro^-, His^-, or Ade^-). Mutants reverting to prototrophy showed the loss of the neomycin-resistant phenotype, which is likely attributed to the precise excision of Tn*5* as found in *Pseudomonas facilis* (50).

Mutants impaired in thiosulfate oxidation were found to belong to three different classes, which gave us some useful information on the metabolism of thiosulfate of this organism.

The class I mutant DNK-10 does not utilize thiosulfate and may have a defect in the regulation of thiosulfate oxidase or sulfite oxidase. Alternatively, a genetic change may have occurred in the biosynthesis of common components involved in the electron transport system. The role of cytochrome $c_{550\text{-}552}$ is probably essential, as the mutant with defects in this cytochrome was unable to utilize thiosulfate. Significantly, in this mutant detectable cytochrome $c_{550\text{-}552}$ was absent (Fig. 2). This result provides evidence that electrons generated by thiosulfate oxidation were transferred to cytochrome *c* and that there is no oxygenase involved in the conversion of thiosulfate to sulfate. So, the biochemical lesions leading to the loss of $c_{550\text{-}552}$-type cytochrome resulted in a negative effect for the induction of

thiosulfate in this mutant. The above observation for mutant DNK-10 appears to follow the same pattern reported from *Thiobacillus versutus* by Lu and Kelly (23). Remarkably, ferricyanide-coupled sulfite oxidase activity found in vitro is in contrast with the result obtained from the respirometric study in which neither sulfite- nor thiosulfate-dependent oxygen uptake was recorded for this mutant (Table 3). The possible reason for ferricyanide-coupled sulfite oxidation in vitro may be the involvement of free radicals, i.e., superoxide anion (10, 24) in sulfite oxidation, or other mechanisms which remain unclear.

The class II mutants DNK-11 and DNK-12 expressed all the enzymes studied here that are involved in thiosulfate metabolism, although at reduced levels compared with those of the wild-type strain. The possible reasons for reduced thiosulfate oxidation are (i) sulfite formed by enzymic cleavage of thiosulfate may accumulate in the cells, thus inhibiting the thiosulfate-oxidizing enzyme, and (ii) sulfane-sulfur produced from thiosulfate may oxidize to sulfite at a rate which is lower than the rate of thiosulfate cleavage, resulting in the excretion of the sulfur moiety outside of the cells (Fig. 1). Suzuki (40) observed a similar phenomenon in *Thiobacillus thioparus.*

Furthermore, the reduced rates of thiosulfate oxidation in mutants DNK-11 and DNK-12 reflect the synthesis of low amounts of cytochrome $c_{550\text{-}552}$ compared with that of the wild-type BI-50 strain (Table 4). Nevertheless, it seems reasonable to conclude that cytochrome $c_{550\text{-}552}$ is apparently part of a macromolecular complex exhibiting thiosulfate-oxidizing activity in this bacterium. Similar findings have been reported for *Thiobacillus* A_2 from studies in which the cytochromes of lithotrophic and organotrophic cultures differ mainly because the lithotrophic cultures contain much larger amounts of cytochrome *c* (21).

The class III mutant DNK-13 lacked both ferricyanide-coupled thiosulfate oxidase and sulfite oxidase activities. This is evidenced by the results obtained from the oxygen-uptake study (Table 3). The possibility remains that this mutant has a lesion in a regulatory gene whose product is necessary for the expression of enzymes involved in thiosulfate oxidation.

The constitutive expression of rhodanese in all the mutants and in the wild-type strain suggests that this enzyme is not involved in thiosulfate metabolism, although the induction of thiosulfate somehow causes a moderate increase in activity levels (Table 4). This is in agreement with the observation of Lu and Kelly (22) on *Thiobacillus versutus*. Significantly, low levels of rhodanese activity in heterotrophically grown cells of mutant DNK-10 may be due to the absence of cytochrome *c*, though other factors may also be involved.

A critical parameter in evaluating the usefulness of a system to produce transposon mutagenesis is the occurrence of single, random insertions. It is possible that the production of mutants defective in thiosulfate metabolism was due to transposon insertion at a unique site on the chromosome in each of these mutants. However, this was not the case. For the four thiosulfate-negative mutants analyzed by Southern hybridization, DNAs from two of these Thi^- mutants (DNK-10 and DNK-13) showed similar hybridization patterns while DNAs from the other mutants did not, suggesting that their transposons were inserted very close to each other but not at identical sites on the chromosome (Fig. 3).

As the transposable elements in transposon-induced mutants are physically linked to the mutated gene, it is obvious that, apart from their usefulness in physiological studies, the mutants obtained may be used as tools in the isolation of genes involved in the thiosulfate metabolism of such chemolithoheterotrophic bacteria as *B. thiooxidans.*

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