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Separation and Distribution of Thiosulfate-Oxidizing Enzyme, Tetrathionate Reductase, and Thiosulfate Reductase in Extracts of Marine Heterotroph Strain 16B†

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Thiosulfate-oxidizing enzyme (TSO), tetrathionate reductase (TTR), and thiosulfate reductase (TSR) were demonstrated in cell-free extracts of the marine heterotrophic thiosulfate-oxidizing bacterium strain 16B. Extracts prepared from cells cultured aerobically in the absence of thiosulfate or tetrathionate exhibited constitutive TSO and TTR activity which resided in the soluble fraction of ultracentrifuged crude extracts. Constitutive TSO and TTR cochromatographed on DEAE-Sephadex A-50, Cellex D, Sephadex G-150, and orange A dye-ligand affinity gels. Extracts prepared from cells cultured anaerobically with tetrathionate or aerobically with thiosulfate followed by oxygen deprivation showed an 11- to 30-fold increase in TTR activity, with no increase in TSO activity. The inducible TTR resided in both the ultracentrifuge pellet and supernatant fractions and was readily separated from constitutive TSO and TTR in the latter by DEAE-Sephadex chromatography. Inducible TTR exhibited TSR activity, which was also located in both membrane and soluble extract fractions and which cochromatographed with inducible TTR. The results indicate that constitutive TSO and TTR in marine heterotroph 16B represent reverse activities of the same enzyme whose major physiological function is thiosulfate oxidation. Evidence is also presented which suggests a possible association of inducible TTR and TSR in strain 16B.

The marine heterotrophic pseudomonad strain 16B is a facultative anaerobe which oxidizes thiosulfate to tetrathionate, with oxygen or nitrate as a terminal electron acceptor (31, 32). In this respect the bacterium is physiologically similar to a large number of bacterial strains which have been isolated from a variety of habitats, including soil, fresh water reservoirs, deep sea sediments, anoxic marine basins, and deep sea hydrothermal vent environments (10, 21, 23, 24, 28, 32, 33, 35, 36, 39). Thiosulfate oxidation increases both the growth rate and yield of 16B in dilute organic media (31) due to a carbon-sparing effect; i.e., energy derived from thiosulfate oxidation permits carbon to be conserved for anabolic metabolism rather than respired (30). This effect would be expected to give heterotrophic thiosulfate oxidizers such as 16B a growth advantage over other heterotrophs which do not oxidize thiosulfate, particularly at low concentrations of utilizable carbon sources.

Thiosulfate oxidation in strain 16B is catalyzed by thiosulfate-oxidizing enzyme (TSO). TSO is constitutive, as evidenced by its synthesis under all growth conditions investigated so far, including anaerobiosis and omission of thiosulfate or other reduced sulfur compounds from the medium (29). In cell-free extracts, TSO activity resides only in the 155,000 × *g* ultracentrifuge supernatant and is therefore believed to be a soluble protein (37). In contrast, 70 to 75% of TSO activity in extracts of soil isolate A-50, the only heterotroph besides 16B in which cell-free TSO has been investigated, is associated with particulate material sedimenting between 12,000 and 144,000 × *g* (28). The TSO of strain 16B also appears to be different from the TSOs of *Thiobacillus thioparus* (13), *Thiobacillus neapolitanus* (27), and *Chromatium* sp. strain D (22). Methods which yield high-purity TSO from the latter three bacteria gave, at best, only partially purified preparations of 16B TSO (37).

Under anaerobic growth conditions in defined

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media containing a nonfermentable organic carbon and energy source, strain 16B uses partially reduced inorganic sulfur compounds, including tetrathionate, thiosulfate, and trithionate, as terminal electron acceptors (34). Therefore, under appropriate growth conditions, 16B forms tetrathionate reductase (TTR), thiosulfate reductase (TSR), bisulfite reductase, and trithionate reductase in addition to TSO.

TTR reduces tetrathionate stoichiometrically to thiosulfate in 16B (29). Like TSO, some of the TTR is constitutive, but after growth in media containing thiosulfate or tetrathionate, the cells exhibit induced TTR activity threefold higher than the constitutive level (29). This finding, partial inhibition of constitutive but not inducible TTR by thiosulfate, and differences in inhibition of the TTR activities by cyanide led to the hypothesis that 16B contains two TTRs, one of which may represent the reverse activity of TSO.

Sulfur metabolism in heterotroph 16B is unique in that it represents a composite of the enzyme activities found in obligate aerobes (e.g., thiobacilli), obligate anaerobes (e.g., *Desulfovibrio* spp.), and facultative anaerobes cultured anaerobically (e.g., certain *Enterobacteriaceae*). Although cell-free TSO, TTR, and TSR activities have been at least partially characterized in other bacteria, they have not been studied in a microorganism such as 16B, which not only exhibits all these activities but couples each of them to growth under different culture conditions (30, 31, 34). Strain 16B is also unusual in that intact cells exhibit both inducible and constitutive TTR activities (29). Furthermore, reversibility of TSO in heterotrophic thiosulfate-oxidizing bacteria has not been clearly demonstrated.

This study was undertaken to characterize the cell-free activities of TSO, constitutive and inducible TTR, and TSR in heterotroph 16B in an effort to clarify the relationships existing among them. We report herein the separation of constitutive and inducible TTR, data supporting a constitutive TSO-TTR protein, and evidence for an inducible TTR-TSR enzyme complex.

(This work was presented in part at the 81st Annual Meeting of the American Society for Microbiology [G. M. Whited and J. H. Tuttle, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K49, p. 145].)

MATERIALS AND METHODS

Bacteria, media, and culture conditions. Stock cultures of strain 16B were maintained on TB medium (32) and transferred monthly. Inocula used for uninduced cultures were transferred three successive times and maintained in 16B growth medium (34) lacking reduced sulfur compounds.

Mass cultures of 16B were prepared in 18-liter carboys containing 16B growth medium set at pH 7.0. For induced cultures, sodium thiosulfate or potassium tetrathionate was added at a final concentration of 6.5 or 5 mM, respectively. Phosphates, sulfates, and lactate or lactate-thiosulfate mixture were autoclaved separately. Lactate, lactate-thiosulfate mixture, or filter-sterilized (0.22-μm membrane) tetrathionate was added to the medium just before inoculation to decrease the possibility of contamination.

Inocula consisted of 1.5% (vol/vol) precultures grown for 24 to 48 h in 16B growth medium appropriately supplemented to match the mass culture medium. Aerobically grown cultures were sparged with sterile, cotton-filtered air and mixed with a magnetic stirrer. For anaerobic cultures, sterile nitrogen replaced air for culturing and harvesting. In addition to growing the cells anaerobically in the tetrathionate-supplemented growth medium, induction of enzymes was also accomplished by growing cells aerobically in thiosulfate-supplemented medium in which aeration was terminated 2 h before and during harvesting. Cells cultured in this manner are hereafter referred to as oxygen deprived. This procedure produced a much larger yield of induced cells than anaerobic culture and was used routinely to provide cells for extracts subjected to extensive purification methods. All mass cultures of 16B were grown at 30°C and harvested at mid- to late-exponential phase. Culture purity was confirmed by streaking a portion of the culture onto nutrient agar (Difco Laboratories, Detroit, Mich.) plus 2% NaCl before harvesting.

Desulfovibrio vulgaris 8303 was used to prepare hydrogenase for TTR and TSR assays (see below). Liquid cultures and 10 g of lyophilized cells were a generous gift of J. M. Akagi. The organism was maintained and grown in mass culture as described previously (1).

Mass cultures were harvested by centrifugation at 20,000 to 30,000 × *g* at 4°C and a flow rate of 125 to 175 ml/min with a KSB continuous flow apparatus (DuPont-Sorval, Newton, Conn.). During harvesting, aerobic cultures were sparged with air, anaerobic cultures were sparged with nitrogen, and oxygen-deprived cultures were treated as described above. 16B cell packs were washed three times in several volumes of pH 7.5 500 mM sodium phosphate–2% (wt/vol) NaCl buffer. Cells were stored at −20°C and, if grown anaerobically, under an atmosphere of hydrogen.

Enzyme assays. TSO activity was measured by a modification of methods described by Trudinger (27) and Tuttle et al. (37) in which thiosulfate oxidation is coupled to ferricyanide reduction. One mole of thiosulfate reduces 1 mol of ferricyanide. Reaction mixtures contained in a 3-ml volume: 2.0 mM potassium ferricyanide, 7.5 mM sodium thiosulfate, 50 mM potassium phthalate buffer (pH 6.25), and cell extract. Reaction mixtures were incubated at 30°C. Ferricyanide reduction was determined colorimetrically by following a decrease in absorbance at 420 nm. One unit of TSO activity is defined as 1 μmol of ferricyanide reduced per min.

TTR activity was determined by the method of Pichinoty and Bigliardi-Rouvier (19), in which the oxidation of H_2 is coupled to tetrathionate reduction through hydrogenase and the intermediate electron carrier benzyl viologen. One mole of H_2 reduces 1 mol

of tetrathionate. H_2 consumption was measured manometrically in conventional Warburg flasks fitted with gassing plugs. The flasks were calibrated by the ferricyanide-hydrazine method of Umbreit et al. (38). Reaction mixtures consisted of 1.0 mM benzyl viologen, 0.5 mg of *D. vulgaris* hydrogenase per ml of reaction mixture (prepared as described below), and various amounts of 16B cell extract in the main well; 10 mM potassium tetrathionate in the side arm; and 0.1 ml of 20% (wt/vol) cadmium chloride in the center well to absorb hydrogen sulfide. Reaction volumes varied from 2 to 6 ml. All reactions were run at 30°C in 50 mM Tris-hydrochloride buffer (pH 7.5). Manometers were shaken at 60 strokes per min and a 4.5-cm amplitude. A complete reaction mixture lacking 16B cell extract was run with each series of assays to correct for variable, low-level residual TTR activity in hydrogenase preparations. One unit of TTR activity is defined as 1 μmol of H_2 consumed per h.

TSR activity was measured manometrically by the same method as that for TTR, except that 10 mM thiosulfate replaced tetrathionate, and 0.2 ml of 20% (wt/vol) cadmium chloride was placed in the center well. One unit of TSR activity is defined as 1 μmol of H_2 consumed per h.

Chromatography. DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was equilibrated in 100 mM Tris-hydrochloride buffer (pH 8.0) and packed into a glass column (2.6 cm [diameter] by 26 cm [height]). 16B protein (82 to 165 mg) was applied to the column. After a wash with 1 void volume of starting buffer, proteins were eluted with a linear gradient of NaCl (0 to 600 mM or 0 to 1 M) prepared in starting buffer. Fraction volumes were 5 ml.

Cellex D (Bio-Rad Laboratories, Richmond, Calif.) was also prepared in 100 mM Tris-hydrochloride (pH 8.0). The resin was packed into columns (0.9 by 14.0 cm). 16B protein (2.8 mg) was applied to the gel, and the column was washed with starting buffer. Fractions of 2.2-ml volume were collected.

Affinity chromatography was done with five types of textile dyes linked to agarose. These gels are commercially available in a Dye-Matrix screening kit (Amicon Corp., Lexington, Mass.) which contains the five gels in small prepoured columns and a control column of agarose without an attached dye ligand. The columns were equilibrated with 20 mM Tris-hydrochloride (pH 7.5), 0.51 mg of 16B protein was applied to each, and the resins were washed with starting buffer. Bound protein was eluted in one step with 1.5 M KCl prepared in starting buffer. In preliminary experiments one of the gels, orange A was found to yield the highest specific activity of TSO and TTR. This gel was subsequently purchased preswollen (Amicon Corp.), packed into a column (1.9 by 5.5 cm), and equilibrated in 20 mM Tris-hydrochloride (pH 7.5). 16B protein (1 mg) was layered onto the gel, and the column was washed with starting buffer. Elution was carried out with a linear gradient of KCl (0 to 2.0 M) prepared in starting buffer.

Molecular weight determinations were made by gel filtration on columns (1.5 by 95 cm) of coarse-grade Sephadex G-150 (Pharmacia) equilibrated in 50 mM Tris-hydrochloride (pH 7.5) for 2 days at 4°C with a pumped flow rate of 7 ml/h. Blue dextran (Sigma Chemical Co., St. Louis, Mo.) was used to determine the void volume of the column. Marker proteins (50 mg of protein each) consisted of bovine serum albumin (molecular weight, 68,000 [25]), hexokinase (molecular weight, 99,000), lactate dehydrogenase (molecular weight, 150,000), and pyruvate kinase (molecular weight, 237,000 [9]). 16B extract was applied to the column as 0.6 mg of OAPFII protein (described below). Eluate was collected in 1.0-ml volumes. Marker proteins were detected by measuring the absorbance of the eluate fractions at 280 nm with a Beckman 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) and TSO and TTR were detected by enzyme activity as described above.

Enzyme preparation and purification. Hydrogenase from *D. vulgaris* was prepared by the method of Akagi (personal communication) at 5°C. A 25 to 50% (vol/vol) suspension of *D. vulgaris* in 50 mM sodium phosphate buffer (pH 7.5) was passed twice through a chilled French pressure cell (American Instrument Co., Silver Spring, Md.) at 18,000 to 22,000 lb/in^2. A small amount of DNase I (Sigma) was added to the crude extract, which was allowed to stand for 30 min and then centrifuged at 27,000 × *g* for 20 min. The supernatant was removed and centrifuged at 164,000 × *g* for 2 h. The resulting supernatant was lyophilized and stored under H_2 at −20°C until used.

The preparation of 16B extracts is summarized in Fig. 1 (The letters in parentheses below refer to the steps shown in Fig. 1.) All procedures were carried out at 5°C. Frozen cells, thawed slowly at 5°C, or freshly harvested cells were diluted to a 25% (vol/vol) suspension in 50 mM sodium phosphate buffer (pH 7.5) containing 2% sodium chloride. This suspension was passed twice through a chilled French pressure cell at 18,000 to 22,000 lb/in^2 to disrupt the cells (A). A small amount of DNase I was added to the extract and allowed to react for 30 min (B), and whole cells were then removed by centrifugation at 10,000 × *g* for 20 min (C). The supernatant, fraction SI, was then centrifuged at 164,000 × *g* for 65 min (D), and the pellet (E) was resuspended in 50 mM Tris-hydrochloride buffer (pH 7.5) to a volume which was 50% of the original SI

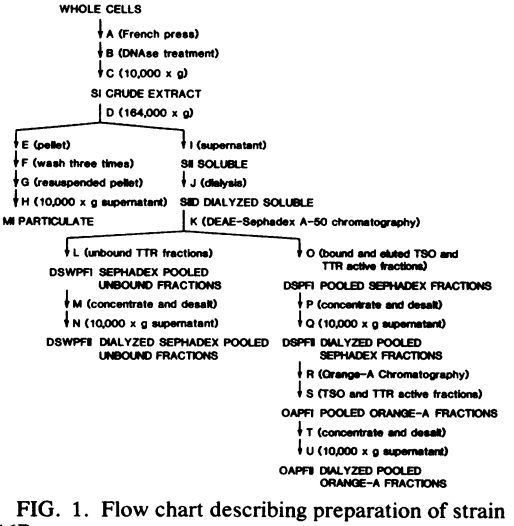

FIG. 1. Flow chart describing preparation of strain 16B extracts.

volume. This material was recentrifuged at 164,000 × g for 30 min, and the procedure was repeated three times (F). The final pellet was resuspended as before and centrifuged at 10,000 × g for 10 min (G). The supernatant (H) was designated the MI particulate fraction.

The supernatant from D (I) was designated fraction SII. This was dialyzed overnight against 100 mM Tris-hydrochloride buffer (pH 8.0) (J), and the resulting extract was designated fraction SIID. This fraction was applied to a DEAE-Sephadex A-50 column (K) as described above, and eluted fractions containing TTR activity in the void volume were pooled (L) and designated DSWPFI. This fraction was concentrated to approximately 5 ml by ultrafiltration with an XM-10 membrane (molecular weight exclusion, 100,000; Amicon Corp.) and desalted with 50 mM Tris-hydrochloride buffer (pH 7.5) (M). Some protein precipitation occurred during this procedure. It was removed by centrifugation at 10,000 × g for 15 min (N), which yielded the supernatant fraction DSWPFII.

The fractions of DEAE eluant which were active for TSO and TTR were pooled and used as DSPFI (O). This was concentrated to approximately 5 ml and desalted by ultrafiltration with an XM-10 membrane and 20 mM Tris-hydrochloride buffer (pH 7.5) (P). Precipitated protein was removed by centrifugation at 10,000 × g for 15 min (Q). The supernatant DSPFII was applied to an orange A column as described above (R), and eluate fractions exhibiting TSO and TTR activities were pooled and designated OAPFI (S). This fraction was desalted by ultrafiltration with an XM-10 membrane and 50 mM Tris-hydrochloride buffer (pH 7.5) (T). Precipitated protein was removed by centrifugation at 10,000 × g for 15 min (U). The resulting supernatant was designated OAPFII.

Electrophoresis. Polyacrylamide gel electrophoresis of fraction OAPFII was done on an 8.0% acrylamide slab gel, prepared by the method of Ogita and Markert (15). Approximately 80 μl (containing approximately 100 μg of protein) of fraction OAPFII was applied to the gel. Electrophoresis was initiated at a constant current of 8 mA for 1 h to allow the proteins to enter the gel, and then the current was increased to 15 mA until the tracking dye (brilliant blue G) reached a point 1.5 cm from the bottom of the gel. The slab was then fixed in 12.5% (wt/vol) trichloroacetic acid for 30 min, stained in a 0.05% (wt/vol) solution of Coomassie blue for 30 min, and transferred to 10.0% (vol/vol) acetic acid to destain (4).

Spectroscopy. Difference spectra of extract fractions MI, SII, and OAPFI were made with a Cary 219 (Varian Associates, Inc., Palo Alto, Calif.) or Aminco DW-2a (American Instrument Co.) spectrophotometer. Untreated (air-oxidized) extracts were compared to sodium dithionate-reduced or sodium thiosulfate-reduced extracts. Dithionate was added as a powder, and thiosulfate was added as a concentrated solution to give a final concentration exceeding 1 μM. Carbon monoxide difference spectra were made by comparing dithionate-reduced extracts bubbled with carbon monoxide with dithionate-reduced extracts.

Chemicals and chemical determinations. Commercially obtained chemicals were of reagent-grade quality or better. Brilliant blue G, yeast hexokinase, rabbit muscle lactate dehydrogenase, rabbit muscle pyruvate kinase, noncrystalline DNase I, and bovine serum albumin were purchased from Sigma Chemical Co.

Tetrathionate and trithionate were synthesized by the method of Roy and Trudinger (20). Thiosulfate, tetrathionate, and trithionate were determined by the cyanolysis method of Kelly et al. (8). Protein was determined by the method of Bradford (2), with bovine serum albumin as the standard. Measurements of pH were made with a Metrohm expanded-scale pH meter (Brinkman Instruments, Inc., Westbury, N.Y.).

RESULTS

Stoichiometry of the TTR assay. The stoichiometry of the cell-free assay for TTR was confirmed by measuring tetrathionate and thiosulfate in reaction mixtures containing SII extract prepared from aerobically cultured cells. After incubation for 76 min, 90% of the tetrathionate consumed during the reaction was recovered as thiosulfate, and hydrogen consumption accounted for 99% of the thiosulfate formed.

Influence of culture conditions on TSO, TTR, and TSR. Extracts prepared from cells cultured aerobically with or without thiosulfate exhibited virtually the same specific activity of TTR (Table 1). This activity remained almost entirely with the soluble fraction SII when SI crude extract was treated by ultracentrifugation (Fig. 1 and Table 1). Oxygen deprivation of the culture in the absence of reduced sulfur compounds had no effect on TSO or TTR activity. However, cells grown anaerobically with tetrathionate or aerobically with thiosulfate and deprived of oxygen exhibited greatly increased TTR specific activity (Table 1). In terms of total activity, TTR increased 11- to 30-fold. A greater percentage of TTR activity in induced cell extracts remained with the soluble SII fraction, but the specific activity of the enzyme was about two- to four-fold higher in the particulate (MI) fraction. Only 27% of fraction SI TTR activity in anaerobically grown cells was recovered in fractions SII and MI, whereas 79% of the initial SI activity was recovered in SII and MI fractions of thiosulfate-grown, oxygen-deprived cell extracts. The reason for this discrepancy is not clear but could have been due to lability of the enzyme with storage of the extract before assay (see below).

Culture conditions which led to induction of increased TTR activity also resulted in induction of TSR (Table 2). Extracts of aerobically grown cells contained virtually no TSR activity. The distribution of TSR in soluble and particulate fractions prepared from induced cells was similar to that of TTR; i.e., the greatest portion of the total TSR activity was found in fraction SII, whereas the highest specific activity was located in fraction MI.

TTR and TSR were always found in the same extract fractions of induced cells. However, the ratio of the specific activities of the two enzymes varied in different extract fractions, particularly

TABLE 1. Distribution of TTR in 16B extracts from cells cultured under different conditions

Growth conditions			Extract fraction	Spec act (U/mg of protein)		% Total activity[c]
O_2	$S_2O_3^{2-}$ (mM)	$S_4O_6^{2-}$ (mM)		$\bar{x}$[a]	n[b]	
Aerobic			SI	5.3 ± 1.9	6	100
			SII	5.5 ± 1.6	4	85
			MI	1.8	2	1
	6.5		SI	7.3 ± 3.3	15	100
	6.5		SII	6.1 ± 1.3	9	70
	6.5		MI	0.0 ± 0.0	10	0
Aerobic/oxygen deprived			SII	5.2	1	—[d]
			MI	0.0	1	—
	6.5		SI	83.6 ± 14.0	4	100
	6.5		SII	68.4 ± 23.0	3	63
	6.5		MI	355.5	2	16
Anaerobic		5.0	SI	160.6 ± 8.5	3	100
		5.0	SII	68.1 ± 2.3	3	17
		5.0	MI	130.8 ± 10.3	3	10

[a] Variance values are 1 standard deviation from the mean.
[b] n, Number of determinations.
[c] Compared to total activity of fraction SI.
[d] —, SI activity not determined.

in fraction DSWPFII (Fig. 1). However, this is based on only one measurement of both TTR and TSR activities in this fraction. The ratio of specific activity (TTR to TSR) in fractions SII and MI was 1.0 and 1.3, respectively, for extracts of anaerobically grown cells and 1.8 and 2.3, respectively, for aerobically grown, oxygen-deprived cells. The variation was due at least in part to the instability of the extract with time and the fact that both enzymes were not assayed simultaneously. TTR was always determined before TSR.

Attempts to separate TSO and constitutive TTR. Previous work with intact cells suggested that TSO and constitutive TTR might represent reversible activities of the same enzyme (29). To test this hypothesis, we attempted to separate the two activities by column chromatographic techniques. Fraction SIID prepared from aerobically grown cells was chromatographed on DEAE-Sephadex A-50 (Fig. 2). A similar technique had been used previously to partially purify TSO in 16B extracts (37). Eluted fractions were tested for TSO and TTR activities in the groups of 5 or 10, and individual fractions of those groups exhibiting enzyme activity were subsequently assayed separately. Since crude extracts of aerobically grown cells had virtually

TABLE 2. Distribution of TSR in 16B extracts from cells cultured under different conditions

Growth conditions			Extract fraction	Spec act (U/mg of protein)	% Total activity[a]
O_2	$S_2O_3^{2-}$ (mM)	$S_4O_6^{2-}$ (mM)			
Aerobic	6.5		SI	1.2	
Aerobic/oxygen deprived	6.5		SII	38.0	83
Aerobic/oxygen deprived	6.5		MI	155.9	17
Anaerobic		5.0	SII	70.2	69
Anaerobic		5.0	MI	99.8	31

[a] Compared to sum of total activity in SII and MI fractions.

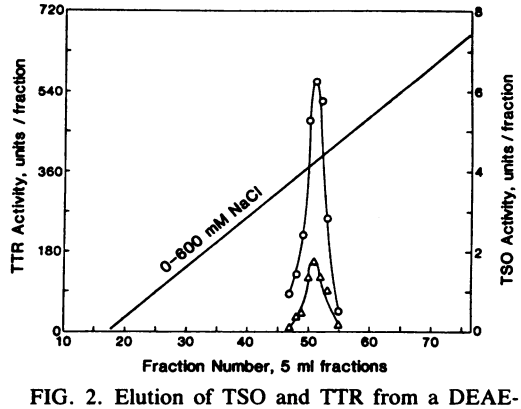

FIG. 2. Elution of TSO and TTR from a DEAE-Sephadex A-50 column. Cell-free extracts were prepared from aerobically grown cells. Symbols: O, TSO activity; △, TTR activity.

no TSR activity (Table 2), the eluate fractions were not examined for TSR.

TSO and TTR cochromatographed on the DEAE column (Fig. 2). The highest specific activities for TSO and TTR, 12.64 and 290.8 U/mg of protein, respectively, were located in fraction 50. Compared to the crude extract, the purification in this eluate fraction was 37-fold for TSO and 36-fold for TTR.

Fraction SIID from aerobically grown cells was also chromatographed on a Cellex D column. Both TSO and TTR were recovered in the wash fractions after elution with starting buffer, indicating that neither TSO nor TTR was bound by the resin.

Further attempts to separate the two activities were made by chromatographing the partially purified DSPFII extract (Fig. 1) on dye-agarose affinity columns. TSO and TTR were bound by all five types of resin tested and were eluted from each gel with 1.5 M KCl. Orange A resin gave the highest purification of both activities, 5.3-fold and 5.6-fold for TSO and TTR, respectively, compared to the specific activities present in the original DSPFII extract applied to the column. Fully 97% of each enzyme activity was recovered.

Orange A columns were subsequently used as a final preparatory step in the purification of TSO and constitutive TTR. When proteins bound to the column were eluted within a linear gradient of 0 to 1.5 M KCl rather than in one step, TSO and TTR cochromatographed, and all the activity was found in only two eluate fractions.

Electrophoresis of TSO and constitutive TTR. Fraction OAPFII, which contained TSO and constitutive TTR, was orange in color. Electrophoresis resolved the color into two distinct orange bands. One edge of the gel was removed and stained for protein. Three darkly staining bands, two of which corresponded to the orange bands, and at least 14 lighter staining bands were revealed. The remaining unstained gel was cut into strips corresponding to the most prominent protein bands. Each strip was then homogenized in 50 mM Tris-hydrochloride buffer (pH 7.0). The acrylamide was removed by centrifugation at 1,000 × *g*, and the supernatants were assayed for TSO and TTR activity. Neither could be detected.

Stability of TSO and constitutive TTR. TSO and constitutive TTR activities in extract fraction OAPFII were tested for heat stability and loss of activity due to storage. After the extract was heated at 55°C for 1 h, 33% of the original TSO activity and 56% of the original TTR activity remained. Upon storage for 6 days at 5°C, about 75% of TSO activity and 52% of TTR activity could be recovered. The activity of both TSO and TTR decreased rapidly for the first 3 days of storage and then remained nearly constant. It should be emphasized that activities for TSO, constitutive and inducible TTR, and TSR could not be determined simultaneously. This was particularly a problem during lengthy purification procedures in which each of the various fractions needed to be assayed for the different enzyme activities.

Molecular weight determination of TSO and constitutive TTR. The molecular weight of TSO and constitutive TTR was estimated by gel filtration of extract fraction OAPFII. TSO and constitutive TTR also cochromatographed on this column, with their activity peaks at an elution volume of 103 ml (Fig. 3). An approximate molecular weight for TSO and TTR of 132,000 was estimated from a linear regression plot of values for the calibration proteins (Fig. 4). The correlation coefficient (r^2) of the regression was 0.97.

Purification comparison of TSO and constitutive TTR. The activities of TSO and TTR were assayed during purification through fraction OAPFI (Fig. 1). Purification of TSO and OAPFI was 60.3-fold, with a yield of 69% based on the total TSO activity of fraction SI. Purification of constitutive TTR from uninduced cells was not carried further than fraction DSPFII, in which the enzyme was purified 9.9-fold from fraction SI. However, the specific activity of constitutive TTR prepared from a mixture of uninduced and induced cells was increased 7.9-fold by orange A chromatography of fraction DSPFII. The purification of constitutive TTR calculated from the two extract preparations was estimated to be 78.2-fold.

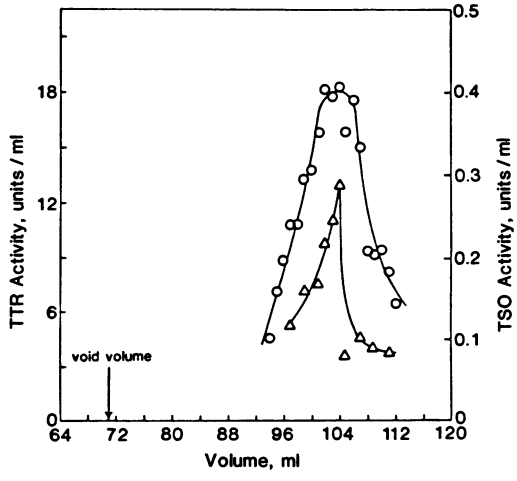

FIG. 3. Elution of TSO and constitutive TTR from a Sephadex G-150 column. Symbols: ○, TSO activity; △, TTR activity.

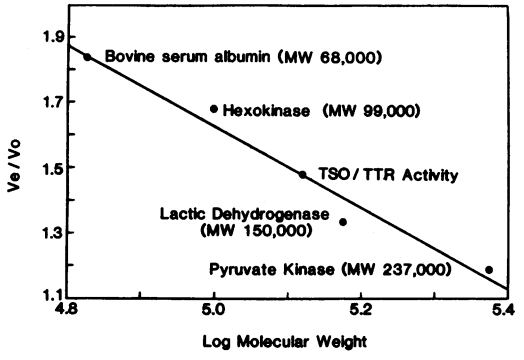

FIG. 4. Molecular calibration curve for the Sephadex G-150 gel filtration column. TSO/TTR activity represents the elution peak of extract fraction OAP-FII. Ve, Elution volume of the protein; Vo, void volume of the column. MW, Molecular weight.

Separation of inducible and constitutive TTR. In contrast to TTR in extracts of aerobically grown cells (Fig. 2), soluble TTR in fraction SIID extracts of anaerobically grown or thiosulfate-grown, oxygen-starved cells was resolved into two peaks by DEAE-Sephadex chromatography (Fig. 5). TSO, however, was found in only one peak, coincident with the lower-activity TTR peak. The higher-activity TTR peak, A, did not absorb to the column and contained several times the activity of TTR peak B. Peak A had no TSO activity.

In subsequent purification steps (Fig. 1), TTR peak B protein cochromatographed with TSO as described above, whereas pooled peak A protein contained TSR activity. Fraction DSWPFII and the ultrafiltrate collected during its preparation (Fig. 1) were tested for TTR and TSR. The activities were found only in the membrane-retained DSWPFII fraction. This suggests that these enzymes have a molecular weight greater than 100,000, the molecular weight exclusion size of XM-100 membranes (Amicon Corp. specifications).

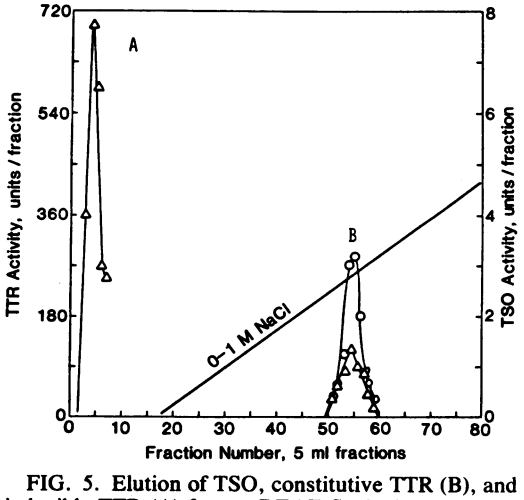

FIG. 5. Elution of TSO, constitutive TTR (B), and inducible TTR (A) from a DEAE-Sephadex A-50 column. Cell-free extracts were prepared from cells grown in the presence of 6.5 mM thiosulfate and deprived of oxygen 2 h before harvesting. Symbols: ○, TSO activity; △, TTR activity.

Kinetics of TTR and TSR activity. H_2 consumption by extracts containing inducible TTR (fractions SII, DSWPFI, DSWPFII, and MI) proceeded linearly to at least 25% of the theoretical completion based on the initial tetrathionate concentration. Hydrogen consumption catalyzed by inducible TTR in the presence of equimolar tetrathionate and thiosulfate was also linear, but the reaction rate remained almost the same as rates observed in the presence of tetrathionate or thiosulfate alone (Table 3). However, when thiosulfate or thiosulfate plus tetrathionate were used as electron acceptors, the cadmium chloride used in center wells of reaction flasks turned yellow almost immediately, indicating production of hydrogen sulfide. Unlike intact cells (34), it appears that cell-free extracts prepared from induced cells reduce tetrathionate and thiosulfate simultaneously. Since hydrogen consumption but not tetrathionate disappearance was measured, the possibility that only thiosulfate was reduced when both electron acceptors were supplied cannot be ruled out. However, this possibility seems unlikely. Kinetics of reactions with tetrathionate alone indicated no apparent inhibition by the product, thiosulfate, and redox potential strongly favors tetrathionate reduction ($S_4O_6^{2-}/2S_2O_3^{2-}$, $E_o^1 = +170$ mV; $S_2O_3^{2-}/SO_3^{2-} + S^{2-}$, $E_o^1 = -423$ mV [17]).

In contrast to inducible TTR, hydrogen consumption catalyzed by constitutive TTR was characterized by a 2- to 10-min linear response, followed by a sharp break in the slope and a gradually decreasing reaction rate. This observation suggested thiosulfate inhibition of constitutive TTR. Consequently, constitutive TTR ac-

TABLE 3. Hydrogen consumption in the presence of thiosulfate, tetrathionate, or both catalyzed by fraction SII extract prepared from induced cells

Electron acceptor		Reaction rate[a] (U)
$S_2O_3^{2-}$ (mM)	$S_4O_6^{2-}$ (mM)	
0	10	26.19
10	0	28.06
10	10	24.89

[a] Standard assay conditions; each reaction flask contained 0.4 mg of extract protein.

TABLE 4. Influence of inhibitors on TTR and TSO activities

Inhibitor	% Concn[a]	% Inhibition		
		TSO	TTR (constitutive)[b]	TTR (inducible)[c]
$S_3O_6^{2-}$	50	52	51	ND[d]
$S_3O_6^{2-}$	100	69	75	ND
NO_3^-	50	22	9	ND
NO_3^-	100	4	32	ND
CN^-	50	87	85	56
CN^-	100	97	67	68
$S_4O_6^{2-}$	50	21		
$S_4O_6^{2-}$	100	18		
$S_2O_3^{2-}$	50		86	ND
$S_2O_3^{2-}$	100		100	5

[a] Percent concentration with respect to initial thiosulfate concentration of 7.5 mM (TSO) and initial tetrathionate concentration of 10.0 mM (TTR).
[b] Extract fraction OAPFI.
[c] Extract fraction DSWPFI.
[d] ND, Not determined.

tivities were determined only during the linear portion of the reaction.

Inhibition of TSO and TTR. The effects of suspected inhibitors of TSO and TTR (Table 4) were in general agreement with similar experiments done previously with intact cells (29). Trithionate inhibited TSO and constitutive TTR nearly equally. Nitrate was not strongly inhibitory to either constitutive enzyme. The reason for decreased nitrate inhibition of TSO at equimolar concentrations is not clear. Cyanide was strongly inhibitory to all three enzyme activities but appeared to inhibit TSO more than TTR. TSO was only weakly inhibited by its product, tetrathionate. In contrast, constitutive TTR was strongly inhibited by thiosulfate. Inducible TTR was hardly affected by thiosulfate, perhaps reflecting the presence of TSR activity in the extract.

Cytochromes. A carbon monoxide difference spectrum of extract fraction MI (Fig. 6A) revealed absorbance maxima at 414, 537, and 570 nm, which are characteristic of cytochrome *o*, a cytochrome *b*-type pigment (3). This absorption pattern was not present in carbon monoxide difference spectra of the soluble extract fraction SII. However, the spectrum of dithionate-reduced fraction SII (Fig. 6B) revealed the presence of *c*-type cytochrome, with absorption maxima at approximately 425, 524, and 553 nm (11). Fractions MI and OAPFII had similar spectra (Fig. 6C), which indicates the presence of cytochrome *c* in both membrane and soluble fractions of strain 16B and tentatively identifies at least one of the two orange bands separated electrophoretically from fraction OAPFII as cytochrome *c*. Thiosulfate reduced the *c*-type cytochrome in fraction SII but not in fraction MI (Fig. 6D).

DISCUSSION

We have described the initial cell-free characterization of TTR and TSR in marine heterotroph 16B. Cell-free TSO in this bacterium has been studied previously, but the reported purification was no greater than 16-fold (37). Improved techniques used in the present study

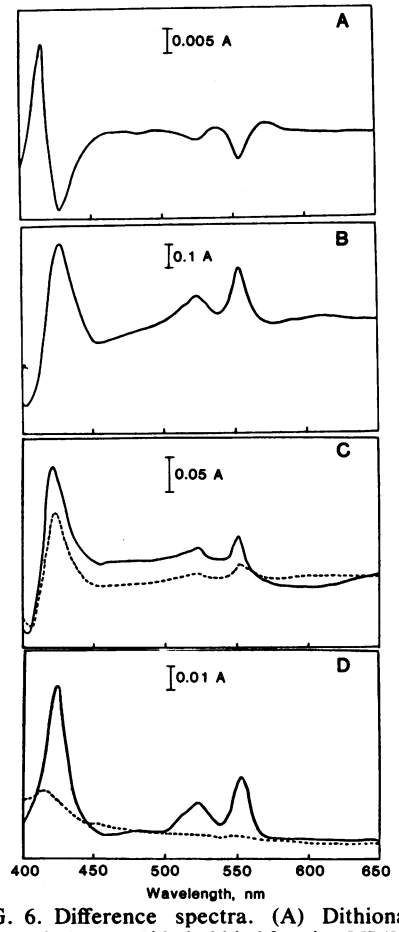

FIG. 6. Difference spectra. (A) Dithionate-reduced, carbon monoxide-bubbled fraction MI (0.74 mg of protein per ml) minus dithionate-reduced fraction MI. (B) Dithionate-reduced fraction SII (14.2 mg of protein per ml) minus air-oxidized fraction SII. (C) Dithionate-reduced fraction OAPFI (0.46 mg of protein per ml) minus air-oxidized fraction OAPFI (solid line); dithionate-reduced fraction MI (0.74 mg of protein per ml) minus air-oxidized fraction MI (dashed line). (D) Thiosulfate-reduced fraction SII (14.2 mg of protein per ml) minus air-oxidized fraction SII (solid line); thiosulfate-reduced fraction MI (0.74 mg of protein per ml) minus air-oxidized fraction MI (dashed line). The light path was 1 cm, and baseline absorbance has been subtracted.

resulted in increased purification of crude extract TSO to about 60-fold.

Native cytochrome *c* in soluble extracts of 16B was reduced in the presence of thiosulfate (Fig. 6D). Membrane preparations which also contain cytochrome *c* (Fig. 6C) but lack TSO (37) did not exhibit reduction of cytochrome *c* by thiosulfate (Fig. 6D). The most purified fraction of TSO, extract OAPFII, still contained the *c*-type cytochrome (Fig. 6C). Therefore, cytochrome *c* appears to be the native electron acceptor for TSO in strain 16B. TSOs from *T. neapolitanus* (27), *T. thioparus* (13), and heterotrophic soil isolate A-50 (28) also reduce native cytochrome *c* in the presence of thiosulfate.

Intact cells of 16B couple thiosulfate oxidation to oxygen (29). However, we have been unable to demonstrate oxygen uptake by crude extracts (SI protein) of 16B in the presence of thiosulfate (unpublished data). Cell breakage or the in vitro assay conditions we used apparently irreversibly uncouple cytochrome *c* from the terminal oxidase. Reduction of endogenous cytochrome *c* by the addition of thiosulfate to TSO-containing extracts, the presence of cytochrome *c* in the most highly purified TSO preparation, and spectral evidence for cytochrome *o* (Fig. 6A) but no other potential terminal oxidases (e.g., cytochrome *a* or *d* in membrane preparations) suggest a possible electron transport chain in 16B from thiosulfate to oxygen. 16B TSO may be a thiosulfate-cytochrome *c* oxidoreductase which passes electrons to cytochrome *o*, either directly or through another intermediate electron carrier, in an energy-yielding step. Intact 16B cells have shown to couple thiosulfate oxidation to ATP generation (30). Further experiments, perhaps with purified components, will be needed to verify the electron transport chain.

Our results clearly demonstrate that 16B contains at least two TTRs. One of these enzymes is constitutive, and its activity was always found with TSO (also constitutive [37]) in soluble extract preparations of 16B. TSO and constitutive TTR cochromatographed from DEAE-Sephadex (Fig. 2 and 5), Cellex D, and orange A affinity agarose. Furthermore, TSO and constitutive TTR had the same retention volume from Sephadex G-150 (Fig. 3); both activities were relatively constant under a variety of culture conditions, were unrelated to the amount of inducible TTR formed in induced extracts, were relatively heat stable, and were purified to approximately the same degree by the identical purification procedure. Although electrophoretic examination of our most purified preparation revealed more than one protein band, the evidence clearly indicates that TSO and constitutive TTR are, as previously suggested (29), reversible activities of the same enzyme.

TSO has a K_m for thiosulfate of 1.6 mM (37). The specific activity of TTR in extract fraction OAPFI remained approximately constant, 277 ± 58 U/mg of protein, over a range of 1 to 20 mM tetrathionate. Therefore, the K_m must be less than 1 mM. Lack of sufficient sensitivity in the TTR assay prevented use of tetrathionate concentrations less than 1 mM.

Cell-free activity of both TSO and TTR in the same bacterium has been demonstrated only for soil isolate A-50 (28) and 16B. In A-50, the TSO and TTR activities must be induced, whereas TSO-TTR is constitutive in 16B. However, the major physiological role of TSO-TTR activity in both microorganisms appears to be the same, i.e., thiosulfate oxidation (29). Observations that constitutive TTR activity in 16B is strongly inhibited by thiosulfate but that tetrathionate hardly affects TSO (Table 4) and that 16B synthesizes a second, more active inducible TTR at appropriate culture conditions are consistent with this suggestion. Nevertheless, constitutive TTR in 16B may provide a means by which the bacterium is able to survive under anaerobic conditions until the inducible TTR can be synthesized.

The soluble, constitutive TTR of 16B differs from the TTRs of *Citrobacter freundii* (19) and *Proteus mirabilis* (16), which are both membrane bound. However, the estimated molecular weight of 132,000 for 16B constitutive TSO-TTR compares with previously reported molecular weights of 115,000 for TSO from *T. thioparus* (12) and 120,000 for TTR from *P. mirabilis* (18). The K_m of tetrathionate estimated for 16B constitutive TTR is lower than the value of 2.1 mM reported for TTR from *C. freundii* (19).

A second TTR activity associated with strain 16B is inducible (Table 1). The inducible activity was easily separated from constitutive TTR in soluble extract fractions by DEAE chromatography (Fig. 5). TSR activity was induced concurrently with TTR (Table 2). Tetrathionate or thiosulfate were necessary for induction of both enzyme activities and are therefore potential inducers. Whether one or the other of the potential inducers is solely responsible for induction of only one or both of the enzymes cannot be determined from our experiments. For example, under aerobic, oxygen-deprived culture conditions, thiosulfate is rapidly oxidized to tetrathionate by TSO during aerobic growth (31), but during oxygen deprivation, constitutive TTR reduces tetrathionate to thiosulfate (29). Under anaerobic growth conditions, tetrathionate is reduced to thiosulfate (29, 34). Therefore, neither tetrathionate nor thiosulfate could be excluded from the growth medium.

Inducible TTR and TSR synthesis was repressed when cultures were aerated, even in the

presence of inducer (Tables 1 and 2). This indicates repression of the 16B enzymes by oxygen and is consistent with oxygen repression of inducible TTRs from *Salmonella* sp. (19) and *C. freundii* (7) as well as TTR and TSR from *P. mirabilis* (5). Both TTR and TSR in 16B are involved in anaerobic respiration (34). Soil isolate A-50 is the only bacterium reported so far as having an inducible TTR whose synthesis is not repressed by oxygen (28). However, this TTR has a low affinity for tetrathionate, and the bacterium is unable to use tetrathionate as a terminal electron acceptor for anaerobic growth (28).

Inducible TTR and TSR activities were located in both soluble and particulate fractions of 16B cell extracts (Tables 1 and 2). In contrast, TSRs in the obligately anaerobic sulfate-reducing bacteria are located exclusively in soluble fractions of cell extracts (26). This generalization includes *Desulfovibrio gigas* TSR, which also has TTR activity (6). However, the soluble nature of a large portion of inducible TTR activity in 16B differs from TTRs of other facultatively anaerobic bacteria. *C. freundii* TTR is chiefly membrane bound (14, 19), and the TTR of *P. mirabilis* is associated with membrane particles (16).

Several observations indicate a possible association of inducible TTR and TSR in 16B. Both activities were induced and repressed under the same conditions; neither was bound by a DEAE-Sephadex column, both were retained by an ultrafiltration membrane which allows passage of molecules smaller than 100,000 daltons, and both were always found in the same extract fractions. With the possible exception of the differences in the ratios of their activity in different extract fractions which were likely due in part to extract instability, our observations suggest that inducible TTR and TSR activities may be catalyzed by the same protein, similar to the TTR-TSR of *P. mirabilis* (18).

Unlike intact cells of 16B which reduce all available tetrathionate before reducing thiosulfate (34), TTR-TSR extracts appeared to reduce both substrates simultaneously (Table 3). These results are in contrast to purified TTR-TSR from *P. mirabilis*, which first reduces tetrathionate and then thiosulfate (17). The discrepancy between 16B TTR and TSR activity in whole cells and cell extracts may be explained by the Eh (redox potential) of the assay conditions. Since reduced benzyl viologen was always saturating in the reaction mixtures due to regeneration, the Eh conditions of the in vitro assay were likely lower than physiological conditions. This may have allowed reduction of both substrates simultaneously. Under physiological conditions, electrons may reduce tetrathionate, when present, before the redox potential is low enough to reduce thiosulfate.

A significant amount of inducible TTR activity was lost from extracts when the membranes were removed (Table 1). For example, the additive total of the activities in SII, SIID, and MI fractions from induced extracts was much lower than the values for the crude SI extracts. This loss of activity could be explained by separation of the endogenous electron transport system from the enzyme. If this is so, the activity measured in fraction SI may represent electrons passed through both the native and the artificial electron transfer systems. The thiosulfate reductase system from *D. gigas* contains four components: two cytochromes, hydrogenase, and a terminal reductase (6). This type of multicomponent complex is common among electron transport systems. A multicomponent electron transfer complex for TTR may be present in 16B.

LITERATURE CITED

1. **Akagi, J. M., and L. L. Campbell.** 1962. Studies on thermophilic sulfate-reducing bacteria. J. Bacteriol. **84**:1194–1201.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Castor, L. N., and B. Chance.** 1959. Photochemical determination of the oxidases of bacteria. J. Biol. Chem. **234**:1587–1623.
4. **Chramback, A., R. A. Resifeld, M. Wyckoff, and J. Zaccari.** 1967. A procedure for rapid and sensitive staining of proteins fractioned by polyacrylamide electrophoresis. Anal. Biochem. **20**:150–154.
5. **deGroot, G. N., and A. H. Stouthamer.** 1970. Regulation of reductase formation in *Proteus mirabilis*. III. Influence of oxygen, nitrate and azide on thiosulfate and tetrathionate reductase formation. Arch. Mickrobiol. **74**:326–339.
6. **Hatchikian, E. C.** 1975. Purification and properties of thiosulfate reductase from *Desulfovibrio gigas*. Arch. Microbiol. **105**:249–256.
7. **Kapralek, F., and F. Pichinoty.** 1970. The effects of oxygen on tetrathionate reductase activity and biosynthesis. J. Gen. Microbiol. **62**:95–105.
8. **Kelly, D. P., L. A. Chambers, and P. A. Trudinger.** 1969. Cyanolysis and spectrophotometric estimation of trithionate in mixture with thiosulfate and tetrathionate. Anal. Chem. **41**:898–901.
9. **Klotz, I. M., and D. W. Darnell.** 1969. Protein subunits: a table. Science **1661**:126–128.
10. **Kuenen, J. G.** 1975. Colorless sulfur bacteria and their role in the sulfur cycle. Plant Soil **43**:49–76.
11. **Lenhoff, H. M., and N. O. Kaplan.** 1955. Cytochrome *c* and cytochrome peroxidase from *Pseudomonas fluorescens*. Methods Enzymol. **2**:758–764.
12. **London, J. P., and S. C. Rittenburg.** 1964. Path of sulfur in sulfide and thiosulfate oxidation by thiobacilli. Proc. Natl. Acad. Sci. U.S.A. **52**:1183–1190.
13. **Lyric, R. M., and I. Suzuki.** 1970. Enzymes involved in the metabolism of thiosulfate by *Thiobacillus thioparus*. III. Properties of thiosulfate-oxidizing enzyme and proposed pathway of thiosulfate oxidation. Can. J. Biochem. **48**:355–363.
14. **Novotny, C.** 1978. Instability of the tetrathionate respiratory chain of *Citrobacter freundii*. Folia Microbiol. **23**:428–432.

15. **Ogita, Z., and C. L. Markert.** 1979. A miniatured system for electrophoresis on polyacrylamide gels. Anal. Biochem. **99**:233–241.
16. **Oltmann, L. F., G. S. Schenmaker, and A. H. Stouthamer.** 1974. Solubilization and purification of a cytoplasmic membrane bound enzyme catalyzing tetrathionate and thiosulfate reduction in *Proteus mirabilis*. Arch. Microbiol. **98**:19–30.
17. **Oltmann, L. F., and A. H. Stouthamer.** 1975. Reduction of tetrathionate, trithionate and thiosulfate, and oxidation of sulfide in *Proteus mirabilis*. Arch. Microbiol. **105**:135–142.
18. **Oltmann, L. R., E. G. Van Der Beek, and A. H. Stouthamer.** 1975. Reduction of inorganic sulfur compounds by facultatively anaerobic bacteria. Plant Soil **43**:153–169.
19. **Pichinoty, F., and J. Bigliardi-Rouvier.** 1963. Recherches sur la tetrathionate-reductase d'une bacterie anaerobie facultative. Biochim. Biophys. Acta **67**:366–378.
20. **Roy, A. B., and P. A. Trudinger.** 1970. The biochemistry of inorganic compounds of sulfur. Cambridge University Press, London.
21. **Ruby, E. G., C. O. Wirsen, and H. W. Jannasch.** 1981. Chemolithotrophic sulfur-oxidizing bacteria from the Galapagos rift hydrothermal vents. Appl. Environ. Microbiol. **42**:317–324.
22. **Smith, A. J.** 1966. The role of tetrathionate in the oxidation of thiosulfate by *Chromatium* sp. strain D. J. Gen. Microbiol. **42**:371–380.
23. **Starkey, R. L.** 1934. Isolation of some bacteria which oxidize thiosulfate. Soil Sci. **39**:197–219.
24. **Starkey, R. L.** 1950. Relations of microorganisms to transformations of sulfur in soils. Soil Sci. **70**:55–65.
25. **Tanford, C., K. Kawahara, and S. Lapanje.** 1967. Proteins as random subcoils. I. Intrinsic viscosities and sedimentation coefficients in concentrated guanidine hydrochloride. J. Am. Chem. Soc. **89**:729–736.
26. **Thauer, R. K., K. Jungerman, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100–180.
27. **Trudinger, P. A.** 1961. Thiosulfate oxidation and cytochromes in *Thiobacillus X*. 2. Thiosulfate-oxidizing enzyme. Biochem. J. **78**:680–686.
28. **Trudinger, P. A.** 1967. Metabolism of thiosulfate and tetrathionate by heterotrophic bacteria from soil. J. Bacteriol. **93**:550–559.
29. **Tuttle, J. H.** 1980. Thiosulfate oxidation and tetrathionate reduction by intact cells of marine pseudomonad strain 16B. Appl. Environ. Microbiol. **39**:1159–1166.
30. **Tuttle, J. H.** 1980. Organic carbon utilization by resting cells of thiosulfate-oxidizing marine heterotrophs. Appl. Environ. Microbiol. **40**:516–521.
31. **Tuttle, J. H., P. E. Holmes, and H. W. Jannasch.** 1974. Growth rate stimulation of marine pseudomonads by thiosulfate. Arch. Microbiol. **99**:1–14.
32. **Tuttle, J. H., and H. W. Jannasch.** 1972. Occurrence and types of thiobacillus-like bacteria in the sea. Limnol. Oceanogr. **17**:532–543.
33. **Tuttle, J. H., and H. W. Jannasch.** 1973. Sulfide and thiosulfate-oxidizing bacteria in anoxic marine basins. Mar. Biol. **20**:64–70.
34. **Tuttle, J. H., and H. W. Jannasch.** 1973. Dissimilatory reduction of inorganic sulfur by facultative anaerobic marine bacteria. J. Bacteriol. **115**:732–737.
35. **Tuttle, J. H., and H. W. Jannasch.** 1977. Thiosulfate stimulation of microbial dark assimilation of carbon dioxide in shallow marine waters. Microbial Ecol. **4**:9–25.
36. **Tuttle, J. H., and H. W. Jannasch.** 1979. Microbial dark assimilation of CO_2 in the Cariaco Trench. Limnol. Oceanogr. **24**:746–753.
37. **Tuttle, J. H., J. H. Schwartz, and G. M. Whited.** 1983. Some properties of thiosulfate-oxidizing enzyme from marine heterotroph 16B. Appl. Environ. Microbiol. **46**:438–445.
38. **Umbreit, W. W., R. H. Burris, and J. F. Stauffer.** 1972. Manometric and biochemical techniques, p. 52–53. Burgess Publishing Co., Minneapolis, Minn.
39. **Vitolins, M. I., and R. J. Swaby.** 1969. Activity of sulphur oxidizing microorganisms in some Australian soils. Aust. J. Soil Res. **7**:171–183.