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Tn5-Induced Mutations Affecting Sulfur-Oxidizing Ability (Sox) of *Thiosphaera pantotropha*

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Mutants of *Thiosphaera pantotropha* defective in chemolithoautotrophic growth were obtained by transpositional mutagenesis with Tn5 coding for kanamycin resistance. The suicide vehicle for introducing Tn5 to *T. pantotropha* was pSUP5011 harbored by *Escherichia coli*. Kanamycin-resistant isolates were screened for the inability to grow with reduced sulfur compounds (Sox⁻). Four classes of Sox⁻ mutants were obtained. Three were of different pleiotropic phenotypes: (i) unable to grow with formate, nitrate, and xanthine; (this class strongly suggested the involvement of a molybdenum cofactor in inorganic sulfur-oxidizing ability); (ii) no growth with hydrogen; (iii) slight growth with hydrogen and formate. Two plasmids, pHG41 (about 450 kilobase pairs) and pHG42 (110 kilobases), were identified in lysates of *T. pantotropha*. In one Sox⁻ mutant pHG41 could not be detected. Revertant analysis suggested that pHG41 and pHG42 were not involved in the Sox character.

Thiobacillus intermedius, *Thiobacillus perometabolis*, *Thiobacillus novellus*, and *Thiobacillus versutus* are known to oxidize and grow with reduced inorganic sulfur compounds. Their metabolism is facultatively chemolithotrophic (20). However, oxidation of reduced sulfur compounds is not restricted to the thiobacilli but is also found with several species of hydrogen-oxidizing bacteria. These are *Aquaspirillum autotrophicum*, *Pseudomonas palleronii*, and several species of the genus *Xanthobacter*. Species able to grow with thiosulfate autotrophically are *Paracoccus denitrificans* (8) and *Pseudomonas pseudoflava* GA3 (this study). Recently a new facultative chemolithoautotrophic bacterium able to grow with thiosulfate or sulfide, *Thiosphaera pantotropha*, has been isolated and described to be distinct from *Thiobacillus versutus* and *Paracoccus denitrificans* (21).

In several hydrogen-oxidizing bacteria the lithoautotrophic character (Hox) is encoded by a megaplasmid (6). Many hydrogen- or sulfur-oxidizing bacteria harbor plasmids (5, 9) which were considered to be cryptic in the thiobacilli (5). Among the thiobacilli *Thiobacillus novellus* and *Thiobacillus versutus* in particular have received intense physiological characterization. The components of the thiosulfate-oxidizing enzyme system have been purified, characterized, and reconstituted from *Thiobacillus versutus* (14, 17). However, the mechanism of thiosulfate cleavage is still unknown (14).

Transpositional mutagenesis has been applied to a number of microorganisms and also to facultatively chemolithoautotrophic bacteria like the hydrogen-oxidizing bacteria *Alcaligenes eutrophus* (25), *Pseudomonas facilis* (27a), as well as to *Thiobacillus novellus* and *Thiobacillus versutus* (4).

The unknown mechanisms of thiosulfate cleavage and the fact that the ability to oxidize thiosulfate is not equivalent to the ability to grow with this sulfur compound as energy source led us to analyze the components involved in the ability to grow with reduced sulfur compounds (Sox). To isolate mutants affected in the Sox character we applied transpositional mutagenesis to *Thiosphaera pantotropha*. Unlike the classical thiobacilli, this strain exhibits alternative lithoautotrophic abilities and favorable growth characteristics. In this report we describe different mutants impaired in the sulfur-oxidizing character.

MATERIALS AND METHODS

Bacterial strains. Strains used in this study are listed in Table 1. Strains TPfd and TPln of *Thiosphaera pantotropha* were from H. Hippe (DSM, Göttingen, Federal Republic of Germany), originating from the Delft Culture Collection. *Escherichia coli* S17-1, constructed by A. Pühler (University of Bielefeld, Bielefeld, Federal Republic of Germany), harbored a vector plasmid (pSUP5011) with a P-type-specific recognition site for mobilization (24) and was used for transposon mutagenesis.

Media and growth conditions. Cells were grown in batch culture at 30°C without pH control. The mineral salts medium of Schlegel was modified as described previously (7). Precultures were grown in 0.4% (wt/vol) fructose minimal medium for 24 h on a rotary shaker (G-76; New Brunswick Scientific Co., Inc., Edison, N.J.) at 220 rpm. Cells that were washed twice with 33 mM sodium phosphate buffer (pH 7.1) were used as inoculum. For lithoautotrophic growth with reduced sulfur compounds, 300-ml sidearm flasks with screw caps contained 30 ml of mineral medium (pH 7.7) supplemented with 20 mM sodium thiosulfate or 10 mM sodium sulfide. Sodium bicarbonate (12 mM) served as carbon source. The final pH was 8.0. Flasks were tightly closed to prevent gas exchange. Lithoautotrophic growth with molecular hydrogen was performed in 30 ml of mineral medium in 500-ml sidearm flasks with a screw cap and an attached valve for gas renewal. The gas mixture contained 80% H_2, 10% O_2, and 10% CO_2. Seed cultures were grown in 0.1% (wt/vol) sodium pyruvate medium with the hydrogen-containing atmosphere. With sodium formate (0.2% [wt/vol]) as carbon source, the initial pH was adjusted to 6.8. Utilization of haloaromatic compounds was examined on solid media at a concentration of 2 mM. Bacteria were grown anaerobically in nutrient broth with potassium nitrate (0.3% [wt/vol]) or sodium nitrite (0.1% [wt/vol]) in screw-cap tubes. Aerobic

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TABLE 1. Bacterial strains used in this study

Species and strain	Relevant phenotype[a]	Reference or source
Thiosphaera pantotropha		
GB17	Sox^+ Hox^+ Fox^+ Arg^- Kan^s	L. Robertson, Delft
TPfd	Sox^- Hox^- Fox^- Arg^+ Kan^s	H. Hippe, DSM
TPln	Sox^- Hox^- Fox^- Arg^+ Kan^s	H. Hippe, DSM
TP19	Sox^- Kan^r	This study
TP37	Sox^- Kan^r	This study
TP57	Sox^- Kan^r	pHG41-cured mutant; this study
TP43	Sox^- Hox^- $Nitd^-$ Kan^r	This study
TP62	Sox^- Hox^- $Nitd^-$ Kan^r	This study
TP61	Sox^- Fox^- Nar^- Xan^- Kan^r	This study
TP67	Sox^- Fox^- Nar^- Xan^- Kan^r	This study
TP71	Sox^L Hox^L Fox^L Kan^r	This study
Paracoccus denitrificans 381	Sox^+ Hox^+	Wild type, DSM 65
Thiobacillus versutus	Sox^+ Hox^-	Wild type, DSM 582
Pesudomonas pseudoflava GA3	Sox^+ Hox^+	Wild type, DSM 1034
Escherichia coli S17-1(pSUP5011)[b]	Kan^r Cm^r Pro^-	A. Pühler, University of Bielefeld

[a] Sox, Hox, and Fox = ability to grow with thiosulfate, H_2, and formate as energy sources, respectively. Xan and Arg = ability to grow with xanthine or with arginin as carbon source, respectively. Kan^s and Kan^r = sensitivity and resistance, respectively, to 300 μg of kanamycin per ml. $Nitd^-$ = inability of anaerobic nitrite respiration, Nar = ability to use nitrate as electron acceptor.

[b] Resistance to 50 μg of kanamycin per ml.

assimilation of nitrate or nitrite was examined in fructose mineral salts medium; with nitrite the initial pH was 7.5. Growth with xanthine (0.1%) was examined on solid minimal medium. Agar (1.5% [wt/vol]) was used for solidification of media.

For cultivation under mixotrophic growth conditions, cells were pregrown on fructose minimal medium for 24 h, washed once, and inoculated to mineral medium (pH 7.2) supplemented with 0.1% (wt/vol) sodium pyruvate–0.5% (wt/vol) $Na_2S_2O_3 \cdot 5H_2O$ and agitated for 17 to 20 h.

Isolation of Tn*5* insertional mutants. *Escherichia coli* S17-1 was grown for 9 h; and *Thiosphaera pantotropha*, *Paracoccus denitrificans*, or *Pseudomonas pseudoflava* GA3 was grown for 15 h in nutrient broth. The donor *Escherichia coli* and the recipients were given in a 1:1 ratio on a nitrocellulose filter (diameter, 30 mm) and incubated for 20 h on nutrient agar. Cells were washed from the filter in 3 ml of 22 mM sodium phosphate buffer (pH 7.1). Cells (0.1 ml) were plated on 0.4% (wt/vol) sodium succinate agar and incubated for 2 days. The cells were washed off with 1 to 2 ml of buffer, and 0.1 ml of the dilutions (up to 10^{-4}) was plated on succinate agar plus 300 μg of kanamycin (Kan) per ml. Putative transconjugants appeared as Kan-resistant single colonies after 2 days. These were picked onto master plates of the same composition and used for postmating selection.

Identification of Sox^- mutants. Growth on thiosulfate mineral agar plates was extremely poor. When thiosulfate also was omitted, some growth occurred from impurities in the agar. Therefore, the colony size did not allow any distinction of the inability to grow with thiosulfate. To identify mutants with the Sox character, cell material from the master plates was transferred to microtiter wells filled with 0.2 ml of thiosulfate medium described above containing phenol red (10 μg/ml). Sox^- mutants exhibited only an insignificant color change after 1 to 2 days of incubation. They were purified from the master plates and stored on fructose-Kan slants. Identification of formate-oxidizing ability (Fox) was done by the same principle, using formate mineral medium with phenol red.

Enzyme assays. Oxidation rates of reduced sulfur compounds were determined by standard manometric techniques following oxygen uptake. Cells grown in induction medium (see above) were washed twice in 50 mM Tris hydrochloride buffer (pH 8.0) at 4°C and concentrated to 6 mg of cell (dry weight) per ml. The Warburg flasks contained, in a final volume of 3 ml, 0.1 ml of 1 M Tris hydrochloride (pH 8.0) and whole cells equivalent to 0.67 mg of protein. The side arm contained, in 0.4 ml, 10 μmol of reduced sulfur compounds to start the reaction. The center well contained 0.2 ml of 10% (wt/vol) KOH. Oxygen uptake rates were corrected for endogenous respiration. The whole cell assays for the determination of the activities of ribulosebisphosphate carboxylase (RuBPCase; EC 2.7.1.19), the NAD-linked formate dehydrogenase, and the membrane-bound hydrogenase of *Thiosphaera pantotropha* were identical to those described for cell extracts of *Alcaligenes eutrophus* (7). Assays for RuBPCase and for formate dehydrogenase contained, in addition, 20 μl of 0.25% (wt/vol) cetyltrimethylammonium bromide. One unit of enzyme activity is defined as 1 μmol of substrate converted per min at 30°C.

Plasmid analysis. Plasmid DNA profiles were obtained by the procedure of Casse et al. (2), involving alkaline lysis and phenol-chloroform extraction. Direct lysis in agarose gel pockets was done by the method of Rosenberg et al. (23) and by the method of Kado and Liu (13) with 0.7% (wt/vol) agarose vertical slab gels. For determination of plasmid sizes, the following reference plasmids were used: pMOL30 and pMOL28 of *Alcaligenes eutrophus* CH34 (239 and 163 kilobase pairs [kb], respectively) *Paracoccus denitrificans* 381 (76 kb) and RP4 (54 kb) (9). The semilogarithmic plot of plasmid size versus migration was linear, and the middle-sized plasmids were within the limits of linearity. Gels were

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TABLE 2. Growth of *Thiosphaera pantotropha* and Sox$^-$ mutants under various growth conditions

Group	Strain	Growth[a] (Klett units) of:					
		Thiosulfate[b]	Formate[b]	Fructose-nitrate-aerobic[c]	NB-nitrate, anaerobic[c]	NB-nitrite, anaerobic[c]	Fructose-nitrite, aerobic[c]
	GB17	48	75	449	319	109	220
I	TP19	3	63	ND[d]	ND	ND	ND
	TP37	6	63	426	293	104	217
	TP57	6	64	440	297	107	222
II	TP43	5	45	455	56	4	212
	TP62	6	56	456	48	4	219
III	TP61	4	3	13	13	103	225
	TP67	2	0	16	26	120	225
IV	TP71	37	34	426	264	106	ND

[a] Media and growth conditions are given in the text.
[b] Readings after 48 h.
[c] Readings after 24 h of growth. NB, Nutrient broth.
[d] ND, Not determined.

stained for 45 min in aqueous ethidium bromide (0.5 μg/ml) and destained for 1 h before photography with a UV transilluminator with a red filter.

Chemicals. Nutrient broth and Bacto agar were purchased from Difco Laboratories, Detroit, Mich. Kanamycin sulfate and agarose type V were from Sigma Chemical Co., Munich, Federal Republic of Germany. Antibiotic sensitivity disks were obtain from Oxoid Ltd., London, United Kingdom. NAD^+ was from Boehringer, Mannheim, Federal Republic of Germany. All other chemicals were purchased from E. Merck AG, Darmstadt, Federal Republic of Germany.

RESULTS

Autotrophic growth. To characterize lithoautotrophic abilities of *Thiosphaera pantotropha*, simple growth conditions were developed for mutant analysis (Table 2). Autotrophic growth with thiosulfate as energy source (doubling time, 4 h) ceased with the acidification of the medium and reached about 150 mg of cell (dry weight) per liter. For growth with hydrogen (doubling time, 7 h), cells were pregrown under mixotrophic conditions with sodium pyruvate and an H_2-containing atmosphere. Heterotrophically pregrown cells exhibited a 4- to 5-day lag phase. *Thiosphaera pantotropha* was reported not to grow with formate as the sole energy and carbon source (21). To discriminate lithotrophic abilities from autotrophic carbon dioxide fixation (Cfx), growth of *Thiosphaera pantotropha* on formate was reexamined. *Thiosphaera pantotropha* grew well with formate as carbon and energy source (doubling time, 2.5 h) when the initial pH was 6.8 (Fig. 1, lane B). Furthermore, growth on formate led to the induction of the key enzyme of autotrophic CO_2 fixation, RuBPCase (Table 3). This suggests that the formate carbon is assimilated via the Calvin cycle, as in other facultatively chemolithotrophic bacteria.

Tn*5* transfer frequencies to thiobacilli. Matings to transfer transposon Tn*5* from *Escherichia coli* to facultatively autotrophic thiobacilli were conducted to select a suitable recipient for transposon mutagenesis. All strains used in this study exhibited excellent characteristics with respect to lithotrophic growth with thiosulfate, hydrogen, or formate (8; this study). Kan^r transconjugants resulting from matings with *Escherichia coli* S17-1 and *Pseudomonas pseudoflava* GA3 occurred at a frequency of about 10^{-8} per recipient. With *Paracoccus denitrificans* 381 and *Thiobacillus versutus* the frequency was 1.2×10^{-8}. The highest frequency of 1.5×10^{-6} transconjugants per recipient cell was obtained with *Thiosphaera pantotropha*. Therefore, this strain was selected for further investigation. No spontaneous Kan resistance ($<10^{-9}$) was observed in the recipients.

Mutant isolation and characterization. Two independent

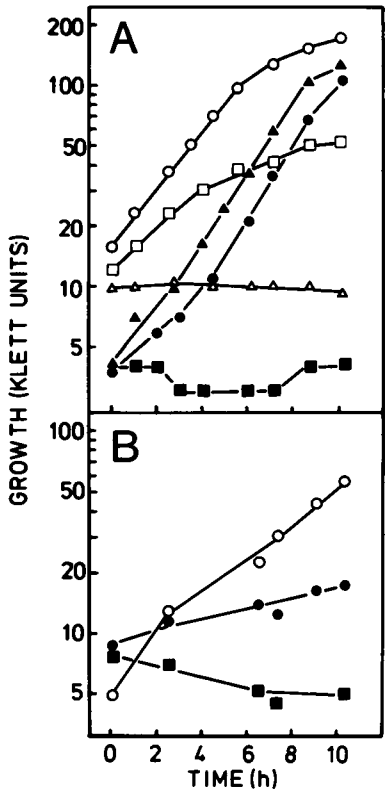

FIG. 1. Growth of *Thiosphaera pantotropha* GB17 and Sox$^-$ mutants. Cells were pregrown on identical media. (A) For anaerobic growth of GB17 (○, ●), TP43 (□, ■), and TP67 (△, ▲) nutrient broth was supplemented with nitrate (open symbols) or nitrite (closed symbols). (B) Growth on formate of GB17 (○), TP67 (■), and TP71 (●). One hundred Klett units are equivalent to 200 μg of cell (dry weight) per ml.

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TABLE 3. Specific activities of key enzymes of lithoautotrophic metabolism of *Thiosphaera pantotropha* after growth on different induction media

Strain	Sp act (U/mg of protein) under the following growth conditions:						
	Pyruvate-H_2		Pyruvate-formate		Pyruvate-thiosulfate		
	Hydrogenase	RuBPCase	Formate dehydrogenase	RuBPCase	Thiosulfate oxidation	Sulfide oxidation	Rhodanese
GB17	1.367	0.091	0.428	0.051	0.451	0.397	0.038
TP37	1.572	0.071	0.326	0.068	0	0.006	0.043
TP43	0.171	0.033	0.303	0.022	0.164	0.191	0.048
TP57	1.373	0.098	0.286	0.056	0	0.003	0.037
TP67	1.173	0.109	0	0	0.008	0.025	0.039
TP71	0.976	0.078	0.280	0.081	0.345	0.303	0.034

crosses were performed with *Escherichia coli* S17-1 and *Thiosphaera pantotropha* GB17. Kan^r transconjugants were isolated on succinate-kanamycin mineral agar, transferred to the same agar, and examined for the ability to oxidize thiosulfate on microtiter plates. Of 5,440 Kan^r isolates, 52 proved to be defective in oxidation of thiosulfate to sulfuric acid. These isolates were unable to grow with thiosulfate as energy source in liquid medium (Sox^-). The Sox^- mutants were analyzed for other litho- or autotrophic growth characteristics and grouped by these properties. Of the phenotypic characters examined four groups were obtained. Group I, represented by three strains (TP19, TP37, and TP57), only lost the sulfur-oxidizing ability (Tables 1 and 2). Group II Sox^- mutants were pleiotropic and did not grow with H_2 as the electron donor. Of the 46 strains representing this group, strain TP43 was examined in detail. Nitrate as electron acceptor allowed some growth to TP43, while nitrite did not (Fig. 1A); strain TP43 grew well with nitrate and with nitrite as nitrogen source (Table 2). Therefore, only anaerobic nitrite utilization was impaired, besides growth with hydrogen. Growth on formate was not affected. Group III mutants (TP61 and TP67) did not grow with formate as carbon and energy source (Fig. 1, lane B). Nitrite but not nitrate could serve as nitrogen source (Table 2). Anaerobic growth with nitrate as electron acceptor did not occur, while nitrite was utilized well anaerobically (Fig. 1A, and Table 2). These mutants also did not utilize xanthine as carbon or nitrogen source (data not shown). Strain TP71 represented the only mutant of group IV and was characterized by slight autotrophic growth with formate (Fig. 1B), thiosulfate, and H_2. Nitrate or nitrite utilization was not affected (data not shown).

Enzymatic analysis of Sox^- mutants. It is evident from the Sox^- mutants that other functions than simply the ability to oxidize thiosulfate are needed to grow with reduced sulfur compounds as energy source. Therefore, key enzymes of lithoautotrophic metabolism such as thiosulfate or sulfide oxidases, hydrogenase, and RuBPCase were assayed in the parent GB17 and the Sox^- mutants grown under mixotrophic conditions. In pyruvate mineral medium with molecular hydrogen in the gas atmosphere, the parent and all Sox^- mutants formed both hydrogenase and RuBPCase activity (Table 3). In this medium thiosulfate-oxidizing activity was not detected (data not shown). In contrast to other mutants the Sox^- Hox^- strain TP43 exhibited only about 10% of the hydrogenase and about 30% of the RuBPCase activity expressed by the parent.

After mixotrophic growth with pyruvate-formate, activities of formate dehydrogenase and RuBPCase were found to be about identical in the parent and in mutants TP37, TP57, and TP71. Strain TP43 had high formate dehydrogenase activity but distinctly less RuBPCase activity. Neither of the enzyme activities was detected in the pleiotropic mutant TP67 (Table 3). The parent did not express hydrogenase- or thiosulfate-oxidizing activity with pyruvate-formate as the growth substrate (data not shown). In pyruvate-thiosulfate mineral medium the thiosulfate-oxidizing activity was formed by strain GB17 (specific activity, 0.451 U/mg of protein). Activities of RuBPCase, hydrogenase, or formate dehydrogenase were not observed. Sodium sulfite or tetrathionate were not oxidized (data not shown). No thiosulfate-oxidizing activity was detected in mutants TP37 or TP57. Marginal activity was present in TP67 (specific activity, 0.008 U/mg of protein). Surprisingly, strain TP43 exhibited 36% of the wild-type activity (0.164 U/mg of protein) and TP71 exhibited even higher activity (0.345 U/mg of protein). Sodium sulfide was oxidized to sulfate by whole cells of GB17 grown in pyruvate-thiosulfate medium. The rate of sulfide oxidation by the parent and the various Sox^- mutants gave activities similar to those for thiosulfate oxidation (Table 3).

Rhodanese cleaves thiosulfate with cyanide to form thiocyanate and sulfite. Rhodanese activity was present in all Sox^- mutants at levels similar to those in the parents (Table 3). Rhodanese activity was also present in pyruvate-grown cells without added thiosulfate (data not shown). From these results we share the opinion of Lu and Kelly (16) that rhodanese is not involved in thiosulfate utilization.

Plasmid analysis. In the facultative chemolithotroph *Alcaligenes eutrophus*, genes for hydrogen-oxidizing ability (Hox), denitrification (Nitd), and autotrophic carbon dioxide fixation are located on the plasmid pHG1 (1, 10, 22). We examined *Thiosphaera pantotropha* and its Sox^- mutants for the presence of plasmids by different techniques (2, 13, 23). *Thiosphaera pantotropha* harbored two plasmids that differed in size from those found in *Paracoccus denitrificans* and *Thiobacillus versutus* (Fig. 2). The approximate size of the *Thiosphaera pantotropha* megaplasmid pHG41 was about 450 kb. The middle-sized plasmid pHG42 was determined to be 110 kb. In mutant TP57, a representative of group I, plasmid pHG41 was not detected by the different methods. The strain still contained pHG42 (Fig. 3). Other group I mutants contained both plasmids (data not shown). An alteration in physical properties of pHG42 of strain TP57 and other Sox^- mutants of groups I, III, and IV, in comparison with the parent, was not observed. Thus, the insertion of Tn*5* into pHG42 of these Sox^- mutants was not likely. However, pHG42 of strain TP43 was slightly smaller than that of the parent and mutants of the other groups (Fig. 3). Sox^+ Kan^s revertants of TP43, obtained after incubation in

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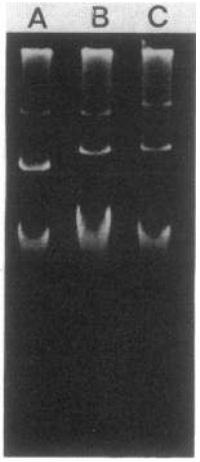

FIG. 2. Plasmid profiles of facultatively lithoautotrophic thiobacilli. Lane A, *Paracoccus denitrificans* 381; lane B, *Thiosphaera pantotropha*; lane C, *Thiobacillus versutus*. Lysis was done by the method of Casse et al. (2).

thiosulfate medium for 7 days, exhibited the same size of pHG42 as strain TP43 (data not shown).

Besides *Thiosphaera pantotropha* GB17 obtained from L. Robertson (21), the strain originating from the Delft Culture Collection and stored by DSM under liquid nitrogen (TPln) or the strain originating as a freeze-dried culture (TPfd) was included in these studies. Both strains were Kan^s and exhibited numerous pleiotrophic effects. They were unable to grow with thiosulfate, hydrogen, formate, and fructose. They did not grow anaerobically with nitrate or nitrite as electron acceptor but grew well aerobically. These compounds were used as nitrogen source (data not shown). The spontaneous mutant TPln harbored, besides pHG41 and pHG42, three new plasmids, pHG411, pHG412, and pHG413. These three plasmids were also harbored by strain TPfd, while pHG41 and pHG42 could not be detected (Fig. 4).

The genetic information located on pHG41 is unknown. Different phenotypic properties have been correlated with plasmids such as resistances against antibiotics or heavy

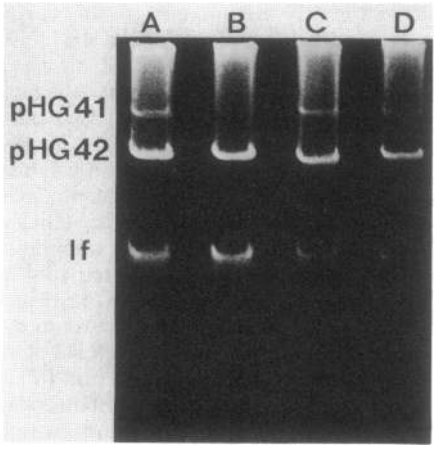

FIG. 3. Plasmid profiles of Tn*5*-induced Sox^- mutants of *Thiosphaera pantotropha*. Lane A, GB17; lane B, TP57; lane C, TP43; lane D, TP67; lf indicates the linear fraction of DNA.

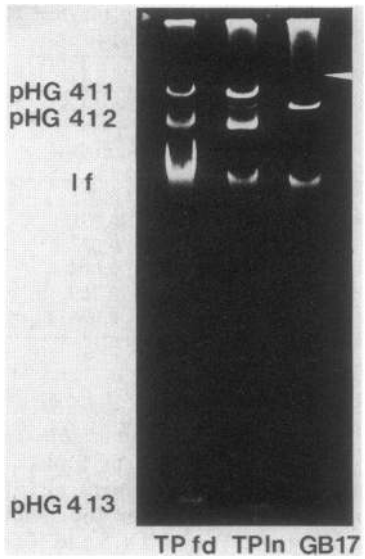

FIG. 4. Plasmid profiles of spontaneous Sox^- mutants of *Thiosphaera pantotropha*. The white arrowhead indicates the position of the faint band of pHG41 of strains GB17 and TPln; lf indicates the liner fraction of DNA.

metals or degradation of haloaromatic compounds (19). The parent and the $pHG41^-$ strain TP57 were investigated for antibiotic sensitivity by the disk method. Both strains were resistant to streptomycin, trimethoprim, gentamicin, lincomycin, 5-fluorocytosine, and clindamycin. Both strains were identically sensitive to rifampin, tetracycline, ampicillin, carbenicillin, chloroamphenicol, nitrofurantoin, vancomycin, erythromycin, nalidixic acid, tobramycin, oleandomycin, and spectinomycin. The neomycin resistance of TP57 was due to Tn*5*. The heavy metal sensitivity of TP57 against mercury, copper, zinc, tellurate, cobalt, nickel, cadmium, thallium, and chromate ions was identical to that of the parent. Both strains were highly resistant to lead and arsenate. *Thiosphaera pantotropha* GB17 and TP57 grew well on 2-fluoro- and 2-aminobenzoate and poorly on 4-fluoro-and 4-hydroxybenzoate. Otherwise, substituted benzoic acid could not be utilized. Therefore, plasmid pHG41 of *Thiosphaera pantotropha* currently must be considered cryptic.

DISCUSSION

By Tn*5* insertional mutagenesis of *Thiosphaera pantotropha* we obtained different groups of mutants that shared the inability to grow lithoautotrophically with thiosulfate. The group I mutants (e.g., TP37 and TP57) were also unable to oxidize sulfide. Therefore, both thiosulfate and sulfide may be oxidized by the same system or they may be under the same regulation of formation.

The group II mutant TP43, defective in anaerobic nitrite metabolism, grew well with nitrite as nitrogen source. Although a low rate of thiosulfate oxidation and hydrogenase activity was expressed, no growth occurred with H_2 or thiosulfate. Therefore, an electron acceptor common to thiosulfate and hydrogen oxidation and to nitrite reduction might have been abolished. This conclusion may be in agreement with the finding of Lu and Kelly (17), who have described cytochrome c_{551} and $c_{552.5}$ to be part of the thiosulfate-oxidizing system of *Thiobacillus versutus*.

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RuBPCase activity was significantly decreased under mixotrophic growth conditions with hydrogen or formate in TP43 (Table 3). In many autotrophic bacteria the synthesis of RuBPCase requires a reductant to allow the highly energy-requiring autotropic CO_2 fixation to operate. Reductant is supplied by high light intensities in *Chromatium vinosum* (15), by reduced substrates such as butyrate during photoheterotrophic growth of *Rhodospirillum rubrum* (26), and by hydrogen or formate metabolism in *Alcaligenes eutrophus* (11). In analogy, the mutation of strain TP43 that caused the inability to grow with H_2 or thiosulfate may have caused the inability to generate energy from this oxidation, leading to decreased RuBPCase formation. Because formate dehydrogenase was linked to NAD, energy generation from formate oxidation was not affected, leading to fully expressed RuBPCase formation.

The Sox^- mutants of group III (TP61 and TP67) were defective in formate oxidation, growth on xanthine, and dissimilatory or assimilatory nitrate reduction. Formate dehydrogenase, xanthine dehydrogenase, and nitrate reductase are molybdenum-containing enzymes in procaryotic and eucaryotic cells (3, 12, 18). In fact, molybdenum is necessary for lithoautotrophic growth on thiosulfate (C. Friedrich, unpublished data). Sulfite oxidase of *Thiobacillus novellus* contains a pterin molybdenum cofactor (27). Although in *Thiobacillus versutus* added sulfite oxidase enhanced the in vitro thiosulfate oxidation rate by 15 to 30%, the enzyme was not considered to be essential (17). At present the mechanism of thiosulfate cleavage is unknown (14). Despite the unknown role of sulfite oxidase, we propose a molybdenum cofactor common to nitrate reductase, formate dehydrogenase, and xanthine dehydrogenase of *Thiosphaera pantotropha* to be involved in thiosulfate oxidation. Thus, thiosulfate cleavage and oxidation may represent a new reaction of molybdenum hydroxylases:

$$S_2O_3^{2-} + H_2O \rightarrow S^{2-} + SO_4^{2-} + 2H^+$$

The observation that neither thiosulfate nor sulfide was not converted in the group I Sox^- mutants supports this model. This model is further supported by the conclusions of Lu and Kelly (16, 17), who have characterized the thiosulfate-oxidizing system of *Thiobacillus versutus* and proposed a reaction mechanism that exclusively involved hydroxylation reactions of the sulfane sulfur rather than oxygenation (16, 17).

The group IV mutant TP71 exhibited only slight growth on thiosulfate, hydrogen, and formate. The key enzymes to metabolize these energy sources were expressed (Table 3). Also, energy generation from this metabolism did not seem to be affected because RuBPCase was expressed as in the parent. Therefore, phosphoribulokinase or a ribulose-phosphate-regenerating sequence like sedoheptulose-bisphosphate activity might have been deficient in this strain.

Two plasmids (pHG41 and pHG42) were found in *Thiosphaera pantotropha*. In strain TP57, cured of pHG41, no difference in the size of the 110-kb pHG42 was observed, indicating that the 7-kb Tn*5* may not be inserted into pHG42. However, restriction analysis will be necessary. Tn*5* insertion into pHG41 cannot be identified by an alteration in the physical properties of the plasmid. However, preliminary reversion analysis reveals that TP57 reverts to the Sox^+ Kan^s phenotype. In case pHG41 did not represent a multimer of pHG42, pHG41 did not seem to be involved in the Sox character. Although in some hydrogen-utilizing bacteria the lithotrophic ability is located on a megaplasmid (6), other strains like *Pseudomonas pseudoflava* GA3 do not harbor any plasmid (9) but carry the Hox and the Sox character. Despite the fact that strain TP43 harbored Tn*5*, it shared common properties with strains TPln and TPfd: (i) the inability for anaerobic nitrite respiration and no growth with H_2 or thiosulfate, and (ii) either a deletion of pHG42 or a gene rearrangement that decreased the size of pHG42 (Fig. 3 and 4). The denitrifying ability (Nitd) of *Alcaligenes eutrophus* is located on the megaplasmid pHG1 (22), and $Nitd^-$ strains of *Alcaligenes eutrophus* are also Hox^-. However, it is doubtful whether pHG42 is involved in the Sox Hox character because strain TP43 was able to revert to the Sox^+ Hox^+ phenotype. Furthermore, a mutant cured of pHG42 but which still harbors pHG41 has been isolated (C. Friedrich, unpublished data), and this mutant exhibits identical lithotrophic properties as the parent strain GB17.

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