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Organic Carbon Utilization by Obligately and Facultatively Autotrophic *Beggiatoa* Strains in Homogeneous and Gradient Cultures

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Marine *Beggiatoa* strains MS-81-6 and MS-81-1c are filamentous gliding bacteria that use hydrogen sulfide and thiosulfate as electron donors for chemolithotrophic energy generation. They are known to be capable of chemolithoautotrophic growth in sulfide gradient media; here we report the first successful bulk cultivation of these strains in a defined liquid medium. To investigate their nutritional versatilities, strains MS-81-6 and MS-81-1c were grown in sulfide-oxygen gradient media supplemented with single organic compounds. Respiration rates and biomass production relative to those of controls grown in unsupplemented sulfide-limited media were monitored to determine whether organic compounds were utilized as sources of energy and/or cell carbon. With cells grown in sulfide gradient and liquid media, we showed that strain MS-81-6 strongly regulates two enzymes, the tricarboxylic acid cycle enzyme 2-oxoglutarate dehydrogenase and the Calvin cycle enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, in response to the presence of organic carbon (acetate) in the growth medium. In contrast, strain MS-81-1c lacked 2-oxoglutarate dehydrogenase activity and regulated ribulose-1,5-bisphosphate carboxylase/oxygenase activity only slightly in response to organic substrates. Tracer experiments with radiolabeled acetate showed that strain MS-81-1c did not oxidize acetate to CO_2 but could synthesize approximately 20% of its cell carbon from acetate. On the basis of these results, we conclude that *Beggiatoa* strain MS-81-1c is an obligate chemolithoautotroph, while strain MS-81-6 is a versatile facultative chemolithoautotroph.

The role of organic compounds in the nutrition of filamentous bacteria of the genus *Beggiatoa* has been debated for more than 100 years. These colorless gliding bacteria are found in marine and freshwater habitats where hydrogen sulfide and oxygen coexist at low concentrations. A defining feature of the genus is the presence of microscopically visible globules of elemental sulfur which are deposited in the periplasm when the cells are exposed to hydrogen sulfide or thiosulfate (30). The concept of chemolithotrophic energy generation (terminology is according to Brock and Schlegel [4]) arose from Winogradsky's early studies with freshwater enrichment cultures of *Beggiatoa* spp. (33); dilute organic compounds were assumed to be sources of cell carbon (4).

Over the last century, the ability of freshwater *Beggiatoa* strains to use organic compounds has been firmly established. Freshwater strains can grow chemoheterotrophically (15, 20, 31, 32) by using a limited number of organic substrates as sources of carbon and energy (17). They possess a functional tricarboxylic acid cycle with a glyoxylate bypass (15, 20). Although these strains deposit internal globules of elemental sulfur when grown in the presence of reduced sulfur compounds, they have not been shown to derive energy from the oxidation of sulfur compounds; the suggestion (8, 27, 31) that they can grow chemolithoheterotrophically or mixotrophically has not yet been confirmed (13, 17).

In contrast, the marine *Beggiatoa* strains available in pure culture can grow as microaerophilic chemolithoautotrophs in media containing hydrogen sulfide and carbon dioxide. Strain MS-81-6, for example, derived more than 90% of its cell carbon from CO_2 when grown in a sulfide-oxygen gradient medium containing [^{14}C]bicarbonate and exhibited ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC/O) activity comparable to that of autotrophic bacteria that fix CO_2 by the Calvin cycle (21). Preliminary studies with this strain (17, 21) implied that it, like the freshwater strains, was able to utilize organic compounds as sources of both carbon and energy. In the current study, we have investigated the nutritional versatilities of two marine *Beggiatoa* strains, MS-81-6 and MS-81-1c. The data presented here indicate they differ considerably with regard to their abilities to utilize organic compounds as anabolic and catabolic substrates for growth.

MATERIALS AND METHODS

Strains. Marine *Beggiatoa* strains MS-81-6 and MS-81-1c were isolated from the Great Sippewissett salt marsh in 1981 (24). Freshwater *Beggiatoa* strain OH-75-2a was isolated from a hot spring in Oregon in 1975 (20). Stock cultures were maintained in marine (JG8) or freshwater sulfide gradient media (18).

Gradient culture methods. Enzyme assays were performed with strain MS-81-6 and MS-81-1c filaments grown at room temperature for 3 to 4 days in tubes of unsupplemented JG8 sulfide gradient medium or in JG8 supplemented with acetate to a final concentration of 5 mM. In this and all other soft-agar gradient media, these microaerophilic bacteria grow as a thin "plate" below the air-agar interface.

Supplemented sulfide gradient media were used to determine the effects of single organic compounds on the growth yields and respiration rates of marine strains. Acetate, pyruvate, oxalacetate, propionate, fumarate, succinate, DL-lactate, L-(−)malate, 2-oxoglutarate, D-glucose, or ethanol was added to the top agar layer (2.5 g of Bacto-agar [Difco Laboratories, Detroit, Mich.] per liter) to a final concentration of 3 mg · atom of C per liter. Yeast extract was added to a final concentration of 0.01% (wt/vol). The initial sulfide concentration of the bottom agar layer was reduced from 8 mM to the minimum concentration which would support growth (2 and 4 mM for strains MS-81-6 and MS-81-1c, respectively). Unsupplemented sulfide gradient media containing Difco Bacto-agar or extensively washed Bacto-agar (3) were used as controls. Blended cells (200 μl; approximately 10 μg of protein) harvested from 7-day-old gradient cultures were uniformly applied to the surface of each tube, and the cultures were incubated at room temperature. At 4-day intervals, the vertical distance from the top of the *Beggiatoa* plate to the agar surface was determined for replicate tubes of each type. The cell plate from each tube was then harvested with a Pasteur pipette, and its protein content was determined. Oxygen consumption was determined

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from the depth of the plate as described previously, with the surface saturation value for O_2 taken as 250 μM (23) and the diffusion coefficient for O_2 taken as 2.03×10^{-5} cm^2 s^{-1} at 23°C (22).

The acetate-supplemented gradient medium used for microelectrode studies was initially constructed from a 10-ml lower layer (8.5 mM sulfide) overlaid with 30 ml of mineral medium (no sulfide). The assembly of the medium in a Leighton tube, its aging to establish an initial sulfide gradient, and its inoculation with strain MS-81-6 were exactly as described previously (22) except that 22 h prior to inoculation a 100-μl aliquot of sterile 120 mM acetate was added dropwise to the surface of the medium. By previously described procedures (22, 23), microprofiles of total soluble sulfide, pH, and oxygen were determined at 24°C with microelectrodes (147 to 149 h after inoculation) and used to calculate sulfide and oxygen fluxes. The total protein content of the *Beggiatoa* plate was determined 152 h after inoculation (22).

Liquid culture methods. Freshwater strain OH-75-2a filaments for enzyme assays were grown at 28°C in DHAT medium (5 mM acetate, 5 mM thiosulfate) which contained, per liter, 50 ml of ND stock (a nitrogen-free mineral base plus trace elements) (6), 0.13 g of $(NH_4)_2SO_4$, 2.60 g of HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid)], 0.035 g of K_2HPO_4, 1.36 g of $CH_3COONa \cdot 3H_2O$, and 1.24 g of $Na_2S_2O_3 \cdot 5H_2O$. The pH was adjusted to 7.2 prior to autoclaving. Cultures (250 ml) were inoculated with filaments from two 7-day-old freshwater gradient cultures (18) and shaken at 150 rpm on a rotary shaker until the cells reached late log phase (approximately 72 h).

Marine strains MS-81-6 and MS-81-1c were grown at room temperature in a 1-liter flask fitted with a butyl rubber stopper and a fritted glass gas dispersion tube. The liquid T5A1 medium developed for the bulk cultivation of these strains was a modification of JG8 marine gradient medium (18) in which artificial seawater (28) replaced natural seawater, glycylglycine (10 mM) was added as a buffer, and sulfide, bicarbonate, and agar were omitted. Sodium thiosulfate and sodium acetate were added to final concentrations of 5 and 1 mM, respectively. The medium was prepared in three portions: part 1 contained 14.55 g of NaCl, 2.65 g of $MgCl_2$, 2.17 g of $MgSO_4 \cdot 7H_2O$, 0.35 g of $CaCl_2 \cdot 2H_2O$, 0.53 g of KCl, and 1.00 g of glycylglycine dissolved in 530 ml of distilled water and adjusted to pH 8.2; part 2 contained 0.06 g of NH_4NO_3, 1.00 g of $Na_2S_2O_3 \cdot 5H_2O$, and 0.11 g of $CH_3COONa \cdot 3H_2O$ in 215 ml of distilled water; and part 3 contained 54 ml of mineral stock (18) plus 0.8 ml of SL8 trace element solution (26) prepared without $FeCl_2$ and EDTA. The pH of part 3 was adjusted to approximately 8.0 with 1 M KOH. After being autoclaved, the three portions were cooled and combined, and 0.32 ml of a filter-sterilized vitamin solution (18) was added. After inoculation with filaments from four 7-day-old gradient cultures (150 to 250 μg of total protein), the medium was aerated at a rate of 150 ml/min with a sterile, humidified gas mixture consisting of 0.25% oxygen, 0.25% carbon dioxide, and 99.5% nitrogen. The culture was continuously stirred (150 rpm) with a Teflon stir bar and stir plate. Cells for enzyme assays were grown in T10A1 medium, in which the thiosulfate concentration was increased to 10 mM, while T5 and T10 media for growth experiments lacked acetate.

Incorporation of acetate by growing MS-81-1c *Beggiatoa* filaments. T10A1 medium (800 ml) was supplemented with 0.20 μmol of [2-^{14}C]acetate (8.0 μCi/μmol; ICN Radiochemicals, Irvine, Calif.) for acetate incorporation experiments. Exit gasses from the growth flask were bubbled sequentially through three stoppered test tubes containing 10 ml of 10% benzethonium hydroxide (Sigma Chemical Co., St. Louis, Mo.) used to trap CO_2. For all samples, radioactivity was measured by liquid scintillation counting (Beckman Instruments, Fullerton, Calif.). The initial radioactivity present was determined from replicate samples (1.0 ml) taken immediately after inoculation. These samples and 0.5-ml aliquots periodically removed from the CO_2 traps were added to vials containing 10 ml of Aquasol-2 liquid scintillation cocktail (NEN Research Products, Boston, Mass.). When the culture reached stationary phase (pH 5.5), 1.0-ml aliquots were removed for final protein determinations. To determine the total radioactivity incorporated into cells, 10.0-ml samples were filtered onto Whatman GF/F filters, washed three times with sterile medium, and placed in vials with scintillation fluid. The culture was then divided into three equal volumes, and the cells were sedimented by centrifugation for 15 min at 8,000 × *g*. Unincorporated radioactivity was measured for two 1.0-ml supernatant aliquots taken from each bottle. The first was placed directly into a scintillation vial containing Aquasol-2 plus 0.3 ml of benzethonium hydroxide. The second was placed into an empty vial and shaken on an orbital shaker at 100 rpm for 4 h to allow the release of any dissolved radiolabeled CO_2; benzethonium hydroxide and Aquasol-2 were then added. Control experiments with [^{14}C]bicarbonate (ICN Radiochemicals) and [2-^{14}C]acetate showed that CO_2 was rapidly volatilized by this method: when 0.095 μCi of radiolabeled bicarbonate was added to 1.0 ml of T10A1 medium, less than 0.1% remained after 4 h when the pH of the medium was 7.0 or lower. Under the same conditions, radiolabeled acetate (0.007 μCi) was volatilized only when the pH of the medium was lower than 3.0.

Fractionation of radiolabeled cell pellets. Cell pellets (0.18 to 0.21 g [wet weight]) were fractionated by the extraction method of Cuhel (7). Four subcellular fractions (soluble pools, lipids, nucleic acids plus polysaccharides, and proteins) were obtained. Aliquots (1.0 ml) of each fraction were placed in scintillation vials, and Aquasol-2 (16.0 ml) was added prior to determination of radioactivity. Three replicate quench control vials were prepared for each fraction. The controls contained a known quantity of radiolabeled acetate (0.0087 μCi), 16.0 ml of Aquasol-2, and 1.0 ml of an appropriate mixture of all solvents present in the fraction. The total counts per minute of the quench control vials were never reduced by more than 3% relative to those of vials containing only 0.0087 μCi of acetate plus 16.0 ml of Aquasol-2.

Enzyme assays. RuBPC/O was assayed as described previously (25) with the following modifications. *Beggiatoa* filaments grown in liquid media were harvested by centrifugation at 8,000 × *g* and 4°C, resuspended in assay buffer, and broken with two passages through a chilled French pressure cell at 100,000 kPa. Crude cell extracts were centrifuged for 10 min at 15,000 × *g*, and the resulting supernatant was used for RuBPC/O assays. Filaments harvested from sulfide gradient tubes were centrifuged for 10 min at 15,000 × *g* to pellet the cells and agar solids. After the supernatant was discarded, the pellets were combined and resuspended in an equal volume of assay buffer. The cells were broken, and the crude extract was clarified as described above. Rates of ribulose-1,5-bisphosphate-dependent [^{14}C]bicarbonate incorporation were measured at 25°C and were corrected by subtracting any nonspecific CO_2 fixation by controls lacking ribulose-1,5-bisphosphate (<2% of the total).

2-Oxoglutarate dehydrogenase activity was assayed colorimetrically by the method of Brown and Perham (5). The methods used to prepare cell extracts were identical to those described above. After the addition of 25 to 200 μg of cell protein to the reaction mixture, the reaction was initiated with 2-oxoglutarate. The reduction of NAD^+ at 25°C was followed spectrophotometrically for 1 min. Rates were corrected by subtraction of any nonspecific rate obtained with heat-inactivated extract.

Protein determinations. Protein was determined by the Coomassie brilliant blue G-250 dye binding assay of Bradford (2). Ovalbumin (Sigma) was used as a protein standard. Concentrated dye reagent was purchased from Bio-Rad Laboratories (Richmond, Calif.). Details of the assay and of the method used to hydrolyze agar and precipitate protein from gradient tube samples with hot 10% trichloroacetic acid have been previously described (24).

Thiosulfate determinations. Samples (1.0 ml) taken during growth experiments were placed in microcentrifuge tubes and centrifuged at 15,000 × *g* for 10 min to remove cells and elemental sulfur. The thiosulfate concentration of the supernatant was determined colorimetrically by the cyanolysis method of Sörbo (29). Thiosulfate concentrations as low as 10 μM could be detected.

Acetate analysis. Acetate concentrations were determined by high-performance liquid chromatography with an IBM liquid chromatograph equipped with an Aminex HPX-87H ion exclusion column (300 by 7.8 mm) for organic acids (Bio-Rad Laboratories). Analyses were performed at 25°C at a flow rate of 0.5 ml/min, and detection was at 210 nm. The mobile phase was 3.5 mM H_2SO_4. Samples (1.0 ml) were filtered through 0.45-μm-pore-size Acrodisc filters (Gelman Sciences, Ann Arbor, Mich.), acidified with 40 μl of 0.5 M H_2SO_4, and immediately injected onto the column. Acetate standards prepared in acetate-free medium were treated in an identical manner. The peak areas of unknown samples were calculated, and acetate concentrations were determined by comparison with peak areas of acetate standards.

Elemental sulfur analysis. Replicate samples for elemental sulfur analysis were filtered and extracted with CS_2 as described previously (19) and were assayed by the colorimetric method of Bartlett and Skoog (1). The assay was linear to 100 μg of S^0 per ml, and quantities as low as 5 μg/ml could be detected.

RESULTS

Cultivation of marine *Beggiatoa* strains in liquid media. Strains MS-81-1c and MS-81-6 grew under microoxic conditions in liquid medium (T10) containing thiosulfate as a sole energy source and CO_2 as a sole source of carbon. Under these conditions, 5 to 7 μg protein was produced per μmol of thiosulfate consumed. The growing filaments rapidly acidified the medium and formed a thin film which coated the walls of the growth flask. Microscopic examination showed that individual filaments were filled with sulfur globules during all stages of growth.

The addition of acetate to cultures of MS-81-1c prevented the adherence of the filaments to the flask walls and decreased the lag period before exponential growth; thus, detailed growth studies with this strain were performed with thiosulfate-supplemented medium supplemented with acetate (T5A1). In this medium, MS-81-1c filaments grew in small, well-dispersed clumps. Final protein concentrations of 26 to 39 μg/ml were achieved. (For values at the lower end of this range, significant quantities of thiosulfate remained in the medium.) In contrast, strain MS-81-6 filaments growing in medium containing acetate often formed a mat at the interface between the liquid and the overlying gas mixture. Repeated attempts to disperse the cells by swirling the contents of the growth flask were unsuccessful. The maximum biomass achieved with strain MS-81-6 in

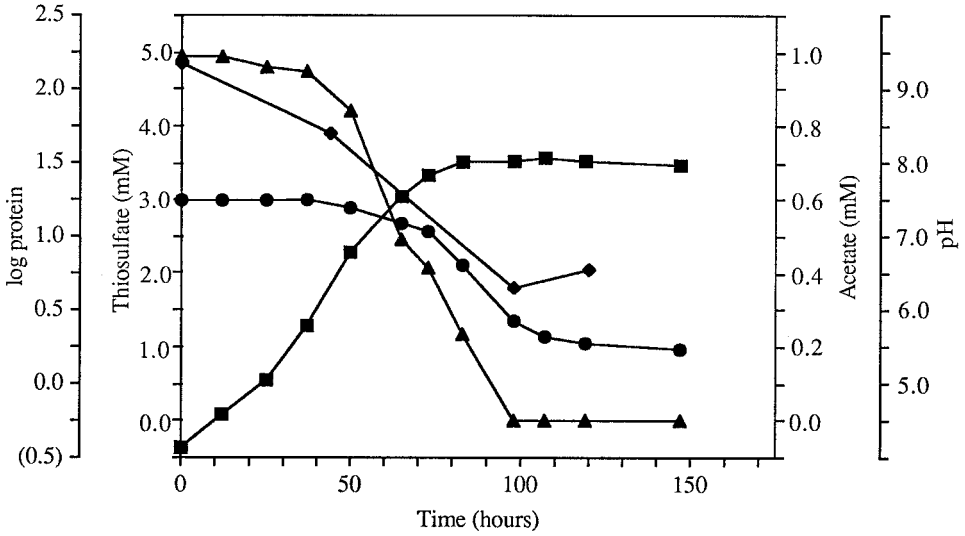

FIG. 1. Growth of *Beggiatoa* strain MS-81-1c in 800 ml of T5A1 liquid medium. ■, protein; ◆, acetate; ▲, thiosulfate; ●, pH.

medium containing acetate (32 μg of protein per ml) was within the range achieved with MS-81-1c.

A representative growth curve for strain MS-81-1c grown in T5A1 medium is shown in Fig. 1. The protein concentration began to increase within 12 h following inoculation and reached a maximum value of 34.2 mg/liter at 107 h (6.9 mg of protein produced per mmol of thiosulfate consumed). Consumption of the 4.0 mmol of thiosulfate initially present was complete by 98 h; however, only half of the 0.8 mmol of acetate initially added to the medium was consumed over the duration of the experiment. The pH of the medium declined from an initial value of 7.49 to a final value of 5.47; there was no such decline in pH in uninoculated flasks. During exponential growth, the ratio of internal elemental sulfur to protein averaged 0.13; by mid-stationary phase, this value had increased to 0.84 (data not shown).

Incorporation of acetate into strain MS-81-1c filaments during microaerophilic growth in T10A1 medium was monitored by using [2-^{14}C]acetate. The growth flask initially contained 800 μmol of total acetate (1.0 mM). Of the radioactive tracer added, 95.2% was recovered in either the cell pellet or the supernatant, with 36.5% of the total (293 μmol of acetate) incorporated into the *Beggiatoa* filaments. Less than 0.1% of the radioactivity was trapped as CO_2. A total of 84.9% ± 1.72% (mean ± standard deviation for three pellets fractionated separately) was recovered during the fractionation. The approximately 15% of the label that was lost was most likely from the highly volatile lipid fraction during heating steps. Of the radioactivity remaining, approximately one-third (29.1% ± 0.81%) was found in proteins, one-fourth (22.4% ± 1.11%) was found in lipids, and one-fourth (23.5% ± 1.51%) was found in nucleic acids and polysaccharides; 10.0% ± 0.23% was found in soluble pools.

Effect of organic compounds on the growth of marine *Beggiatoa* strains in sulfide gradient media. For strain MS-81-6, the addition of certain organic compounds to gradient cultures (2 mM initial sulfide concentration) had a strong influence on biomass production and on the depth of the *Beggiatoa* plate. Representative data for one such compound, acetate, are presented in Fig. 2. Plate depth accurately predicts the rate of oxygen consumption per gradient culture (23). On day 14, for example, the average depths of the plates in the acetate-supplemented tubes (7.2 mm) and in the control tubes (14.8 mm) correspond to respiration rates of 11.6 and 5.6 pmol of O_2 s^{-1} $tube^{-1}$, respectively, indicating a 2.1-fold enhancement in the total respiration of cells grown in the presence of acetate. The protein content of the acetate-supplemented tubes (86.8 μg per tube) was 2.6-fold higher than that of the control tubes (32.9 μg per tube). In general, the addition of acetate, oxala-

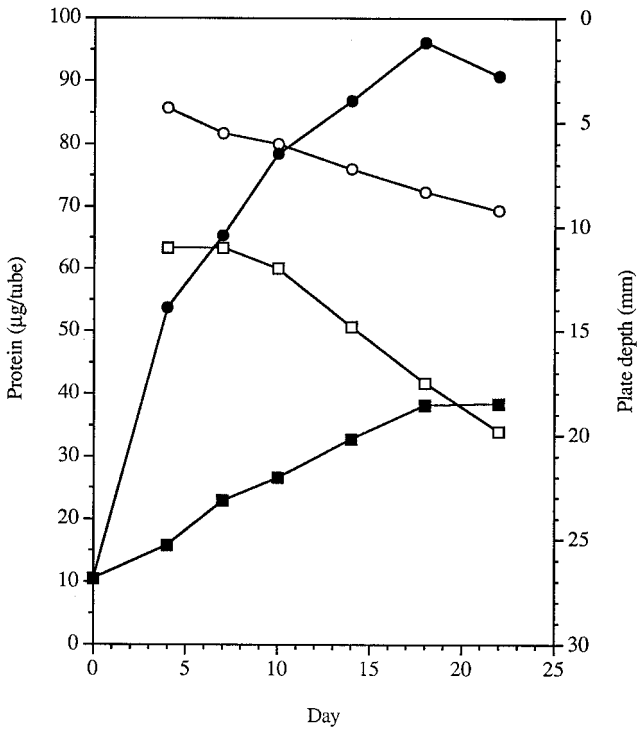

FIG. 2. Representative growth curves and plate depth measurements for *Beggiatoa* strain MS-81-6 in unsupplemented or supplemented (3 mg · atom of C per liter, as acetate) sulfide-oxygen gradient medium. The initial sulfide concentration of the bottom agar layer was 2 mM. ■, protein in unsupplemented medium; □, plate depth in unsupplemented medium; ●, protein in acetate-supplemented medium; ○, plate depth in acetate-supplemented medium.

TABLE 1. Effect of organic compounds on the growth of marine *Beggiatoa* strains in sulfide gradient media

Organic substrate (3 mg · atm of C/liter)	Strain MS-81-6				Strain MS-81-1c			
	Protein (μg/tube)[a]	Plate depth (mm)[b]	Yield enhancement[c]	Respiration enhancement[d]	Protein (μg/tube)	Plate depth (mm)	Yield enhancement	Respiration enhancement
None (control)	38.4	19.8	1.0	1.0	36.2	17.5	1.0	1.0
Acetate	96.1	8.3	2.5	2.4	43.4	16.5	1.2	1.1
Pyruvate	100.9	12.0	2.6	1.7	37.6	16.8	1.0	1.0
Oxalacetate	90.0	13.0	2.3	1.5	38.1	16.8	1.1	1.0
Propionate	88.3	8.3	2.3	2.4	33.1	18.0	0.9	1.0
Fumarate	76.3	11.0	2.0	1.8	33.4	16.8	0.9	1.0
Succinate	79.6	11.0	2.1	1.8	36.6	16.8	1.0	1.0
Lactate	63.3	12.8	1.7	1.6	40.0	18.5	1.1	1.0
Malate	70.2	13.3	1.8	1.5	41.0	18.0	1.1	1.0
Yeast extract	49.8	17.0	1.3	1.2	33.8	19.0	0.9	0.9
2-Oxoglutarate	40.5	17.5	1.1	1.1	36.1	18.0	1.0	1.0
Glucose	38.3	19.8	1.0	1.0	37.4	18.5	1.0	1.0
Ethanol	37.8	20.0	1.0	1.0	34.8	17.3	1.0	1.0

[a] Mean protein content of two sulfide gradient tubes when maximum biomass had been achieved.
[b] Mean plate depth of two tubes at point when maximum biomass had been reached.
[c] Mean protein content of supplemented tubes divided by mean protein content of unsupplemented controls.
[d] Mean plate depth of unsupplemented tubes divided by mean plate depth of supplemented controls.

cetate, propionate, or pyruvate to the medium resulted in a maximum biomass that was 2.3 to 2.6 times greater than that of the MS-81-6 controls (Table 1). A 1.5- to 2.2-fold increase in respiration rate was also observed with these substrates. Fumarate, lactate, malate, succinate, and yeast extract had a lesser effect upon growth. In the presence of these compounds, the maximum biomass achieved was increased 1.3- to 2.0-fold, while respiration rates were increased 1.2- to 1.8-fold. Of the compounds tested, only ethanol, glucose, and 2-oxoglutarate had no effect on biomass or respiration rates. Growth yields (17 day) in unsupplemented control media prepared with unwashed ($n = 11$) or washed ($n = 10$) Difco Bacto-agar were not significantly different by Student's t test ($P > 0.5$; average difference, 1.2 μg of protein per tube).

Organic substrates had little effect on the position of the strain MS-81-1c plate or on the maximum biomass achieved by this strain (Table 1). At a 4 mM initial sulfide concentration, only the addition of acetate resulted in a small but significant increase in the maximum biomass relative to that in control tubes which lacked organic substrates. The slight increase in respiration in the presence of acetate is within the range of experimental error. The 11 other organic substrates tested had no significant effect upon biomass production or respiration rates of strain MS-81-1c. Again, growth yields (20 day) in unsupplemented media containing unwashed ($n = 10$) or washed ($n = 10$) agar were not significantly different by Student's t test ($P > 0.2$; average difference, 3.6 μg of protein per tube). As with strain MS-81-6, the slightly higher yield was observed with the washed agar medium. All organic substrates tested appeared to inhibit the growth of MS-81-1c relative to that of unsupplemented controls when initial sulfide concentrations were 3 mM or lower (data not shown).

For strain MS-81-6 grown in acetate-supplemented sulfide gradient medium, the microprofiles of oxygen, pH, and total soluble sulfide (Fig. 3) show two features also seen in control cultures grown in the absence of added organic compounds (22). First, the sulfide and oxygen profiles terminate in the 850-μm-thick bacterial plate, demonstrating that the sulfide flux was quantitatively oxidized by the bacteria. Second, the depth at which the pH minimum occurs aligns closely with the sulfide-oxygen interface. The protein content of the entire culture (166 μg per tube, 152 h postinoculation) was 1.43-fold that of a nonacetate control (116 μg per tube, 153 h postinoculation). The ratio of oxygen consumption to sulfide consumption, deduced from the linear portions of their respective profiles below and above the bacterial plate (Fig. 3), was 2.25:1, while the value for a nonacetate control interpolated for 148 h postinoculation was 1.8:1 (22).

Enzyme activities. RuBPC/O activities were determined with extracts from marine and freshwater *Beggiatoa* filaments grown in liquid and gradient media (Table 2). When marine *Beggiatoa* strains were grown in unsupplemented gradient medium, comparable average RuBPC/O activities of 23 and 20.4 nmol of CO_2 fixed min^{-1} mg of $protein^{-1}$ were measured in extracts from MS-81-6 and MS-81-1c filaments, respectively. The presence of acetate affected each strain differently. With strain MS-81-6, RuBPC/O activities in gradient and liquid cultures supplemented with acetate were comparable but equalled only 15 to 17% of those detected in extracts from unsupplemented gradient cultures. In contrast, when strain MS-81-1c was grown in acetate-supplemented gradient and liquid media, RuBPC/O activities were roughly 70 and 150%, respectively, of those in unsupplemented gradient medium. Freshwater strain OH-75-2a grown in the presence of acetate and thiosulfate exhibited a very low RuBPC/O activity of 0.3 nmol of CO_2 fixed min^{-1} mg of $protein^{-1}$, which is nonetheless significantly above the limit of detection (16).

For strain MS-81-1c, the activity of 2-oxoglutarate dehydrogenase was never above the limit of detection regardless of whether the medium contained acetate, a sulfide gradient, or 10 mM thiosulfate (Table 2). For strain MS-81-6, however, growth in the presence of acetate in liquid or gradient medium resulted in 2-oxoglutarate dehydrogenase activity that was high (approximately 100 to 150 nmol min^{-1} mg of $protein^{-1}$) and comparable to that of heterotrophically grown freshwater strain OH-75-2a. Growth of strain MS-81-6 in the absence of acetate resulted in a dramatic reduction in 2-oxoglutarate dehydrogenase activity to below the detection limit.

In liquid cultures, strain MS-81-1c grew exponentially (8.7-h doubling time) between 25 and 60 h (Fig. 1). In sulfide gradient cultures of strain MS-81-1c, linear growth occurred as a result of substrate limitation (22), with the biomass approximately doubling between day 3 and day 6 (data not shown).

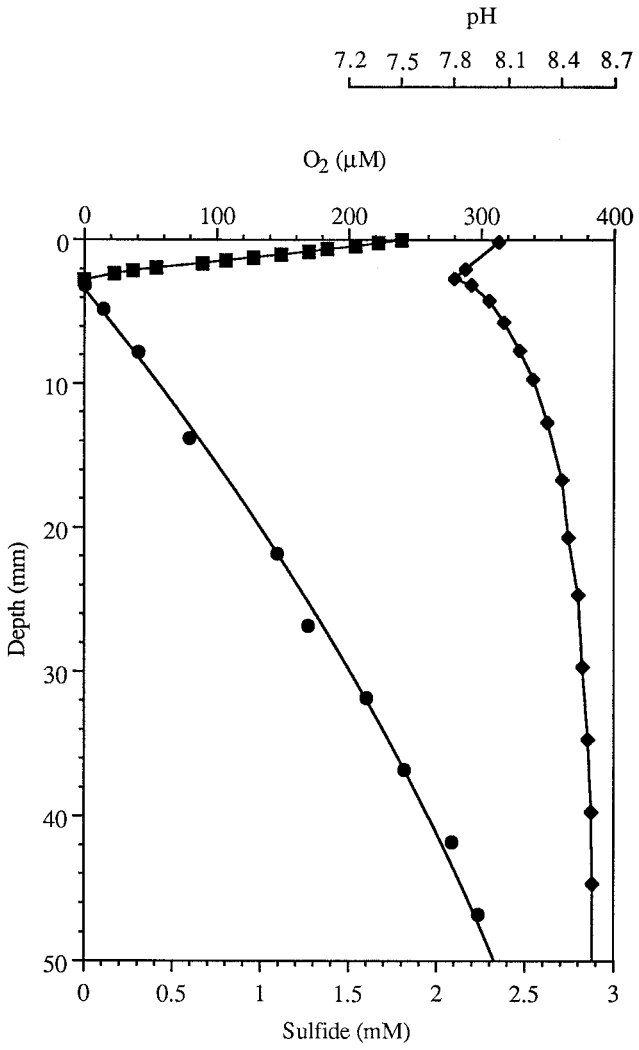

FIG. 3. Microprofiles of total soluble sulfide (●), oxygen (■), and pH (◆) in an acetate-supplemented gradient culture of *Beggiatoa* strain MS-81-6 148 h after inoculation. One day prior to inoculation, sufficient sodium acetate was added to the surface (depth = 0) to give a concentration of 0.3 mM (0.9 mg · atom of C per liter) if it were uniformly distributed throughout the semisolid medium. At 148 h strain MS-81-6 grew in a dense plate 850 μm thick (depth, 2.50 to 3.35 mm). Oxygen and sulfide microprofiles terminated within the plate and coincided with the pH minimum.

DISCUSSION

Insights from gradient culture studies. To date virtually all physiological studies with chemolithoautotrophic sulfur bacteria have been performed with homogenous liquid cultures. This study demonstrates two novel uses of sulfide gradient media for studies of these bacteria. First, the abilities of single carbon sources, when added to otherwise mineral medium, to increase respiration or biomass production can be readily assessed. Detection of increased oxygen consumption by a microaerophilic gradient organism such as a *Beggiatoa* sp. requires only measurements of plate depth; periodic protein determinations from a subset of the cultures complete the analysis. As demonstrated here, the technique can clearly distinguish an obligate chemolithoautotroph from a facultative strain that can respire and incorporate certain organic substrates; it should also be able to detect an organism capable of chemolithoheterotrophy.

A second insight obtained from this study concerns the relevance of enzyme activities measured with cells grown under different culture conditions. In previous studies, chemolithoautotrophic sulfur bacteria (primarily thiobacilli) have almost always been propagated in homogenous liquid cultures with thiosulfate as the oxidizable substrate. Hydrogen sulfide is presumed to be a dominant energy source for *Beggiatoa* spp. and thiobacilli in their natural habitats, where steep gradients of their electron donor and acceptor are the rule (10, 11); therefore, it is instructive to compare enzyme activities from homogenous thiosulfate-oxidizing cultures with those from cells grown in sulfide gradient media. For 2-oxoglutarate dehydrogenase, there appears to be good agreement between activities in the two media; likewise, for strain MS-81-6, RuBPC/O activity is equally down-regulated by acetate in gradient and homogenous cultures. For strain MS-81-1c, however, the regulation of RuBPC/O activity in response to the presence of acetate differed for gradient and homogenous media (Table 2). In acetate-supplemented gradient medium, the RuBPC/O activity in MS-81-1c extracts was approximately 30% lower than that in unsupplemented gradient medium, consistent with the utilization of acetate as approximately 20% of total cell carbon. The higher RuBPC/O activity measured when MS-81-1c was grown in liquid medium containing acetate is consistent with the shorter doubling time of cells in liquid medium where diffusional limitation of substrates does not occur.

Strain MS-81-1c is an obligate chemolithoautotroph. The data indicate that marine *Beggiatoa* strain MS-81-1c is an "obligate" chemolithoautotroph. In contrast to the highly restrictive definition of Winogradsky (34), it is now evident that many

TABLE 2. Activities of RuBPC/O and 2-oxoglutarate dehydrogenase in cell extracts from marine and freshwater *Beggiatoa* strains grown in the presence of a reduced sulfur compound with or without acetate

Strain	Growth conditions	RuBPC/O activity (nmol of CO_2 fixed min^{-1} mg of protein^{-1})[a]	2-Oxoglutarate dehydrogenase activity (nmol of NAD^+ reduced min^{-1} mg of protein^{-1})[a]
MS-81-1c	Liquid (thiosulfate + acetate)	31.7 ± 3.39 (2)	0 (2)
	Gradients (sulfide only)	20.4 ± 2.48 (2)	0 (2)
	Gradients (sulfide + acetate)	14.1 ± 1.56 (2)	0 (2)
MS-81-6	Liquid (thiosulfate + acetate)	3.35 ± 0.21 (2)	103.3 ± 3.62 (3)
	Gradients (sulfide only)	23 ± 9.2 (6)	0 (2)
	Gradients (sulfide + acetate)	3.9 ± 0.78 (3)	153 ± 11.3 (3)
OH-75-2a	Liquid (thiosulfate + acetate)	0.3 (1)	99.6 ± 1.56 (2)

[a] Data are reported as means ± standard deviations, with sample sizes shown in parentheses.

bacteria classified as "obligate" chemolithoautotrophs actually exhibit greater nutritional versatility; in fact, if grown in thiosulfate-limited chemostat culture, they typically increase their total protein or dry weight by 10 to 35% in response to supplements with certain single organic compounds (14, 16). Since the flux of the oxidizable energy source, sulfide, becomes limiting within a few days of inoculating a gradient culture of a *Beggiatoa* sp. (22), our yield enhancement experiments (Table 1) are conceptually similar to chemostat studies with the thiobacilli (14). The yield increment observed for strain MS-81-1c with acetate is, comparably, 20%, with no evidence of additional stimulation by the diverse organic contaminants of unwashed agar. Like many obligate chemolithoautotrophs, this strain was unable to utilize a variety of other organic substrates to any detectable extent. It also appeared to lack the enzyme 2-oxoglutarate under a variety of growth conditions and regulated levels of RuBPC/O activity only moderately compared with strain MS-81-6 in response to the presence or absence of acetate.

Strain MS-81-1c utilized acetate only as a supplemental carbon source. Although approximately 300 μmol of radiolabeled acetate was incorporated when this strain was grown in T5A1 medium, the absence of radiolabeled CO_2 in the medium or the exit gasses implies that no acetate was respired to CO_2. The following calculations demonstrate that CO_2 must have supplied more than three-fourths of all protein carbon. In the experiment whose results are shown in Fig. 1, 27.4 mg of total protein was produced. If the average protein is assumed to be 54% carbon by weight (21), the 27.4 mg of protein contained 14.8 mg of carbon. If, in line with the results of cell fractionation following incorporation of [^{14}C]acetate during growth (see results), 29% of the carbon in the 0.46 mmol of acetate assimilated was used to synthesize protein, acetate could have supplied at most 21.5% (3.18 mg) of the carbon required.

In gradient cultures of strain MS-81-1c, respiration was not enhanced significantly by the tested organic compounds compared with the increases for MS-81-6 (Table 1). These data imply that strain MS-81-1c cannot respire glucose, ethanol, the organic acids, or the amino acids (yeast extract) tested in this study. The maximum enhancement of oxygen consumption (approximately 10%) should be viewed in the following context. When growing as a strict chemolithoautotroph in completely inorganic medium, a *Beggiatoa* sp. utilizes approximately one-sixth of the electrons available from sulfide to reduce CO_2 (22), with the remainder being passed through the respiratory electron transport system. Therefore, if exogenous organic compounds could not be respired at all but could supply all of the biosynthetic needs of the *Beggiatoa* sp., a 20% increase in respiratory oxygen consumption is the maximum anticipated. Replacing roughly one-fourth of fixed CO_2 with exogenous organic compounds should, therefore, increase sulfide respiration and oxygen consumption by approximately 5%. This analysis, within the limits of experimental error, is entirely consistent with the cell fractionation data and the data in Table 1. In summary, on the basis of both gradient and liquid culture studies, strain MS-81-1c seems to respire only inorganic energy sources; that is, it is an obligate chemolithotroph. Although it is technically capable of mixotrophic carbon metabolism, the contribution of organic compounds is limited to 10 to 20% regardless of growth conditions; therefore, it is an obligate chemolithoautotroph.

Since glycylglycine had no effect upon growth yields in gradient medium (data not shown), a molar growth yield on thiosulfate ($Y_{S_2O_3}$) can be calculated for MS-81-1c from the biomass production and thiosulfate consumption data for cells grown in T5 medium. Assuming that protein is 50% of average cell dry weight (9), $Y_{S_2O_3}$ for strain MS-81-1c is estimated to be 10 g (dry weight) per mol, excluding elemental sulfur. This value falls within the range of 6 to 17 g (dry weight) per mol reported for chemoautotrophic thiobacilli grown aerobically on thiosulfate (12) and is close to the value of 8.4 g (dry weight) per mol of H_2S reported for strain MS-81-6 grown in a chemoautotrophic sulfide gradient medium (22). If, at the conclusion of the experiment, 30% of the dry weight of the cells was S^0 (19), then the true growth yields, exclusive of elemental sulfur, would be underestimated by approximately 20% because of incomplete oxidation of the electron donor. While Y_{H_2S} has not yet been determined for strain MS-81-1c, it is possible that the value obtained for thiosulfate represents this strain's molar growth yield on H_2S as well, since the complete oxidation of either thiosulfate or sulfide yields eight electrons per molecule.

Strain MS-81-6 is a facultative chemolithoautotroph. *Beggiatoa* strain MS-81-6, like MS-81-1c, is capable of strict chemolithoautotrophic growth. This conclusion stems from its ability to grow with typical growth yields in a completely inorganic gradient medium containing sulfide as the only oxidizable substrate (22) and from an analysis of carbon balance (21) showing that at least 90% of cell carbon could be derived from CO_2. However, strain MS-81-6 is a facultative chemolithoautotroph. The significant increases in respiration rate and biomass production noted when it was grown in sulfide-limited gradient media supplemented with acetate, lactate, oxalacetate, propionate, pyruvate, fumarate, malate, and succinate (Table 1) are, as documented below, consistent with the simultaneous use of each as a carbon and an energy source. The very modest respiration and biomass enhancement by yeast extract argues against any general ability to utilize peptides or amino acids. This, along with the inability to utilize glucose, may reflect catabolic or permeability limitations; the inability to utilize 2-oxoglutarate probably reflects the latter because of the strong presumption of an active tricarboxylic acid cycle.

The ability of strain MS-81-6 to strongly regulate RuBPC/O and 2-oxoglutarate dehydrogenase activities in response to the presence of organic compounds (Table 2) also supports the view that this strain is a facultative chemoautotroph. Organic substrates might theoretically support one of the following modes of growth: (i) chemolithoheterotrophy, in which cell carbon is obtained solely from organic sources and inorganic substrates provide energy; (ii) mixotrophy, in which cell carbon is simultaneously obtained from CO_2 and organic sources and/or energy is simultaneously derived from the oxidation of inorganic and organic substrates; or (iii) strict chemoorganoheterotrophy, in which organic substrates provide all cell carbon and energy. In the last case, reduced sulfur compounds such as hydrogen sulfide might theoretically simply provide the microoxic conditions required for growth. While strain MS-81-6 has been reported to be capable of strict heterotrophic growth with acetate in the absence of reduced sulfur compounds (21), it is unlikely that it grew that way in the T5A1 medium used in this study. There, its growth yield, estimated at 32 to 34 g (dry weight) per mol of acetate (assuming that protein is 50% of the dry weight of the average cell [9]), was much greater than the yield of 22 g (dry weight) per mol of acetate previously reported for this strain (21) and than the heterotrophic yields of 16 to 21 g (dry weight) per mol of acetate reported for freshwater *Beggiatoa* strains (8, 20).

There is likewise evidence against strict chemoorganoheterotrophic growth of strain MS-81-6 in acetate-supplemented sulfide gradient medium because the sulfide flux continued to be quantitatively consumed by the *Beggiatoa* plate (Fig. 3). This and the pH minimum suggest catabolic oxidation of hydrogen

sulfide to sulfuric acid, thereby ruling out strict chemoorganotrophy. As discussed above (for strain MS-81-1c), respiration enhancement by 1.2-fold due to uptake of organic compounds as the sole source of cellular carbon is the maximum predicted for strict chemolithoheterotrophic growth. For MS-81-6, the data (Table 1) suggest that, at a minimum, one-third (malate and oxalacetate) to approximately one-half (acetate, fumarate, lactate, pyruvate, propionate, and succinate) of the oxygen consumption is driven by organic respiration. Microelectrode studies of this strain in the presence of a lower acetate concentration (averaging 0.9 mg · atom of C per liter) show an O_2/total sulfide consumption ratio of 2.25:1. Because this ratio is above the theoretical maximum (2.0:1) observable if sulfide is the only substrate being oxidized, it is evidence for simultaneous oxidation of acetate and sulfide. Assuming that sulfide is also oxidized in the presence of the other stimulatory organic compounds (Table 1), strictly chemolithoheterotrophic growth is ruled out for these compounds, and mixotrophy, at least with respect to energy generation, is established. It is not proven that strain MS-81-6 can grow mixotrophically with respect to its carbon source as well. However, in liquid or gradient medium supplemented with organic compounds, a residual RuBPC/O specific activity of 17% of the level found in cells growing strictly chemoautotrophically suggests a continued contribution from CO_2 fixation.

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