Use of Reduced Sulfur Compounds by *Beggiatoa* spp.: Enzymology and Physiology of Marine and Freshwater Strains in Homogeneous and Gradient Cultures

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The marine *Beggiatoa* strains MS-81-6 and MS-81-1c are filamentous, gliding, colorless sulfur bacteria. They have traditionally been cultured in very limited quantities in sulfide gradient media, where they grow as chemolithoautotrophs, forming a thin horizontal plate well below the air-agar interface. There, the facultatively chemolithoautotrophic strain MS-81-6 quantitatively harvests the flux of sulfide diffusing from below and oxidizes it to sulfate by using oxygen as the electron acceptor. Only recently have these strains been cultivated in bulk in defined liquid media (K. D. Hagen and D. C. Nelson, *Appl. Environ. Microbiol.* 62:947–953, 1996). In the current study, the obligately chemolithoautotrophic strain MS-81-1c was shown to have, despite much greater storage of elemental sulfur, an apparent Y_{H_2S} twice that of MS-81-6 when the two strains were grown in identical sulfide-limited gradient media. While the basis of this difference in energy conservation has not been established, differences in sulfur oxidation enzymes were noted. Strain MS-81-1c appeared to be able to oxidize sulfite by using either the adenosine phosphosulfate (APS) pathway or a sulfite:acceptor oxidoreductase. APS pathway enzymes (ATP sulfurylase and APS reductase) were present at relatively high and constant levels regardless of growth conditions, while the sulfite:acceptor oxidoreductase activity varied at least eight-fold, with the highest activity produced in sulfide gradient medium. By contrast, strain MS-81-6 showed no detectable activity of the APS pathway enzymes and possessed a sulfite:acceptor oxidoreductase activity just sufficient to account for its observed rate of growth in sulfide gradient medium. Freshwater strain OH-75-2a showed activity and regulation of sulfite:acceptor oxidoreductase consistent with lithotrophic energy conservation, a feature not yet proven for any freshwater *Beggiatoa* strain.

Filamentous bacteria of the genus *Beggiatoa* are found in sulfur springs, in organic-rich freshwater sediments, in coastal marine sediments and salt marshes, and at deep-sea hydrothermal vents. The filaments grow at the narrow oxic-anoxic interface where hydrogen sulfide and oxygen coexist and can be identified by their gliding motility, by their lack of photosynthetic pigments, and by the presence of periplasmic globules of elemental sulfur (29). The ability to oxidize reduced sulfur compounds places *Beggiatoa* spp. within a large and diverse group of sulfur-oxidizing microorganisms which includes the thiobacilli and other colorless sulfur bacteria, the purple and green photosynthetic sulfur bacteria, and several thermophilic *Archaea*. These organisms gain energy from the oxidation of compounds such as hydrogen sulfide, thiosulfate, polythionates, and elemental sulfur, which are found in nature as a consequence of geological and industrial processes and of the anaerobic mineralization of organic matter by dissimilatory sulfate reduction. It has been estimated that dissimilatory sulfate reduction alone results in the production of as much as 10^{10} tons of hydrogen sulfide per year (17). In coastal sediments, 50% to more than 95% of the H_2S produced is reoxidized by sulfur-oxidizing bacteria or in spontaneous nonbiological reactions (14). Since marine *Beggiatoa* spp. have been shown to oxidize hydrogen sulfide at rates which are several thousand times greater than the rates of spontaneous chemical oxidation when positioned at the oxic-anoxic interface (15, 32), it is likely that these bacteria make a significant contribution to the sulfur cycle in the sediments where they reside.

Difficulties encountered in the isolation and cultivation of

Beggiatoa spp., along with controversy regarding the physiological role of sulfur compound oxidation in freshwater strains (29), have hindered investigations of their metabolism. Consequently, very little is known about the mechanisms used to oxidize hydrogen sulfide and thiosulfate. Other sulfur-oxidizing bacteria, such as the thiobacilli, are known to utilize a variety of complex pathways to produce sulfate and partially oxidized compounds from reduced sulfur compounds (18). Since the pathways and the enzymes catalyzing these oxidations vary greatly from organism to organism, it is important to examine a number of different sulfur-oxidizing bacteria to better understand the potential contribution of each to the global sulfur cycle. In this study, marine and freshwater *Beggiatoa* strains were examined to identify the enzymes catalyzing some steps in the oxidation of reduced sulfur compounds and to determine the relative efficiencies with which obligately or facultatively chemoautotrophic strains produce biomass from H_2S oxidation.

MATERIALS AND METHODS

Strains and media. The chemoautotrophic marine *Beggiatoa* strains MS-81-6 and MS-81-1c (34) were routinely maintained in 16- by 150-mm screw-capped tubes of JG8 sulfide gradient medium (29). This is a semisolid medium which, except for vitamins and agar, contains only inorganic constituents. When initially poured, only the lower third of the agar contained 8 mM neutralized Na_2S , and oxygen was principally in the headspace. After 3 days of curing, opposed gradients of sulfide and oxygen had developed (33) and the medium could be inoculated. Cells for enzyme assays were grown in this medium or in glycylglycine-buffered T10A1 liquid medium (12) containing thiosulfate (10 mM) and acetate (1 mM). Liquid media lacking acetate (T10) or both glycylglycine and acetate (T10B) were also employed. T10B was buffered with bicarbonate (20 mM). All media were inoculated with cells harvested from 7-day-old gradient cultures. Microoxic conditions were maintained in liquid cultures of marine strains by sparging with a humidified gas mixture consisting of 0.25% O_2 , 0.25% CO_2 , and 99.5% N_2 .

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The heterotrophic *Beggiatoa* strain OH-75-2a (30) was grown in freshwater sulfide gradient medium (29) or in liquid DHAT medium containing acetate (5 mM) and thiosulfate (5 mM) (12). Liquid cultures of this strain were aerated by continuous shaking on a rotary shaker (150 rpm).

Thiobacillus novellus was obtained from the American Type Culture Collection (ATCC 8093) and maintained on nutrient agar plates. When required as a positive control for enzyme assays, *T. novellus* was adapted from heterotrophic to autotrophic growth by the method of Charles and Suzuki (8).

Escherichia coli K-12 was obtained from the American Type Culture Collection (ATCC 10798) and was grown aerobically at 37°C in Luria-Bertani medium (40) on a rotary shaker.

Calculation of oxygen consumption by *Beggiatoa* strains growing in gradient media. Oxygen consumption and biomass production data were obtained with filaments grown at room temperature in JG8 sulfide gradient medium. O₂ consumption was determined from the depth of the bacterial plate as described previously (12), with the surface saturation value for O₂ taken as 250 μM and the diffusion coefficient for oxygen taken as $2.03 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ at 23°C (33).

Preparation of cell extracts for enzyme assays. Filaments from liquid cultures were harvested ($8,000 \times g$, 4°C, 10 min), resuspended in several volumes (2 to 3 ml) of ice-cold assay buffer, and passed twice through a chilled French pressure cell at 100 MPa. The crude extract was centrifuged at low speed ($2,000 \times g$, 4°C, 10 min) to remove elemental sulfur. The resulting low-speed supernatant was centrifuged for 30 min at $165,000 \times g$ and 4°C in a Beckman TL-100 ultracentrifuge. The clear, orange high-speed supernatant was retained for enzyme assays. The high-speed pellet, which lacked a visible elemental sulfur layer, was washed and resuspended in assay buffer. This cloudy, reddish-brown suspension was designated the membrane fraction.

Filaments grown in 10 to 20 tubes of gradient media were harvested with a Pasteur pipette and centrifuged for 10 min at $15,000 \times g$ to pellet the cells and agar solids. The pellet was resuspended in assay buffer, broken by passage through the French pressure cell, and centrifuged at $165,000 \times g$ as described above. Because the quantity of cells in each gradient tube was small and because the cells could not be harvested without a portion of semisolid agar (typically 0.75 to 1.0 ml per gradient tube), the protein concentration in cell extract prepared from gradient-grown *Beggiatoa* filaments was typically 50 times lower than that in extract prepared from cells grown in liquid medium. All assays of liquid- and gradient-grown cells were performed on high-speed supernatants unless specifically noted otherwise.

Sulfite:acceptor oxidoreductase, thiosulfate:acceptor oxidoreductase, and APS reductase assays. Sulfite:acceptor oxidoreductase (EC 1.8.2.1), thiosulfate:acceptor oxidoreductase (EC 1.8.2.2), and adenosine 5'-phosphosulfate (APS) reductase (EC 1.8.99.2) activities were determined by the ferricyanide method (1, 21, 26) with minor modifications. The reaction mixture for the sulfite:acceptor oxidoreductase assay contained cell extract, 50 mM Tris-HCl (pH 7.8), 1 mM K₃Fe(CN)₆, 250 μM EDTA, and 3 mM Na₂SO₃ in a final volume of 1.0 ml. When thiosulfate:acceptor oxidoreductase was assayed, 3 mM Na₂S₂O₃ replaced the sulfite. The reaction mixture for the APS reductase assay was identical to that for the sulfite:acceptor oxidoreductase assay but was supplemented with 750 μM AMP. Reactions were initiated with sulfite (or thiosulfate). The reduction of ferricyanide (extinction coefficient = $1.01 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) was monitored at 420 nm with a Gilford Response II UV-VIS spectrophotometer. The assay temperature was maintained at 25°C with a thermoset cuvette holder. For some assays, 50 μM horse heart cytochrome *c* type III or *Saccharomyces cerevisiae* cytochrome *c* (Sigma Chemical Co., St. Louis, Mo.) replaced ferricyanide as the electron acceptor. A molar extinction coefficient of $19.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (550 nm) was used for cytochrome *c*. Any nonenzymatic reduction of electron acceptor was accounted for by assaying cell extract which had been heat inactivated (100°C, 10 min). Controls lacking extract and/or substrate were also assayed. Specific activities were expressed as nanomoles of electron acceptor reduced minute⁻¹ milligram of protein⁻¹.

ATP sulfurylase, ADP sulfurylase, and adenylate kinase assays. Two assays were used to detect ATP sulfurylase (EC 2.7.7.4) activity. The first was the coupled colorimetric assay employed by Kramer and Cypionka (21), which was also used to detect ADP sulfurylase and adenylate kinase activities. The reaction mixture for this assay contained cell extract, 100 mM Tris-HCl (pH 7.5), 220 mM glucose, 8 mM MgCl₂, 0.75 mM NADP⁺, 0.5 mM APS, 3.5 U of hexokinase, and 0.35 U of glucose-6-phosphate dehydrogenase in a final volume of 1.0 ml. The reaction was initiated with 0.5 mM pyrophosphate (PP_i), and the reduction of NADP⁺ was monitored at 340 nm. A molar extinction coefficient of $6.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ was used for activity calculations. In ADP sulfurylase (EC 2.7.7.5) assays, 0.5 mM orthophosphate (P_i) replaced PP_i. ADP formed was converted to ATP and AMP by adenylate kinase. For adenylate kinase (EC 2.7.4.3) assays, 2 mM ADP replaced the APS and PP_i or P_i. Enzyme activities were expressed as nanomoles of NADP⁺ reduced minute⁻¹ milligram of protein⁻¹.

The more sensitive ATP sulfurylase ³⁵SO₄²⁻ release assay of Seubert et al. (42) was used to confirm the data obtained with the coupled assay described above and was particularly useful when cell extracts from gradient cultures were used. The reaction mixture for this assay contained cell extract, 50 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 100 μM PP_i, 20 μM unlabeled APS, and 0.02 to 0.5 μM [³⁵S]APS (10 to 25 mCi/μmol) in a total volume of 200 μl. The [³⁵S]APS, a gift from I. H. Segel, was synthesized as previously described (10). The reaction occurred at 25°C and was initiated with cell extract. At 1-min intervals thereafter,

20-μl aliquots were removed from both the control and experimental tubes and were placed in disposable conical centrifuge tubes containing 2.0 ml of an ice-cold solution of 2% activated charcoal, 100 mM Na₂SO₄, and 50 mM Tris-HCl (pH 7.8). The contents of the conical tubes were mixed immediately, and the tubes were placed on ice. After five aliquots had been removed from the experimental reaction mixture in each tube, two final 20-μl aliquots were taken to determine the total radioactivity added to the assay mixture. These were placed in conical tubes containing 2.0 ml of a 50 mM Tris-HCl-100 mM Na₂SO₄ solution lacking charcoal. The conical tubes containing charcoal were centrifuged for 5 min at $2,000 \times g$ in a clinical centrifuge. Any radiolabeled APS that did not react became adsorbed to the charcoal, while any ³⁵SO₄²⁻ produced remained in the supernatant. Aliquots (1.0 ml) of the supernatant from each conical tube were placed in scintillation vials with 10 ml of Aquasol II liquid scintillation cocktail, and the radioactivity was determined with a Beckman LC60001C liquid scintillation counter. ATP sulfurylase activity was expressed as nanomoles of ³⁵SO₄²⁻ released minute⁻¹ milligram of protein⁻¹.

Pyrophosphatase assay. Pyrophosphatase (EC 3.6.1.1) was assayed by the method of Bias and Trüper (5). The reaction mixture contained cell extract, 50 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, and 1 mM Na₂P₂O₇ in a final volume of 1.0 ml. To determine the rate of P_i formation, replicate tubes containing identical amounts of cell extract were prepared. The reaction was initiated with PP_i, and the mixture was incubated at 30°C. At sequential time points, 100 μl of 20% trichloroacetic acid (TCA) was added to a replicate to stop the reaction. After the reaction had been stopped in the final tube, the TCA concentration in each of the tubes was brought to 11.5%, and they were allowed to stand for 10 min at room temperature. The tubes were then centrifuged for 10 min at $15,000 \times g$ to remove precipitated protein. The phosphorus content of the supernatant was determined by the colorimetric method of Taussky and Shorr (46) and was compared to KH₂PO₄ standards (50 to 100 mg of phosphorus per liter of 11.5% TCA). Reagents and standards were prepared in acid-washed glassware to prevent contamination with exogenous phosphorus. After the addition of reagents, the samples were allowed to stand for 1 min, at which point the absorbance at 650 nm was determined.

Detection of APS by TLC. To detect APS, the product of the APS reductase reaction, APS reductase assays were performed with radiolabeled AMP. Radiolabeled APS was detected with thin-layer chromatography (TLC). For these experiments, the volume of the APS reductase reaction mixture was reduced to 200 μl and 0.18 μCi of [¹⁴C]AMP (ICN Radiochemicals) was added. Experimental reactions were initiated with sulfite. Control tubes initiated with water were incubated in parallel. The reactions were stopped by boiling briefly. The radiolabeled [¹⁴C]APS formed in these mixtures was separated from the AMP by TLC on 20-cm by 20-cm by 0.1-mm Polygram DEAE-cellulose MN300 plates. A 10-μl volume of each sample was spotted at intervals along a line 3.0 cm from the bottom of the plate. Standards (18 to 20 nmol of AMP, ADP, APS, and ATP) were spotted along the same line. The plate was developed for 30 min in 20 mM HCl as described by Randerath (38). Because they absorbed light in the UV range, the position of the adenosine nucleotides could be monitored with a hand-held UV lamp ($\lambda_{\text{max}} = 254 \text{ nm}$). After the chromatogram dried, the positions of the standard spots were measured, and the locations of the radiolabeled APS and AMP in the sample lanes were estimated by comparison to the standards. The TLC plate was cut vertically to separate each 1.5-cm lane, and the individual lanes were then cut in horizontal slices 1.0 to 3.0 cm in length. Each slice was placed in a scintillation vial containing 10 ml of Aquasol II, and the counts in each region of the plate were determined.

Protein determinations. Protein was determined by the Coomassie brilliant blue dye binding method of Bradford (7). Concentrated dye reagent was purchased from Bio-Rad Laboratories (Richmond, Calif.), and ovalbumin (Sigma Chemical Co.) was the protein standard. Details of the assay and of the methods used to hydrolyze agar with hot 10% TCA and precipitate protein from gradient tube samples were described previously (34).

Sulfur content of cells. Replicate tubes of JG8 sulfide gradient medium were inoculated with strains MS-81-1c and MS-81-6 as detailed elsewhere (12), and the bacteria were grown at room temperature. Periodically, entire bacterial plates were harvested from triplicate tubes for each strain, and the protein contents were determined. At each harvest date, an additional three or four replicates were analyzed for elemental sulfur content as follows. Bacterial plates were harvested, and the agar was hydrolyzed as in protein analyses. The pellets containing protein and elemental sulfur were extracted twice with 0.5 ml of carbon disulfide (high-pressure liquid chromatography grade; Sigma), the carbon disulfide extracts were pooled and evaporated, and the residue was assayed for sulfur by cyanolysis as previously described (30a). Results were corrected for the elemental sulfur content of solvent blanks.

RESULTS

Growth studies in gradient media. Biomass production and rates of oxygen consumption were determined for marine strains grown chemolithoautotrophically in JG8 sulfide gradient medium. The protein contents of gradient cultures of both strains increased almost linearly during the first 10 days, after

FIG. 1. Autotrophic, sulfide gradient cultures (JG8 medium) after inoculation ($t = 0$) with marine *Beggiatoa* strain MS-81-1c (2.1 µg of protein/tube) (squares) or strain MS-81-6 (3.3 µg of protein/tube) (triangles). (A) Biomass (per tube) versus time. (B) Specific rate of oxygen consumption versus culture age.

which the rate of growth decreased (Fig. 1A). Stationary phase was reached by day 17 (MS-81-1c; 119 µg of protein/tube) or by day 21 (MS-81-6; 91 µg of protein/tube). Cultures of strain MS-81-1c contained approximately 20 to 30% more protein than those of strain MS-81-6 at all time points shown. The two strains also differed in the rates at which they acidified gradient medium. As indicated by a change in the color of the phenol red indicator, MS-81-6 acidified the medium within 2 to 3 days following inoculation, while MS-81-1c did so only after several weeks of growth. Instantaneous oxygen consumption rates, calculated for each strain from the average depth of the cell plate at each time point, are shown in Fig. 1B. Specific rates of oxygen consumption were greatest for both strains during the early portion of arithmetic growth and declined continuously thereafter. By integrating oxygen consumption rates over a series of time intervals during this period, the average amount

TABLE 1. Molar growth yields for H₂S and O₂ for strains MS-81-1c and MS-81-6 grown in inorganic sulfide gradient medium (JG8)

Time interval (days)	Molar growth yield (g [dry wt] produced/mol of substrate consumed) for strain:			
	MS-81-6		MS-81-1c	
	Y _{O₂} ^a	Y _{H₂S} ^b	Y _{O₂} ^a	Y _{H₂S} ^c
3–5	4.1	7.1	14.3	17.3
3–7	5.0	8.7	13.5	15.9
3–10	5.3	9.1	12.8	14.5
Avg ± SD	4.8 ± 0.4	8.3 ± 0.6	13.5 ± 0.4	15.9 ± 1.4

^a Y_{O₂} values are based on protein values measured at the beginning and end of the time interval (converted by assuming 2.45 g [dry weight]/g of protein [32]) and O₂ consumption integrated over the same interval.

^b Calculated from the corresponding Y_{O₂} by assuming that strain MS-81-6 consumes 0.58 mol of H₂S per 1.00 mol of O₂ (32).

^c Calculated from the corresponding Y_{O₂} as follows (illustrated for data for days 3 to 5). The 14.3 g (dry weight) produced per 1.00 mol of O₂ consumed requires oxidation of 4.00 mol of H equivalents for energy generation. This dry weight corresponds to 0.596 mol of C, assuming that 50% of the dry weight is C. Reduction of CO₂ to the level of cell matter (CH₂O) to provide this dry weight would require 2.38 mol of H equivalents (4×0.596), yielding a total of 6.38 mol of H equivalents ($4.00 + 2.38$) consumed per 1.00 mol of O₂. To convert this into mol of H₂S consumed, we account for the S⁰ content of cells ($\text{H}_2\text{S} \rightarrow \text{S}^0 + 2\text{H}^+ + 2\text{e}^-$) and assume that the remainder of the sulfide is fully oxidized ($\text{H}_2\text{S} + 4\text{H}_2\text{O} \rightarrow \text{H}_2\text{SO}_4 + 8\text{H}^+ + 8\text{e}^-$). The S⁰ content of cells at day 5 (Fig. 2) converts to 0.04 mol (1.28 g of S⁰) per 14.3 g (dry weight). This would provide 0.08 mol of H equivalents, leaving 6.30 mol of H equivalents ($6.38 - 0.08$) to come from complete oxidation of H₂S. This implies an additional 0.788 mol of H₂S consumed ($6.30/8$), for a total of 0.828 mol of H₂S ($0.788 + 0.040$) consumed per 1.0 mol of O₂. This predicts a final Y_{H₂S} of 17.3 g (dry weight)/mol (14.3 g [dry weight]/mol of O₂⁻¹/0.828 mol of H₂S mol of O₂⁻¹).

of oxygen required by each strain for the production of biomass could be determined. For strain MS-81-1c, consumption during arithmetic growth (days 3 to 10) averaged 181 nmol of O₂/µg of protein produced, while for strain MS-81-6 it was 2.8 times greater, averaging 515 nmol of O₂/µg of protein. The corresponding Y_{O₂} values are 13.5 and 4.8 g (dry weight)/mol of O₂, respectively (Table 1). The corresponding Y_{H₂S} values (Table 1) (see Discussion) indicate that strain MS-81-1c produces roughly twice the biomass per unit of H₂S oxidized.

Elemental sulfur content of marine *Beggiatoa* spp. Microscopic observations during growth yield experiments showed that strain MS-81-1c became densely packed with globules of elemental sulfur. Subsequent quantitative experiments showed that the sulfur content of strain MS-81-1c began to increase significantly by day 4 (Fig. 2), reaching a sulfur/protein ratio of almost 1:1 by day 10. By contrast the sulfur content of strain MS-81-6 remained below the detection level of the assay (Fig. 2), although occasional internal globules could be observed microscopically.

Sulfite-dependent ferricyanide reduction by *Beggiatoa* cell extracts. Specific rates of sulfite-dependent ferricyanide reduction detected in extracts from marine and freshwater *Beggiatoa* strains and from two control organisms, *T. novellus* and *E. coli*, are shown in Table 2. In the presence of sulfite and absence of AMP, ferricyanide was reduced by extracts from all *Beggiatoa* spp. tested. Although the specific activities were sometimes low, there was a linear relationship between ferricyanide reduction rate and protein concentration. Specific activities detected in extracts from *Beggiatoa* filaments grown in liquid media containing thiosulfate and acetate were always below 100 nmol min⁻¹ mg of protein⁻¹. When filaments were grown in sulfide gradient media, however, specific activities were increased by four- to sixfold for strains OH-75-2a and MS-81-1c. There was no significant sulfite-dependent reduction of ferri-

FIG. 2. Change in ratio of elemental sulfur to protein for strains MS-81-1c (squares) and MS-81-6 (triangles) during growth in inorganic sulfide gradient medium. The medium and inoculation protocols were as for Fig. 1.

cyanide when boiled cell extract controls were assayed, nor did ferricyanide reduction occur when thiosulfate or nitrate replaced sulfite, when mammalian or yeast cytochrome *c* replaced ferricyanide, or when sulfite or cell extract was omitted from the reaction mixture (data not shown).

Although the addition of AMP to the reaction mixture did not increase the rate of sulfite-dependent ferricyanide reduction by extracts from strains MS-81-6 and OH-75-2a, there was a dramatic increase in rates with extracts from strain MS-81-1c (Table 2). The AMP-dependent activity, i.e., that of APS reductase, in extracts from sulfide gradient-grown MS-81-1c was 2 to 3 times higher than the activity measured in the absence of AMP and was as much as 25 times higher in extracts from filaments grown with thiosulfate in liquid medium. There was no ADP- or ATP-dependent activity (data not shown).

Sulfite-dependent ferricyanide reduction assays with membrane and soluble fractions of strains MS-81-6, MS-81-1c, and

TABLE 2. Sulfite-dependent ferricyanide reduction by extracts of marine and freshwater *Beggiatoa* strains and controls

Organism	Medium ^a	Activity ^b	
		AMP independent	AMP dependent
MS-81-1c	Marine H ₂ S gradient (JG8)	252–312	570–735
	S ₂ O ₃ ²⁻ (T10)	42	741
	S ₂ O ₃ ²⁻ + acetate (T10A1)	57–61	771–1320
MS-81-6	Marine H ₂ S gradient (JG8)	24–99	0
	S ₂ O ₃ ²⁻ + acetate (T10A1)	29–77	0
OH-75-2a	Freshwater H ₂ S gradient + acetate	190–317	0
	S ₂ O ₃ ²⁻ + acetate (DHAT)	29–86	0
<i>T. novellus</i>	S ₂ O ₃ ²⁻ only	141	ND ^c
<i>E. coli</i>	LB ^d	0	ND

^a The medium was a homogeneous liquid unless gradient (agar gelled) is specified.

^b Activities are reported as nanomoles of ferricyanide reduced minute⁻¹ milligram of protein⁻¹. The AMP-dependent activity was calculated after the subtraction of any AMP-independent activity.

^c ND, not determined.

^d LB, Luria-Bertani medium.

TABLE 3. Sulfite-dependent ferricyanide reduction in membrane and soluble fractions prepared from 2,000 × *g* clarified crude cell extracts of *Beggiatoa* strains MS-81-1c, MS-81-6, and OH-75-2a grown in acetate- and thiosulfate-supplemented liquid medium (T10A1 or DHAT)

Strain	Fraction	Activity ^a	
		AMP independent	AMP dependent
MS-81-1c	Crude	74	783
	Membrane	100–107	186–268
	Soluble	60–62	1,098–1,320
MS-81-6	Crude	39–42	0
	Membrane	53–68	0
	Soluble	1–5	0
OH-75-2a	Crude	22	0
	Membrane	23	0
	Soluble	12	0

^a Activities are reported as nanomoles of ferricyanide reduced minute⁻¹ milligram of protein⁻¹. The AMP-dependent activity was calculated after the subtraction of any AMP-independent activity.

OH-75-2a. Membrane and soluble fractions were prepared from cells grown in liquid media and were assayed in an attempt to localize the AMP-independent and AMP-dependent activities (Table 3). The AMP-dependent activity detected in strain MS-81-1c extracts was clearly located in the soluble fraction. After centrifugation, the AMP-dependent activity in the soluble fraction had nearly doubled relative to that in the crude extract, while the AMP-dependent activity in the membrane fraction was three to four times lower. By contrast, the AMP-independent activity detected in all strains appeared to be associated with the membrane, since the activity found in the membrane fraction was always greater than the activity measured in the soluble fraction or crude extract.

To confirm that the AMP-dependent activity detected in soluble extracts from strain MS-81-1c was due to the enzyme APS reductase, APS was identified by TLC of radiolabeled reaction mixtures. Under the conditions used here, the *R_f* values of the UV absorbing adenosine nucleotide standards were as follows: ATP, 0.26; APS, 0.32; ADP, 0.42; and AMP, 0.52. Although [¹⁴C]APS formed during the course of the assay could not be visualized with UV light, its location could be determined from the position of the APS standard. In Table 4, evidence is given for the sulfite-dependent production of APS by extracts from strain MS-81-1c filaments grown in the presence of acetate and thiosulfate. The radioactivity per cellulose plate slice as a percentage of the total radioactivity applied in each lane is shown. In the control lanes, which were spotted with reaction mixtures that lacked sulfite, there was no evidence for APS production. In these lanes, approximately 99% of the total radioactivity applied at the origin was found in the AMP slice, while the remainder was distributed equally among the other six slices. In the lanes spotted with MS-81-1c reaction mixtures that contained sulfite, the results were very different. After 1 min, only 45% of the total counts remained in the AMP slice, while 46% of the counts appeared in APS slice and 7.5% of the counts were found in the region between AMP and APS. APS was not detected for reaction mixtures from strain MS-81-6 filaments grown in either the presence or the absence of acetate.

Characteristics of APS reductase in soluble extracts from MS-81-1c. Under standard assay conditions with MS-81-1c sol-

TABLE 4. TLC for detection of [^{14}C]APS produced in APS reductase reaction mixtures containing soluble fractions from *Beggiatoa* strains grown in liquid medium containing 10 mM thiosulfate

Strain	Growth conditions (medium)	Assay conditions	% Radioactivity in the following region:						
			Below origin	Origin + ATP	APS	ADP	AMP	Above AMP	Below solvent front
MS-81-1c	With acetate (T10A1)	No SO_3^{2-} (control)	0.1	0.0	0.1	0.1	99.0	0.7	0.0
		With SO_3^{2-}	0.0	1.0	45.2	7.5	46.0	0.3	0.0
MS-81-6	No acetate (T10)	No SO_3^{2-} (control)	0.0	0.0	0.0	0.2	97.6	1.9	0.4
		With SO_3^{2-}	0.0	0.0	0.1	0.0	97.1	2.2	0.6
	With acetate (T10A1)	No SO_3^{2-} (control)	0.0	0.0	0.1	0.1	98.8	0.8	0.2
		With SO_3^{2-}	0.0	0.2	0.4	0.2	96.7	2.2	0.3

uble extracts, there was a linear relationship between ferricyanide reduction rate and protein concentration until the concentration of protein in the reaction mixture reached approximately 150 $\mu\text{g}/\text{ml}$. Based on Lineweaver-Burk plots, the apparent K_m s for sulfite and AMP were 1.8 mM and 130 μM , respectively (11). Substrate inhibition occurred at AMP concentrations of greater than 1 mM. APS reductase activity in MS-81-1c extracts was pH dependent, with maximum rates of AMP-dependent ferricyanide reduction occurring between pH 7.8 and 8.0. When soluble extracts were held at 4°C, as they were when routine assays were performed, there was no detectable loss in APS reductase activity over 7 h. Storage at -80°C was preferable, as there was only a 4% loss of activity in soluble extracts that were thawed and assayed after 4 weeks of storage.

ATP sulfurylase, ADP sulfurylase, adenylate kinase, and pyrophosphatase activities in *Beggiatoa* extracts. Assays for the enzyme ATP sulfurylase, which converts APS and PP_i to ATP and sulfate, were performed with soluble extracts from *Beggiatoa* strains MS-81-6, MS-81-1c, and OH-75-2a. The data obtained with both colorimetric and radioactive assays are shown in Table 5. When strain MS-81-1c was grown in liquid medium on thiosulfate in the presence or absence of acetate, ATP sulfurylase activity ranged from 996 to 1,978 $\text{nmol min}^{-1} \text{mg of protein}^{-1}$. The activity was equally high in sulfide gradient-grown cells (1,523 to 1,710 $\text{nmol min}^{-1} \text{mg of protein}^{-1}$). The activities measured in extracts from strain MS-81-6 grown in liquid T10A1 medium or in sulfide gradients were much lower, at 17 to 31 $\text{nmol min}^{-1} \text{mg of protein}^{-1}$. Little or no ATP sulfurylase activity was detected in strain OH-75-2a or *E. coli* extracts. When the colorimetric assay was used, the reaction was sometimes initiated with P_i rather than PP_i to test for the activity of ADP sulfurylase. Little or no activity was detected with P_i when it replaced PP_i in assays performed with extracts from strain MS-81-1c, MS-81-6, or OH-75-2a.

Adenylate kinase assays were performed with cell extracts from *Beggiatoa* strains MS-81-1c, MS-81-6, and OH-75-2a, while pyrophosphatase assays were performed only with extracts from strain MS-81-1c. The specific activities of both of these enzymes in the *Beggiatoa* strains tested here were comparable to the specific activities detected in *E. coli* extracts (data not shown).

DISCUSSION

Growth studies with gradient media. The differences in the rates of oxygen consumption detected in this study are consistent with the view that marine strain MS-81-1c derives more useful energy from the oxidation of reduced sulfur compounds than does strain MS-81-6. At all time points during growth, the

specific rates of oxygen consumption (picomoles of O_2 second $^{-1}$ microgram of protein $^{-1}$) for strain MS-81-6 were two to three times those for strain MS-81-1c (Fig. 1B). On average, strain MS-81-6 produced 2.8 times more biomass per unit of oxygen consumed (Table 1). Since oxygen consumption is directly related to electron transport, these data imply that strain MS-81-1c conserves more energy during sulfide oxidation and is able to produce more biomass per electron consumed.

These relative efficiencies of capturing energy from sulfide can be expressed quantitatively based on the following considerations. It was previously demonstrated (32) that the growth of strain MS-81-6 in sulfide gradient medium is limited by sulfide flux from day 3 onward. This results in arithmetic growth during which the bacteria quantitatively harvest soluble sulfide as it diffuses from the medium reservoir. Via gliding motility they also adjust their vertical position to maintain a flux of oxygen which is optimal for their needs, i.e., 1.00 mol of O_2 per 0.58 mol of H_2S (32). Applying this consumption ratio to the Y_{O_2} data from this study for strain MS-81-6 gives an average $Y_{\text{H}_2\text{S}}$ of 8.3 g (dry weight)/mol (Table 1, days 3 to 10), which is virtually identical to the value calculated by a different

TABLE 5. ATP and ADP sulfurylase activities measured in soluble fractions of cell extracts from several *Beggiatoa* strains

Organism	Medium ^a	ATP or ADP production ^b ($\text{nmol min}^{-1} \text{mg of protein}^{-1}$)		[^{35}S] SO_4^{2-} release ^c ($\text{nmol min}^{-1} \text{mg of protein}^{-1}$) (with PP_i)
		With PP_i	With P_i	
MS-81-1c	H_2S gradient (JG8)	ND ^d	ND	1,523–1,710
	$\text{S}_2\text{O}_3^{2-}$ (T10)	1,978	0	ND
	$\text{S}_2\text{O}_3^{2-}$ + acetate (T10A1)	996–1,480	2	ND
MS-81-6	H_2S gradient (JG8)	ND	ND	23–30
	$\text{S}_2\text{O}_3^{2-}$ + acetate (T10A1)	17–31	0	ND
OH-75-2a	$\text{S}_2\text{O}_3^{2-}$ + acetate (DHAT)	0	0	1
<i>E. coli</i>	LB ^e	0	ND	ND

^a The medium was a homogeneous liquid unless gradient (agar gelled) is specified.

^b Colorimetric assay.

^c Radioactive assay.

^d ND, not determined.

^e LB, Luria-Bertani medium.

method (32). In agreement with previous evidence that strain MS-81-6 completely oxidizes H_2S to SO_4^{2-} (32), the current study demonstrates negligible partial oxidation of H_2S to S^0 by strain MS-81-6 (Fig. 2). For strain MS-81-1c the calculation of $Y_{\text{H}_2\text{S}}$ is more complicated. It involves (i) accounting for the portion of H_2S that is incompletely oxidized to S^0 and (ii) calculating the fraction of total H equivalents consumed that is used for direct reduction of CO_2 (Table 1, footnote c). Both its average yield (15.9 versus 8.3 g [dry weight]/mol of H_2S) and the percentage of its total H_2S consumption that provides for direct reduction of CO_2 (36 versus 14%) are considerably higher than those for strain MS-81-6. The $Y_{\text{H}_2\text{S}}$ for strain MS-81-1c is at the high end of the range tabulated for thiosulfate for other free-living chemolithoautotrophs, while that for MS-81-6 is near the middle (16). This difference in efficiency may reflect either (i) a partial uncoupling of respiration and energy conservation in strain MS-81-6 or (ii) the presence in strain MS-81-1c of electron transport chain components such as *b*-type cytochromes, which, while not universal in chemoautotrophic sulfur bacteria, appear to allow more efficient energy conservation (16).

Because each strain was limited by the availability of sulfide, as is indicated by the nonexponential nature of the growth curves beyond day 3 (Fig. 1A), it is probable that all of the available sulfide was oxidized by the *Beggiatoa* filaments (32). Because the media were identical, it can be assumed that the sulfide fluxes intercepted were the same for both *Beggiatoa* strains. In spite of this, there was earlier medium acidification (phenol red color change at pH 6.4) by strain MS-81-6. Consistent with this observation, both the incomplete oxidation of sulfide (to elemental sulfur) by strain MS-81-1c (Fig. 2) and its greater use of sulfide to provide reductant for CO_2 fixation predict less acidification of the medium. These factors contribute to differences in the predicted $\text{H}_2\text{S}/\text{O}_2$ molar consumption ratio between strain MS-81-1c (0.83:1 to 0.87:1 [Table 1]) and strain MS-81-6 (0.58:1 [32]).

Oxidation of sulfite by extracts of *Beggiatoa* spp. The enzyme sulfite:acceptor oxidoreductase, found in all mesophilic sulfur-oxidizing bacteria examined to date (18), catalyzes the direct oxidation of sulfite to sulfate: $\text{SO}_3^{2-} + \text{H}_2\text{O} + \text{acceptor}_{\text{oxidized}} \rightarrow \text{SO}_4^{2-} + 2\text{H}^+ + \text{acceptor}_{\text{reduced}}$. Electrons from sulfite enter the electron transport chain via cytochrome *c* or cytochrome *b* (17, 45, 49), and ATP is generated via a chemiosmotic mechanism. The data presented here indicate that *Beggiatoa* strains also possess this activity. In the absence of AMP, all of the *Beggiatoa* strains examined here were capable of sulfite-dependent ferricyanide reduction, although the specific activities measured were sometimes low (Table 2). The specificity of the reaction for sulfite was indicated by the fact that little or no activity was detected in the absence of sulfite or in the presence of two sulfite analogs, thiosulfate and nitrite. Although there was a low, but measurable, rate of ferricyanide reduction even with the heat-treated controls (data not shown), this nonbiological rate was subtracted prior to calculation of the specific activities reported (Table 2).

Ferricyanide is the artificial electron acceptor most frequently used in assays for sulfite:acceptor oxidoreductase, although yeast or mammalian cytochrome *c* can often replace it and can sometimes be a more effective electron acceptor for the enzymes isolated from thiobacilli (9, 27). The physiological electron acceptor for sulfite:acceptor oxidoreductase is generally thought to be cytochrome *c* (9, 17, 22, 25, 49). With extracts from *Beggiatoa* strains, specific activities were very low when either yeast or mammalian cytochrome *c* replaced ferricyanide (data not shown), a phenomenon which has also been noted with the sulfite:acceptor oxidoreductases of phototrophic

bacteria (35) and certain thiobacilli (4, 44). The rate of AMP-independent sulfite oxidation measured in cell extracts from *Beggiatoa* strains might be higher if native cytochrome *c* was used as the electron acceptor.

Both soluble (9, 13, 27, 35) and membrane-associated (2, 4, 47) sulfite:acceptor oxidoreductases have been reported for phototrophic and chemoautotrophic sulfur bacteria. In the *Beggiatoa* strains examined here, sulfite:acceptor oxidoreductase activity appeared to be associated with the membrane (Table 3). The fact that some sulfite:acceptor oxidoreductase activity remained in the soluble fractions of strains MS-81-1c and OH-75-2a after centrifugation at $165,000 \times g$ may indicate that the enzymes are loosely associated with the membrane and are easily solubilized.

The low activities of sulfite:acceptor oxidoreductase detected in the *Beggiatoa* strains examined here are not surprising when their different metabolic capabilities and growth conditions are taken into account. Marine strain MS-81-1c grown in sulfide gradients or in liquid medium on thiosulfate does not require a highly active sulfite:acceptor oxidoreductase because it, unlike the other strains examined, has a second, very active enzyme, APS reductase, which can also catalyze sulfite oxidation. Low sulfite:acceptor oxidoreductase activity in the heterotrophic freshwater strain OH-75-2a might also be predicted, since this strain does not require reduced sulfur compounds for growth (30) and can obtain both carbon and energy from the acetate in freshwater liquid and sulfide gradient media. The same is true for marine strain MS-81-6 grown in the liquid T10A1 marine medium used in this study, since its growth in this medium was dependent upon the presence of both acetate and thiosulfate, and very little of the thiosulfate was oxidized (12). Only strain MS-81-6 grown chemoautotrophically in sulfide gradient medium would be expected to have high sulfite:acceptor oxidoreductase activity, since no additional mechanism for sulfite oxidation is known to exist in this strain.

Calculations were made to determine how high sulfite:acceptor oxidoreductase activity would have to be for strain MS-81-6 to support a doubling time of 100 h, approximately that observed between days 5 and 8, when growth was linear (Fig. 1A) (32). The following previously justified assumptions (32) were made: H_2S is completely oxidized to sulfate, with 86% of the electrons passed to O_2 and the remainder participating in CO_2 reduction; 2 mol of ferricyanide is reduced per mol of sulfite oxidized; protein comprises 50% of cell dry weight; and $Y_{\text{H}_2\text{S}}$ is 8.4 g (dry weight). It was then determined that a dry weight increment of 1% per h equates to a minimum sulfite:acceptor oxidoreductase activity of $68 \text{ nmol min}^{-1} \text{ mg of protein}^{-1}$, a value approximately in the middle of the range determined (Table 2) for these cultures grown in sulfide gradient medium. If it were possible to obtain a sufficient quantity of this strain from early growth in gradient medium (exponential doubling time, 11 h [32]), it is predicted that the sulfite:acceptor oxidoreductase activity would be much higher.

The presence in the freshwater strain OH-75-2a of a membrane-associated sulfite:acceptor oxidoreductase that significantly increases in activity in sulfide-grown cells is suggestive. There has never been a clear demonstration of energy generation linked to the oxidation of reduced sulfur compounds in any freshwater *Beggiatoa* strain (29), but these data suggest that at least one requisite enzyme is present and appropriately regulated.

In addition to sulfite:acceptor oxidoreductase, some sulfur-oxidizing bacteria utilize a second set of enzymes, APS reductase and ATP or ADP sulfurylase, that act together in what has been called the APS pathway (16). In the first step in the APS

pathway, the flavin-containing enzyme APS reductase catalyzes the AMP-dependent oxidation of sulfite to APS by using an unknown electron acceptor: $\text{SO}_3^{2-} + \text{AMP} + \text{acceptor}_{\text{oxidized}} \rightarrow \text{APS} + 2\text{e}^- + \text{acceptor}_{\text{reduced}}$. In the second step, the APS participates in a substrate-level phosphorylation reaction catalyzed by either ATP or ADP sulfurylase. In this reaction, PP_i or P_i is consumed, and sulfate and ATP or ADP is released: for ATP sulfurylase, $\text{APS} + \text{PP}_i \rightarrow \text{SO}_4^{2-} + \text{ATP}$, and for ADP sulfurylase, $\text{APS} + \text{P}_i \rightarrow \text{SO}_4^{2-} + \text{ADP}$.

An increase in the rate of sulfite-dependent ferricyanide reduction upon the addition of AMP was noted only with marine *Beggiatoa* strain MS-81-1c (Table 2). Further assays performed with extracts from this strain showed that APS was formed from sulfite and [^{14}C]AMP (Table 4); therefore, the 20-fold increase in rate seen with AMP was attributed to the presence of the enzyme APS reductase. The APS reductase activities were equally high regardless of how strain MS-81-1c was grown (Table 2) and are consistent, along with the correspondingly high ATP sulfurylase activities (Table 5), with the capacity to double in less than 10 h.

The pHs for optimal activity of APS reductases from various sulfur bacteria ranged from 7.2 to 8.7 with ferricyanide as the electron acceptor (6, 19, 20, 28, 37, 43, 48) and from 9.0 to 9.5 with cytochrome *c* (28, 48). The optimal pH in strain MS-81-1c occurred distinctly between 7.8 and 8.0 with ferricyanide, a value which fell in the middle of the range of the pH optima reported for the APS reductases of other sulfur bacteria. Activity with horse heart cytochrome *c* was very low for strain MS-81-1c, even at elevated pH (data not shown).

Kinetic studies with extracts of *Beggiatoa* strain MS-81-1c showed that the apparent K_m values for sulfite and AMP were 1.8 mM and 130 μM , respectively. These figures are similar to those reported for the APS reductases of a number of sulfur bacteria (6, 28, 37, 41, 43, 48). With strain MS-81-1c, APS reductase activity was inhibited by AMP when the concentration of AMP in the reaction mixture exceeded 1.0 mM. This phenomenon was also noted for other sulfur bacteria (19, 28), although the AMP concentration at which inhibition began to occur varied from as low as 100 μM to as high as 2 mM in these studies.

Whether ATP or ADP sulfurylase is the second enzyme of the APS pathway is a question that has been the subject of much debate, and the existence of ADP sulfurylase as an entity separate from ATP sulfurylase has recently been questioned (39). This controversy began with the work of Peck (36), who reported APS reductase activity plus both ATP sulfurylase and ADP sulfurylase activities in cell extracts from *Thiobacillus thioparus*. Questioning the intracellular availability of PP_i for the ATP sulfurylase reaction, he suggested that ADP sulfurylase was the enzyme most likely to be involved in sulfite oxidation and proposed a mechanism involving three enzymes acting in concert to explain the ATP sulfurylase activity he also detected in *T. thioparus* extracts:

By Peck's mechanism, pyrophosphatase (equation 1) converted PP_i to P_i , ADP sulfurylase (equation 2) catalyzed the P_i -dependent release of sulfate from APS, and adenylate kinase (equation 3) converted the ADP formed by ADP sulfurylase to AMP and ATP. Both pyrophosphatase and adenylate kinase activities were shown to be present in *T. thioparus*.

Since ATP was produced from APS and both P_i and PP_i in

T. thioparus, Peck's mechanism was sufficient to explain the results he saw with extracts from this organism. It does not, however, explain the data obtained in this study (Table 5). When cell extracts of strain MS-81-1c were assayed, high levels of ATP sulfurylase activity were detected, but ADP sulfurylase activity was essentially absent. The presence of ATP sulfurylase was confirmed with two different assays performed with reaction mixtures containing strain MS-81-1c extract. The spectrophotometric assay of Kramer and Cypionka (21) showed that PP_i - and APS-dependent ATP production occurred, while the assay of Seubert et al. (42) confirmed that radiolabeled sulfate was released from [^{35}S]APS upon the addition of PP_i to the reaction mixture. Little ATP production could be detected when P_i replaced PP_i , even though adenylate kinase and pyrophosphatase activities were both found in strain MS-81-1c extracts. PP_i was clearly the preferred substrate; therefore, the synthesis of ATP by the combined action of pyrophosphatase, ADP sulfurylase, and adenylate kinase is unlikely. Similar results have also been seen with two strains of *Chlorobium limicola* (5), with *Thiobacillus denitrificans* (3), and with the sulfur-oxidizing symbiont of the hydrothermal vent tube worm *Riftia pachyptila* (39). These organisms all contain ATP sulfurylase but have little or no ADP sulfurylase activity. As discussed in detail elsewhere (39), there are several potential sources of PP_i for the ATP sulfurylase reaction in sulfur bacteria.

While the high specific activities of ATP sulfurylase and APS reductase detected in marine strain MS-81-1c imply a dissimilatory role for these enzymes, the lack of APS reductase activity and the low ATP sulfurylase activity detected in strains MS-81-6 and OH-75-2a imply that their ATP sulfurylases may function in assimilatory sulfate reduction rather than in sulfite oxidation. The specific activities of ATP sulfurylase detected in extracts from strain MS-81-1c (996 to 1,978 $\text{nmol min}^{-1} \text{mg}$ of protein $^{-1}$) were roughly in the range measured for crude extracts from three organisms known to use ATP sulfurylases in dissimilatory sulfur metabolism: *C. limicola* (289 to 524 $\text{nmol min}^{-1} \text{mg}$ of protein $^{-1}$) (5), *Desulfovibrio desulfuricans* (840 to 3,700 $\text{nmol min}^{-1} \text{mg}$ of protein $^{-1}$) (21), and *T. denitrificans* (3,900 to 4,100 $\text{nmol min}^{-1} \text{mg}$ of protein $^{-1}$) (41a).

In contrast, the low levels of ATP sulfurylase (1 to 30 $\text{nmol min}^{-1} \text{mg}$ of protein $^{-1}$) detected in extracts from strains OH-75-2a and MS-81-6 are closer to the levels of ATP sulfurylase detected in extracts from *E. coli* grown in medium lacking organic sources of sulfur (0.2 to 6 $\text{nmol min}^{-1} \text{mg}$ of protein $^{-1}$) (24). Since strains OH-75-2a and MS-81-6 are both able to grow on acetate in medium containing sulfate as the only source of sulfur (30, 31), both strains are assumed to be capable of assimilatory sulfate reduction. Because the dissimilatory ATP sulfurylases of the sulfur-oxidizing and sulfate-reducing bacteria may be very different from the assimilatory enzymes of other organisms, the function of ATP sulfurylases found in various bacteria might best be determined by purification of the enzymes and determination of their kinetic and structural properties. Alternatively, probes specific for assimilatory and dissimilatory ATP sulfurylase genes could be utilized. Such a probe has recently been constructed from a portion of the ATP sulfurylase gene from the sulfur-oxidizing endosymbiont of *R. pachyptila*, a vestimentiferan tube worm found at deep-sea hydrothermal vents (23). The endosymbiont utilizes ATP sulfurylase and APS reductase in dissimilatory sulfur metabolism. Interestingly, the ATP sulfurylase gene probe hybridized with digested total genomic DNA from strain MS-81-1c but not with DNA from strains MS-81-6 and OH-75-2a (23), a result which supports the idea that the latter ATP sulfurylases are strictly assimilatory enzymes.

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