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Growth Pattern and Yield of a Chemoautotrophic *Beggiatoa* sp. in Oxygen-Sulfide Microgradients

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Recently developed techniques involving opposed, gel-stabilized gradients of O_2 and H_2S permit cultivation of a marine *Beggiatoa* strain as a chemolithoautotroph which uses gliding motility to precisely track the interface between H_2S and O_2. In the current study with microelectrodes, vertical profiles of H_2, O_2, and pH were measured in replicate cultures grown for various intervals. After an initial period of exponential biomass increase (doubling time, 11 h), linear growth prevailed throughout much of the time course. This H_2S-limited growth was followed by a transition to stationary phase when the declining H_2S flux was sufficient only to supply maintenance energy. During late-exponential and linear growth phases, the *Beggiatoa* sp. consumed a constant 0.6 mol of H_2S for each 1.0 mol of O_2, the ratio anticipated for balanced lithoautotrophic growth at the expense of complete oxidation of H_2S to SO_4^{2-}. Over the entire range of conditions studied, this consumption ratio varied by approximately twofold. By measuring the extent to which the presence of the bacterial plate diminished the overlap of O_2 and H_2S, we demonstrated that oxidation of H_2S by *Beggiatoa* sp. is approximately 3 orders of magnitude faster than spontaneous chemical oxidation. By integrating sulfide profiles and comparing sulfide consumed with biomass produced, a growth yield of 8.4 g (dry weight) mol^{-1} of H_2S was computed. This is higher than that found for sulfide-grown thiobacilli, indicating very efficient growth of *Beggiatoa* sp. as a chemoautotroph. The methods used here offer a unique opportunity to determine the yield of H_2S-oxidizing chemolithoautotrophs while avoiding several problems inherent in the use of homogeneous liquid culture. Finally, by monitoring time-dependent formation of H_2S profiles under anoxic conditions, we demonstrate a method for calculating the molecular diffusion coefficient of soluble substrates in gel-stabilized media.

Based on the classical study with enrichment cultures by Winogradsky (33) and the subsequent study by Keil (12), the genus *Beggiatoa* has long been suspected to contain chemolithoautotrophic representatives. Despite the negative reports in all recently tested freshwater isolates (3, 7, 16, 22, 23, 26, 27, 29), chemolithoautotrophy was recently proven for a marine strain by using agar-stabilized oxygen-sulfide gradient media (18). Both in gradient media and in nature, *Beggiatoa* spp. typically occur as a dense horizontal "plate" of growth with an apparently strong preference for microoxic environments. The gliding motility of filaments, along with tactic responses to light, oxygen, and possibly sulfide, enable this organism to remain within this narrow, preferred microenvironment even while it migrates rapidly on a diurnal basis (15, 17, 19).

By using natural populations of filaments, Jørgensen and Revsbech obtained data on the oxygen, pH, and sulfide profiles in and around the narrow zone occupied by *Beggiatoa* spp. (11). That study used microelectrodes for obtaining the profiles of these three parameters and indicated that *Beggiatoa* spp. in natural mats proliferated in a layer 500 to 700 μm thick, oxygen and sulfide coexisted within this layer over a vertical distance of approximately 50 μm, and turnover times of oxygen and sulfide within the overlap zone were approximately 4 orders of magnitude faster than under conditions of nonbiological oxidation. Although *Beggiatoa* spp. were the dominant bacteria in this natural mat assemblage, the possibility cannot be ruled out that other bacterial types contributed significantly to the observed consumption of oxygen and sulfide.

The investigation we report here used similar microelectrode techniques in conjunction with biomass measurements to study the axenic growth of *Beggiatoa* sp. in oxygen-sulfide gradient media. By analyzing the overlapping distribution of these two reactive compounds in the presence and absence of *Beggiatoa* sp., we examined the extent to which these bacteria can compete with spontaneous chemical oxidation of sulfide. Because of the strain and the growth medium used (18), this is a quantitative study of chemolithoautotrophic growth under environmentally realistic conditions. It provides a detailed picture of *Beggiatoa* growth, giving information on time-dependent changes in the rates of oxygen and sulfide consumption. Finally, we report chemolithoautotrophic growth yield values which may be the most accurate available for aerobic sulfide oxidizers because of limitations inherent in the use of homogeneous liquid cultures.

MATERIALS AND METHODS

Theory. Gradients of soluble sulfide (designated here collectively as H_2S) were initially formed as step gradients. Agar medium containing H_2S was solidified in the bottom of a vertically oriented culture tube and was overlaid with equivalent medium lacking H_2S. From these initial conditions, diffusion of H_2S may be modeled as occurring only in the vertical direction. In the absence of H_2S oxidation, the sulfide concentration C at depth x in the agar column will change with time t according to:

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$$\partial C/\partial t = D'(\partial^2 C/\partial x^2) \quad (1)$$

where D' is the apparent diffusion coefficient of H_2S (5). With the initial interface between sulfide-agar and nonsulfide agar as $x = 0$, the initial conditions ($t = 0$) for diffusion in our medium are $C = 0$ for $x > 0$ and $C = C_0$ for $x < 0$. After diffusion has proceeded ($t > 0$), the boundary conditions are $C \rightarrow 0$ for $x \rightarrow \infty$; $C \rightarrow C_0$ for $x \rightarrow -\infty$. (When C at the bottom of the tube drops significantly, this last boundary condition is no longer met.) The solution to this differential equation (6, 14) is

$$C = 0.5C_0 \text{ erfc } [x/(4D't)^{0.5}] \quad (2)$$

in which erfc designates the complementary error function. H_2S profiles were determined at times sufficiently short to comply with boundary assumptions. For each profile, plotting $C/(0.5C_0)$ versus x yielded a separate estimate of D' by the method of Duursma and Hoede (6). Generally speaking, the H_2S concentration of any depth will change over time, but equation 2 predicts that the concentration at $x = 0$ will remain at 0.5 C_0 as long as the initial boundary conditions are maintained, assuming that D' is independent of x.

Strain and media. Throughout this study, the marine strain MS-81-6 was used. Its isolation from a salt marsh is detailed elsewhere (20). It was maintained as a chemolithoautotroph in semisolid J2 medium containing oxygen and sulfide gradients (18). This medium (salinity, 2.0%) was based on aged and filtered natural seawater supplemented with sodium bicarbonate, ammonium nitrate, potassium phosphate, and trace elements, and it contained no added organics except vitamins.

Two procedures were used for the initial juxtaposition of two layers of agar, one layer of which contained sulfide. In both cases, sulfide gradients changed temporally due to molecular diffusion. Additionally, with the first procedure (detailed below), nonbiological oxidation contributed to the shape of sulfide gradients, and an oxygen gradient also formed. Aseptic procedures were utilized up to the time of measuring gradients, and each culture or tube of medium was sampled only once.

(i) For studying changes accompanying growth, we used replicate cultures. A 10.0-ml portion of sodium sulfide-supplemented medium (8.5 mM, 0.8% agar) was solidified by cooling (10°C) in the bottom of each Leighton tube (28 by 110 mm, 70-mm window; Bellco Glass, Inc., Vineland, N.J.). While the tube and agar plug were still cool, a 30-ml volume of medium (0.25% agar, no sulfide) was added carefully to minimize convection mixing of sulfide from the lower agar. To allow gradients to form, the medium was aged before inoculation for 41 h at room temperature with humidified air having access through sterile cotton.

(ii) For measuring the apparent diffusion coefficient of H_2S, procedures were carried out under rigorously anoxic conditions (Hungate technique) to ensure that no sulfide oxidation occurred before gradient sampling. In each culture tube (25 by 150 mm), a 25-ml portion of medium (7.38 mM Na_2S, 0.4% agar [pH 8.4]) was solidified. With the tubes in a 10°C water bath, top medium (0.25% agar, no sulfide) was added to each in three sequential portions of 1, 1, and 18 ml, allowing complete solidification before the next portion was added. Uninoculated medium was sealed under N_2 with butyl rubber stoppers and was maintained at 19 ± 1°C until sampled for chemical sulfide determinations.

Microelectrodes. Microelectrodes for measuring oxygen, soluble sulfide, and pH were constructed as described by Revsbech et al. (24, 25). When only the O_2 electrode was used, the procedures were as previously described (19). When all three were used, each was, in turn, secured to the micromanipulator and positioned at appropriate increments within the culture while the electrode current or voltage was recorded. All three profiles were taken along the vertical axis in the center of a tube, and each typically required less than 1 h. While measuring a profile, replicate determinations (n = 4 to 6) were made of the exact micrometer reading for three reference points. Observation with a dissecting microscope through the flat window of a Leighton tube helped to identify the depth at which the electrode tip was aligned with either the upper or lower edge of the dense *Beggiatoa* plate or with the air/agar interface. The three profiles for each culture were aligned with the upper surface of this plate as the reference point, but vertical distance is displayed graphically with the air/agar interface as zero.

Electrodes were calibrated during or after each experiment. Oxygen electrodes were calibrated as described previously (19). Calibration of sulfide and of pH electrodes was as follows. After obtaining a vertical profile of sulfide electrode readings, five to eight agar samples spanning the profile were taken (19) and analyzed chemically for sulfide (4). The sulfide electrode measures only S^{2-}, which becomes a smaller fraction of total soluble sulfide with decreasing pH. Sulfide electrode output, after standardizing to pH 8.4 with a correction of 29.5 mV per pH unit, gave a linear standard curve when plotted versus chemically determined values. Above 100 μM H_2S, slopes ranged from 29.4 to 31.6 mV per decade of sulfide, in good agreement with the Nernst equation. Below 20 to 50 μM H_2S, the slope of the calibration curve began to increase. pH microelectrodes were calibrated in natural seawater (salinity, 2.0%) adjusted to pH 7.0, 7.5, 8.0, and 8.5 (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] or Tris buffer), with a commercial pH electrode and meter (model PHM 62; Radiometer A/S, Copenhagen, Denmark). The slopes of linear calibration curves ranged from 55.6 to 57.9 mV per pH unit.

For the oxygen and sulfide electrodes, vertical resolution was 10 μm or less. Minimal oxygen concentrations which could be differentiated from zero were on the order of 0.2 μM. Differences in pH of 0.01 could be measured, but assignment of a precise vertical location was problematic since a typical electrode had a tip of pH-sensitive glass 100 μm long. The depth recorded for a pH reading was to the tip of the electrode. The sulfide electrode could accurately measure total soluble sulfide at concentrations of a few micromolar under optimal conditions, but sensitivity declined with decreasing pH (see above).

By electrode measurements of oxygen at various depths in seawater-agar following a "step up" from air to 100% O_2 above the agar surface (constant source method, [6]), the diffusion coefficient of oxygen was determined to be 2.03×10^{-5} cm^2 s^{-1} (0.25% agar, 23°C, salinity 2.0%; N. P. Revsbech, unpublished data).

Time course of growth. The inoculum consisted of chemoautotrophically grown filaments harvested (along with adjacent agar) after 6 days of growth in equivalent medium. After mixing well, a 100-μl portion (7.7 μg of protein ml^{-1}) was distributed dropwise, as evenly as possible, across the surface of each tube of aged medium. Samples were then preserved for time zero biomass determinations. Following inoculation, all 12 replicate cultures and three uninoculated controls were returned to a humidified chamber, which minimized desiccation of the medium. The 20-liter chamber was flushed with fresh air twice daily to maintain constant composition of the headspace gases, which had access to the

gradient cultures through sterile cotton. Periodically, throughout the next 11 days, a replicate was measured for oxygen, soluble sulfide, and pH profiles. Then the entire bacterial plate was harvested with a minimum quantity of agar to a tared tube, and known samples were immediately preserved for biovolume and protein determinations. Microscopic observations were also made to confirm culture purity. Throughout culture growth, room temperature ranged from 22 to 25°C.

Biomass parameters. Biovolume was determined on samples preserved with 1.5% neutralized formaldehyde. Subaliquots were stained (acridine orange), evenly distributed by gentle suction onto counterstained Nuclepore filters, and viewed by epifluorescence microscopy (8). Total filament length was estimated by counting intersections of filaments with a superimposed grid (method 5 [21]). From length estimates and filament width (4.0 μm; unpreserved material), the volume formula for a cylinder permitted extrapolation of biovolume per tube.

Total protein was determined by the Coomassie brilliant blue dye-binding technique (2) after acidic hydrolysis of the unwanted agar (90°C, 10% trichloroacetic acid) and dissolution of the precipitated protein in dilute base. Ovalbumin (grade 5; Sigma Chemical Co., St. Louis, Mo.) served as a standard. Blank corrections were applied based on hydrolysis of portions of medium from uninoculated tubes. Details of this and the microprotein assay procedure are given elsewhere (18).

Miscellaneous methods. For samples containing *Beggiatoa* filaments plus agar, elemental sulfur (S^0) content was determined by the method of Troelsen and Jørgensen (32). Combined with microscopic examination of filaments and medium, this made it possible to estimate the intracellular elemental sulfur content.

Slopes of concentration gradients and exponential growth curves were calculated by the method of least-squares linear regression. Unless noted otherwise, confidence limits designate the standard error of the mean.

RESULTS

Diffusion coefficient for soluble sulfide. For this study, knowledge of the apparent diffusion coefficient for soluble sulfide was essential. At the initial pH of these studies (8.4), sulfide will be principally in the HS^- form with a lesser fraction as H_2S and S^{2-} (30); nonetheless, we use H_2S to denote the sum of these soluble forms. For the anaerobic diffusion experiment, profiles after 28.5 and 46.8 h are shown (Fig. 1) along with best-fit values of D' for each data set. The theoretical profile each D' predicts is shown and agrees equally well with the data above or below the initial interface. Two other profiles (23.8 and 37.8 h; not shown) yielded values for D' of 1.40×10^{-5} and 1.36×10^{-5}. This gives an average of $D' = 1.39 \times 10^{-5}$ cm^2 s^{-1} ($\pm 0.05 \times 10^{-5}$; $n = 4$).

Growth. Within 4 h of inoculating the replicate tubes of gradient medium, the filaments had moved via gliding motility to a depth of approximately 5 mm below the air/agar interface. In all 12 tubes, they were distributed quite uniformly in a horizontal plate approximately 0.5 mm in vertical thickness. In spite of having been inoculated only at the surface of the medium, virtually no filaments remained within the top 4 mm of the medium. In preliminary experiments, the completeness and rapidity of this migration depended on the inoculum being from a culture which had not yet reached stationary growth phase.

Growth in the replicate tubes was initially at an exponential rate (Fig. 2A). The specific growth rate for the first 47 h averaged 0.065 h^{-1} (correlation coefficient, ≥0.98) both for protein and for biovolume data. The 60- and 72-h data points indicate that this exponential rate was not sustained, but growth continued for at least 5 days thereafter. Between approximately 60 and 180 h, linear rates of increase were obtained for protein and biovolume data (Fig. 2A). From 180 h on, growth proceeded at a rate which was apparently continually decreasing, but because of the limited number of data points, it is not certain that it had ceased entirely by the end of the experiment.

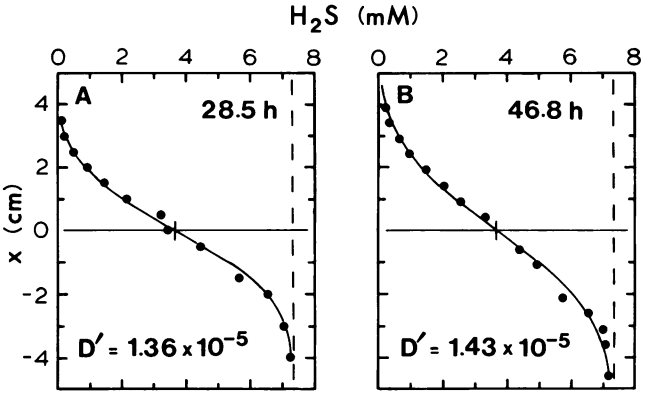

FIG. 1. Computation of apparent diffusion coefficient (D') for H_2S under anoxic conditions starting from initial step gradient. The H_2S concentrations were determined by the method of Cline (4). For each graph, the indicated D' value is the best fit derived by the graphical algorithm of Duursma and Hoede (6). Time is expressed relative to initial formation of the step gradient, and the smooth curve is the theoretical profile for the specified time and D'. At time zero, H_2S (7.36 mM) was present only below $x = 0$. H_2S, total soluble sulfide.

From 37 to 60 h, the *Beggiatoa* plate became more compact (Fig. 2B), even though biomass was increasing (Fig. 2A). Thereafter, the vertical thickness of the plate increased by almost sixfold over the next 190 h, approximately in parallel with biomass increase. The net result of these similar patterns of increase was that *Beggiatoa* biovolume occupied a fairly constant 0.2% of the volume of its growth region, at least during the period of linear increase. This is comparable to the biovolume density in a culture containing 4×10^9 spherical cells per milliliter, each having a diameter of 1.0 μm.

The lowest pH measured within the bacterial plate declined monotonically over the course of the experiment from 8.30 to 7.42. These minima all occurred within the upper 20 μm of the plate of filaments. By contrast, all controls lacked a sharp pH minimum, and the lowest pH values measured were 8.43, 8.37, and 8.36 at 6, 94, and 251 h, respectively.

Interaction between *Beggiatoa* sp. and gradients. In an uninoculated control 6 days after it was poured, the H_2S concentration increased linearly with increasing depth between the depths of approximately 8 to 30 mm (Fig. 3A). Oppositely sloped gradients of H_2S and O_2 overlapped between the depths of approximately 1 to 8 mm. Viewed in more detail, the oxygen profile is linear in the upper 2 to 3 mm, and each profile shows a mirror image curvature throughout most of the rest of the overlap region (Fig. 4A). The other controls (8 and 251 h; profiles not shown) were similar in many respect to this one. Similarities included the location and vertical dimension of the sulfide/oxygen overlap zone, the curvature of each profile within the overlap region,

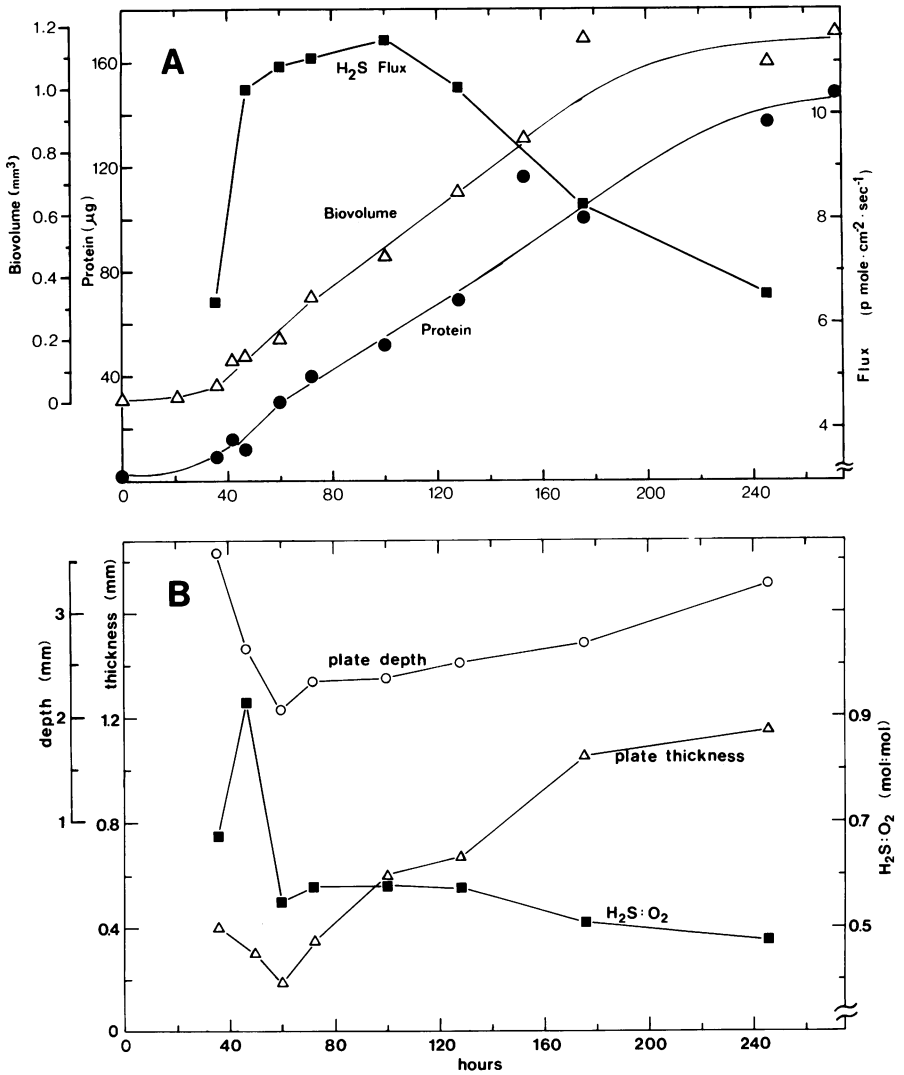

FIG. 2. Time course of *Beggiatoa* (strain 81-6) growth in H_2S/O_2 gradients. After 41 h of aging, replicate tubes of medium received equal inocula (0.77 μg of protein tube^{-1}). Time zero is the hour of inoculation. Biovolume and protein each gave $\mu = 0.065$ h^{-1} by linear regression (up to 47 h, $r = 0.98$). Values are expressed per tube. (A) Symbols: ●, protein; △, biovolume; ■, H_2S flux; (B) Symbols: ■, ratio of fluxes (H_2S/O_2, mol/mol) △, vertical thickness of *Beggiatoa* plate; ○, distance from air/agar interface to top of plate.

and their respective linearities immediately outside this region. In all three controls, H_2S was detected within 1 to 2 mm of the surface, but not at the very surface of the medium.

Even a few hours after inoculation, the above picture was greatly changed by the presence of bacteria. From 47 h onward, when the *Beggiatoa* plate was well established, differences from the controls were as follows. The presence of this bacterium reduced the H_2S/O_2 overlap region to 4% or less of the vertical coexistence in the corresponding control (Fig. 4 and see Table 2). In the experimental culture, no oxygen penetrated to the lower boundary and no sulfide penetrated to the upper boundary of the narrow bacterial plate (Fig. 5; Nelson, unpublished data). The two profiles were apparently curved in the much-reduced overlap region. Immediately above or below the thin plate of filaments, the oxygen or sulfide gradient, respectively, was linear (Fig. 4 and 5).

Some soluble sulfide was detected above the region of *Beggiatoa* growth in the 36-h culture. This implies that an undefined percentage of the sulfide flux at this time was oxidized nonbiologically. However, from 47 h onward, analysis indicates that essentially all oxygen and sulfide consumption were bacterially mediated (see below). Thus, oxygen flux measured immediately above the plate, and sulfide flux measured immediately below, are direct measures of consumption by *Beggiatoa* sp. By 47 h, H_2S flux had risen to 10.5; it remained at approximately 11 pmol cm^{-2} s^{-1} between 60 and 128 h and declined gradually to a value of 6.6 by 246 h (Fig. 2A).

Oxygen consumption data are presented relative to sulfide consumption as a molar ratio of fluxes (Fig. 2B). This ratio reached a value of 0.67 mol of H_2S per mol of O_2 at 36 h, with some oxidation at that time still being nonbiological. By 47 h, the H_2S/O_2 ratio was 0.93, and all oxidation was due to *Beggiatoa* sp. Thereafter, the filaments consumed approximately 0.6 mol of H_2S per mol of O_2 until at least 128 h. By 246 h, the ratio had dropped to just below 0.5.

Sulfide integral and growth yield. When the medium for the replicate cultures was first poured, the sulfide plug contained 85 μmol of H_2S. This was initially confined (Fig. 3B)

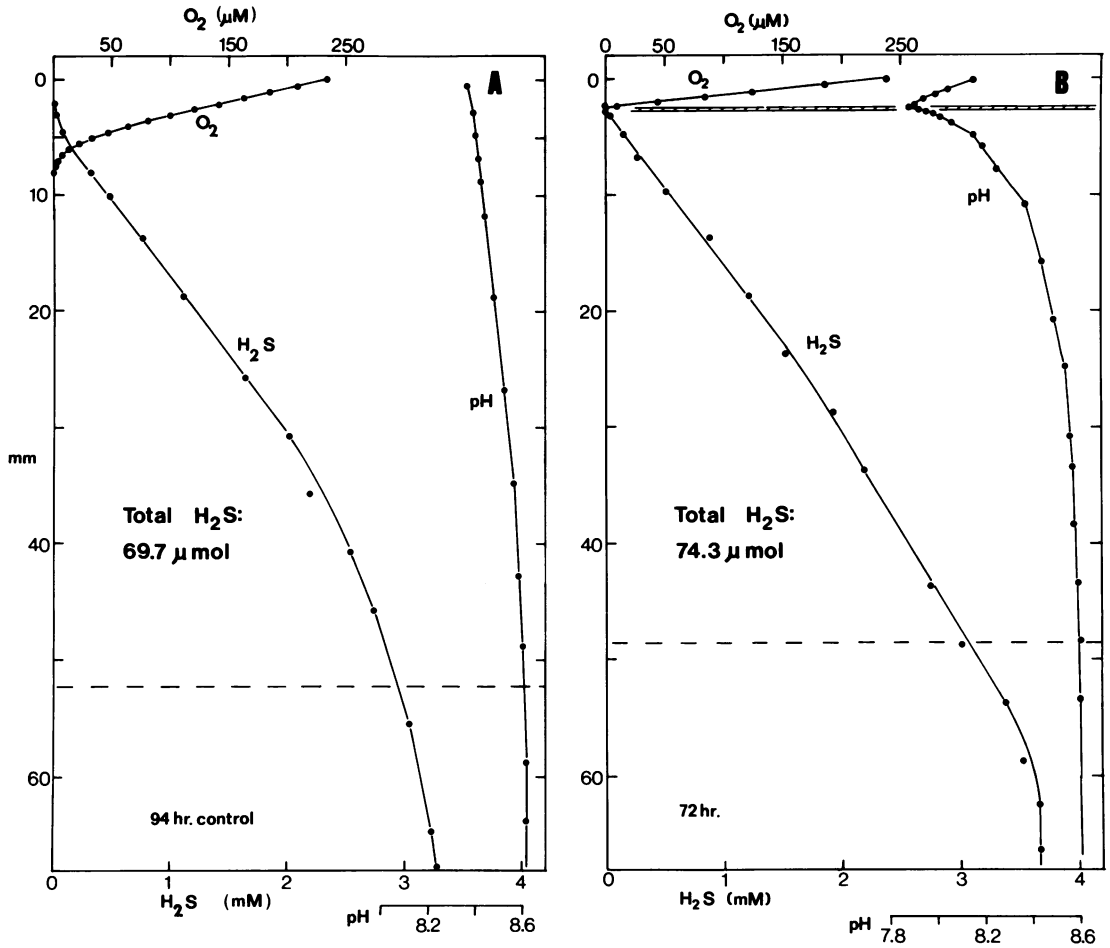

FIG. 3. Overview of H_2S, O_2, and pH profiles in 94-h control medium (A) or inoculated 72-h culture (B). ▨ (2.5 mm, panel B), *Beggiatoa* plate. Inoculation was at time zero, but initial step gradients were poured 41 h previously. Depth zero indicates the air/agar interface. ---, Initial sulfide/nonsulfide interface. All tubes initially contained 10.0 ml of sulfide-agar (C_0 = 8.50 mM) and 30.0 ml of top agar. Slight differences in the tube dimensions resulted in differences in the vertical extent of gradients.

between the depths of 49 and 69 mm (bottom of tube). For each H_2S profile, the integrated total of soluble sulfide remaining in the tube was computed. By 72 h, the value had declined to 74.3 μmol of H_2S (Fig. 3B) and decreased further over time (Table 1).

From 47 h onward, sulfide losses from this reservoir can be ascribed solely to bacterial consumption (see below). Thus, we can compute molar growth yields for *Beggiatoa* sp. growing at the expense of sulfide oxidation (Table 1). Data are presented from 60 h onward because internal sulfur stores were not excessive by that time (approximately 30% [wt/wt]) and declined constantly thereafter. The molar growth yield on soluble sulfide, Y_{H_2S}, averaged 8.4 g (dry weight) mol^{-1}. A value of 7.4 g/mol computed by comparing the 60-h reference point with the 72-h time point was omitted from Table 1 because of the large percent error inherent in comparing two similar integrated sulfide values.

DISCUSSION

Diffusion coefficient of sulfide. When precautions were taken to prevent nonbiological oxidation of H_2S and to ensure that the initial H_2S gradient closely approximated a step gradient, accurate determination of D′, the apparent diffusion coefficient of H_2S, was possible. Although it is specific for our experimental conditions of salinity and pH, correction to another temperature is possible since D′ should vary in direct proportion to absolute temperature and in inverse proportion to viscosity. Our diffusion coefficient incorporates the porosity and adsorption characteristics of the medium, so it is not completely comparable with other published values even though they appear to be in agreement (11).

The experimental method we used is analogous to that used by Krom and Berner (14) for measuring diffusion coefficients of several ions in marine sediments. However, in that study a best-fit value of D' was obtained using a least-squares criterion, which will give maximum weighting to the highest concentrations measured nearest $x = 0$. In our study, these are the values most subject to error from initial convective mixing; therefore, we used the graphical method of Duursma and Hoede (6), which allows the lower concentrations, measured farther from $x = 0$, to be given equal weighting.

Impact of *Beggiatoa* sp. on diffusional gradients. The impact of *Beggiatoa* sp. on diffusional gradients was deduced by comparing rates of sulfide and oxygen turnover in uninoculated controls with rates in equivalent inoculated medium. To make this contrast quantitative, we used the one-dimensional form of Fick's first diffusional law:

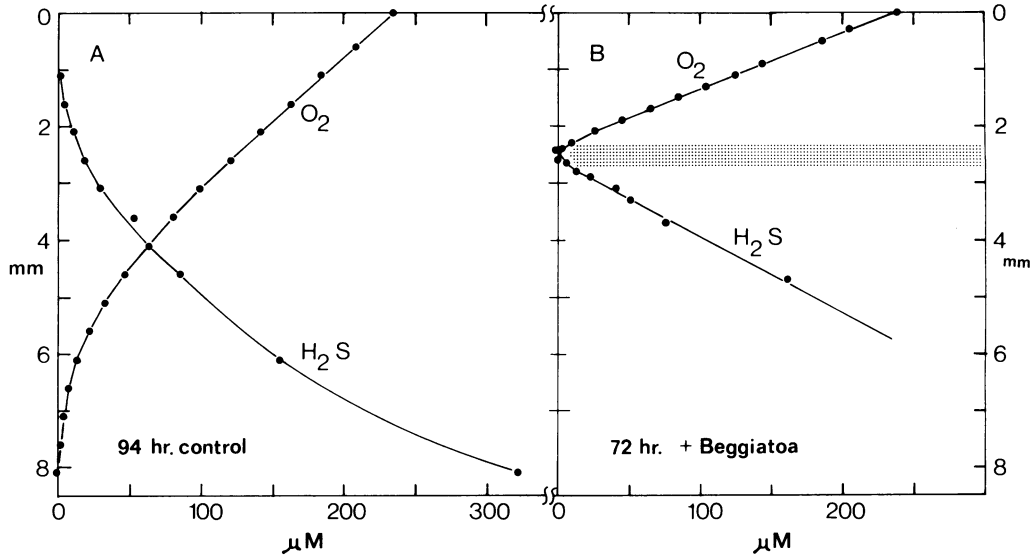

FIG. 4. Expanded view of regions of overlap between O_2 and H_2S for corresponding panels of Fig. 3. Shaded area shows the *Beggiatoa* plate.

$$\text{Flux} = -D'(\partial C/\partial x) \quad (3).$$

where D', C, and x are defined above. Flux designates the quantity of solute which flows across a unit of surface area perpendicular to the concentration gradient per unit of time.

First we apply this equation to an uninoculated control (Fig. 3A and 4A). Assuming a steady state, the linear regions ($\partial C/\partial x$ constant) above and below the region of H_2S-O_2 overlap may be used to measure the flux of H_2S and of O_2 into the overlap region. For the temperature of this experiment, D' for H_2S is 1.57×10^{-5} cm^2 s^{-1} and D' for O_2 is 2.03×10^{-5} (see above). Application of these to Fig. 3A yields flux values (toward the overlap region) of 11.8 and 9.2 pmol cm^{-2} s^{-1} for H_2S and O_2, respectively. Within the region of overlap, the absolute value of $\partial C/\partial x$ became lower while moving down either solute gradient, indicating spontaneous oxidation-reduction within this region, which eventually resulted in a complete consumption of both solutes. For this control, the vertical dimension of H_2S-O_2 overlap was 6.7 mm (Fig. 4A), and the average O_2 and H_2S concentrations computed by trapezoidal approximations within this region were 69 and 109 μM, respectively. Since each compound reacted with the other only in this overlap zone, the total amount within this zone divided by the flux of the compound measures average turnover time. For O_2 and H_2S in the 94-h control, these were 5.2×10^3 and 6.5×10^3 s. These plus equivalent computations for the other controls are entered in Table 2. The range of turnover times for H_2S in the three controls is in line with a half-life of 3.2×10^3 to 7.8×10^3 for H_2S (1 to 60 μM, pH 8.5) in oxygenated seawater (1). Consistent with the assumption that O_2 is the nonbiological oxidant, the turnover time of O_2 is approximately the same.

Comparison of Fig. 4A and B (same scale) illustrates the dramatic way in which sulfide and oxygen gradients are altered by *Beggiatoa* sp. growing at the expense of sulfide oxidation. For the 72-h culture, the concentrations of H_2S and O_2 in the overlap zone averaged 2.1 and 1.9 μM, and the turnover times were 5.2 and 2.7 s, respectively. The turnover times and average concentrations computed for all inoculated cultures were remarkably similar (Table 2). The somewhat higher turnover times and average concentrations observed in the 36-h culture represent a mixture of biological

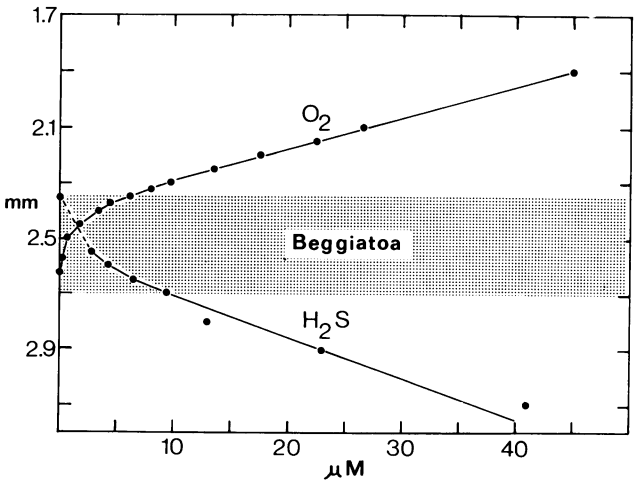

FIG. 5. Expanded view of *Beggiatoa* region shown in Fig. 4B.

TABLE 1. Molar growth yield, Y_{H_2S}, calculated over various time intervals relative to 60-h data[a]

Time (h)	Integrated sulfide[b] (μmol tube^{-1})	Sulfide consumed[c] (μmol)	Biomass[d] (μg [dry wt] tube^{-1})	Biomass gain[c] (μg [dry wt])	Y_{H_2S} (g mol^{-1})
60	77.6	—	73.0	—	—
100	72.0	5.6	125.9	52.9	9.4
128	64.4	13.2	168.6	95.6	7.2
176	59.2	18.4	241.8	168.8	9.2
246	44.3	33.3	335.4	262.4	7.9
					$\bar{x} = 8.4 \pm 0.53$

[a] Sulfide and biomass data are given on a per-tube basis.
[b] Total soluble sulfide present in the *Beggiatoa* layer and below.
[c] With 60-h value as reference point.
[d] Biomass computed from protein data as follows: protein carbon = 54% of protein weight (18); total cell carbon = 2.27 × protein carbon (18); biomass = 2.0 × total carbon (13). Net: biomass = 2.45 × protein (dry weight).

TABLE 2. Concentration, flux, and turnover of substrates in control and experimental (plus *Beggiatoa* sp.) gradient media

Treatment	Time[a] (h)	Substrate	Flux (pmol cm^{-2} s^{-1})	Reaction zone[b] (mm)	Avg[c] concn (μM)	Turnover time[c] (s)
Control	8	O_2	8.0	6.70	76	6.4×10^3
		H_2S	8.7	6.70	36	2.7×10^3
	94	O_2	9.2	7.00	69	5.2×10^3
		H_2S	11.8	7.00	109	6.5×10^3
	251	O_2	8.0	5.70	65	4.6×10^3
		H_2S	6.5	5.70	28	2.5×10^3
Plus *Beggiatoa* sp.	36	O_2	9.5	0.450	3.4	16
		H_2S	6.4	0.450	6.9	48
	47	O_2	11.2	0.280	2.0	5.0
		H_2S	10.5	0.280	2.9	7.8
	72	O_2	19.0	0.270	1.9	2.7
		H_2S	11.1	0.270	2.1	5.2
	128	O_2	18.3	0.180	3.0	3.0
	176	O_2	14.2	0.200	3.7	5.2
	246	O_2	13.9	0.160	3.4	3.9

[a] Inoculation time = time zero. Media were aged 41 h before inoculation.
[b] Vertical dimension of H_2S-O_2 overlap. After 72 h, the coarse vertical resolution of the pH electrode precluded accurate determination of H_2S turnover. After 72 h, reaction zone for O_2 was taken as portion of bacterial plate containing O_2 (see text).
[c] Total substrate within reaction zone was determined by using trapezoidal approximations to integrate substrate profile over this zone. This total (not shown) divided by the height of the reaction zone gave the average concentration. Dividing total substrate by appropriate flux value gave average turnover time.

and nonbiological processes (see above). Taken as a whole, these data indicate that sulfide and oxygen turnover were accelerated 1,000 to 2,000-fold by the presence of bacteria and that average concentrations in the overlap zone were approximately 10- to 50-fold lower due to biological oxidation. It is a priori obvious that a chemolithotrophic *Beggiatoa* sp. must be able to compete successfully with nonbiological H_2S oxidation; however, the degree to which it so completely dominated could not have been predicted.

The appropriateness of our controls might be questioned, since spontaneous oxidation of H_2S in uninoculated medium occurs under higher average concentrations of H_2S and O_2 than does the small amount of nonbiological oxidation which undoubtedly still takes place in the presence of *Beggiatoa* sp. In both cases, however, the H_2S/O_2 ratio is roughly 1, which means that the rates of nonbiological oxidation should be comparable (1).

Jørgensen and Revsbech (11) reached a generally similar conclusion regarding the ability of native *Beggiatoa* mats to successfully compete with chemical oxidations. The combining ratios they observed for O_2 and H_2S gave confidence that much of the observed metabolic activity was chemolithotrophy attributable to the dominant *Beggiatoa* sp. In that study, the turnover times computed for oxygen and sulfide were 5 to 10 times faster than those measured here. However, the O_2 and H_2S gradients of that study were also steeper, and it is possible that faster turnover would have occurred in our experiments if steeper gradients had also been provided.

TABLE 3. Theoretical ratios for consumption of H_2S versus O_2 based on end products and whether CO_2 fixation occurs

End products of H_2S oxidation (mol fraction)		H_2S/O_2 Consumption ratio (mol/mol)		S^0 (% of total biomass[b])
S^0	SO_4^{2-}	H_2S for energy generation only	H_2S for energy + reductant[a]	
1.00	0.00	2.00:1	2.42:1	94
0.50	0.50	0.80:1	0.97:1	75
0.25	0.75	0.62:1	0.75:1	54
0.00	1.00	0.50:1	0.61:1	0

[a] Assuming that Y_{H_2S} (aerobic, complete oxidation) is equal to 8.4 (Table 1) and that molar growth yield for oxidation of sulfide to level of S_0 is one-fourth of Y_{H_2S} value. See text for details of this calculation.
[b] Assuming balanced growth and that all S^0 is deposited intracellularly.

Regulation of growth by diffusional gradients of O_2 and H_2S. By initiating growth in gradient cultures with a small, evenly distributed inoculum, use of replicate tubes provided a satisfactory way to study the chemolithoautotrophic growth of *Beggiatoa* sp. under conditions which mimic essential aspects of the niche of this bacterium. These aspects include: opposed gradients of oxygen and sulfide, and a physical substrate which allows gliding of filaments to their preferred microenvironment.

Since sulfide flux was essentially constant or slowly declining during the period of linear biomass increase (60 to 180 h [Fig. 2A]), this suggests that sulfide availability limited growth. A consequence of biomass increase coupled to an approximately constant sulfide flux is that from 60 h on, an ever-increasing fraction of the energy generated from sulfide oxidation will be used as maintenance energy (31). When no further biomass increase was noted, we postulate that biomass had increased to the point that sulfide flux just satisfied maintenance requirements. In theory, this maintenance energy requirement could be computed from the data of Fig. 2A, assuming that the biomass attained by 246 h truly represents a yield plateau.

The position of the *Beggiatoa* plate was not solely determined by availability of H_2S. Rather, filaments adjusted their vertical location so that they were concurrently limited by the rate of oxygen diffusion from the overlying air-filled headspace (Fig. 2B). During the major portion of linear growth, the flux ratio of H_2S:O_2 was essentially constant at 0.58 (60 to 128 h; Fig. 2B). This ratio is close to the stoichiometry of 0.61 predicted for oxidation of H_2S completely to the level of H_2SO_4 if one considers also the additional H_2S necessary as a reductant for CO_2 fixation. We present here the derivation of this predicted ratio based on the computational approach of Kelly (13). Since we assumed that 50% of *Beggiatoa* dry mass was carbon (Table 1), our computed Y_{H_2S} value of 8.4 g/mol dictates that 0.35 mol of CO_2 was fixed per mol of H_2S oxidized. CO_2 fixation to the

level of cell material can be estimated to require 4 H equivalents per mol of CO_2 fixed. Thus, per mol of H_2S completely oxidized, 1.4 H equivalents (4 × 0.35) would go to CO_2 reduction. This leaves 0.825 mol of H_2S, representing the remaining 6.6 H equivalents, which would require 1.65 mol of O_2 for complete oxidation. Thus, the overall stoichiometry predicted for H_2S to O_2 is 1.00:1.65, or 0.61. Table 3 summarizes these calculations and similar ones for incomplete H_2S oxidation of various degrees.

From 180 to 246 h, when the rate of biomass addition was falling below the previous linear rate, the molar flux ratio (H_2S/O_2) also declined to a value of 0.48. This is close to the ratio of 0.50 predicted for complete oxidation of sulfide to sulfate for energetic purposes only (i.e., no biosynthesis [Table 3]). These flux ratio data strongly support the postulated switch from balanced growth to "maintenance energy only" occurring gradually after 180 h.

Regulation of the H_2S/O_2 consumption ratio is apparently precisely controlled by the vertical migration of *Beggiatoa* sp. For the gradients used in this study, a 1-mm change in the vertical position of the bacterial plate could change the steepness of the oxygen gradient (and thus its consumption rate) by almost 50%. Since sulfide flux changed by approximately twofold over the entire course of this experiment, filaments must migrate only a few millimeters up or down to keep oxygen flux in balance. At the gliding rate of strain 81-6 (approximately 4 $\mu m\ s^{-1}$), such an adjustment could theoretically be accomplished in a few minutes. By balancing these fluxes, *Beggiatoa* sp. grows in an environment where neither substrate is in excess. This is in contrast to the limitation of growth by a single nutrient, as is typically observed in batch or chemostat cultures.

Transient storage of S^0. Early in the experiment, when growth was exponential and no sulfide or oxygen limitation existed, *Beggiatoa* sp. consumed sulfide and oxygen in a ratio as high as 0.93:1 (mol/mol). This ratio reflects incomplete oxidation of sulfide (Table 3) and is in accordance with the observation that S^0 accumulated within the cells during this early growth phase.

If one assumes balanced growth and arbitrarily assumes that one-half or three-fourths of sulfide consumed was oxidized to sulfate and the remainder only to the level of S^0, this predicts an H_2S/O_2 flux ratio of 0.97 or 0.75, respectively (Table 3). Assuming that all of the resultant S^0 is stored intracellularly (periplasmically [28]), the percentage of *Beggiatoa* biomass which will be S^0 can also be computed by assuming steady-state growth (Table 3). It appears, based on the maximum elemental sulfur content of filaments (discussed below), that even 25% oxidation to the level of S^0 is an upper limit for incomplete oxidation of sulfide under such circumstances. As demonstrated here for a transient situation, on the other hand, almost 50% of the sulfide consumed may be oxidized only as far as S^0. In steeper gradients and with cells initially devoid of S^0, the percentage of incomplete oxidation could theoretically go higher, perhaps approaching 100% for a fraction of a doubling.

Beggiatoa sp. has a theoretical flexibility of almost fivefold in H_2S/O_2 combining ratios (Table 3). This potential, coupled with the ability to store S^0 internally, may be of great advantage. For example, filaments depleted of S^0 which suddenly encounter a strong sulfide flux, could process almost five times as much H_2S per unit oxygen flux by oxidizing sulfide only to S^0. In addition to providing energy stored as S^0 for future consumption, this way of obtaining a high rate of sulfide removal may protect filaments from toxicity due to excess H_2S. This hypothesized transition from low to high sulfide flux over a short time is likely to occur on a daily basis in the environment from which strain 81-6 was isolated. When the sun sets, cessation of oxygen production by algae and diminished sulfide consumption by phototrophic bacteria will have the effect of exposing filaments to an increased sulfide flux and diminished oxygen flux in typical shallow water habitants (10, 17).

In pure culture, a freshwater *Beggiatoa* sp. was observed to deposit up to 30% of its biomass as S^0 (16). Observations on field material by Jørgensen (9) indicated an S^0 content of approximately half that percentage. Assuming a Y_{S^0} value equal to three-fourths of Y_{H_2S}, a 30% internal sulfur store would allow, by its complete oxidation to sulfate, a 6% increment in biomass. Thus, storage of S^0 appears to be of little significance, viewed in the light of its ability to support balanced growth. However, as an energy source enabling filaments to follow a rapidly moving sulfide/oxygen interface, it could prove highly valuable.

Winogradsky (33) postulated that S^0 was oxidized only after H_2S was depleted. By contrast, we assert that sulfate was produced from the beginning when strain MS-81-6 was grown chemolithoautotrophically in sulfide gradient medium. Supporting data include the observation of a pH minimum within the bacterial plate as early as 36 h even though sulfide was nonlimiting (deduced from continued exponential growth). Supporting data also include the fact that the H_2S/O_2 consumption ratio (Fig. 2B) never approached the range of 2.0 or 2.4:1, which would indicate oxidation exclusively to S^0.

Molar growth yields. Molar growth yields for other chemolithoautotrophic sulfur bacteria have been determined principally for thiobacilli growing on thiosulfate. At maximum efficiency (graphically extrapolated), the average aerobically grown thiobacillus has a theoretical growth yield of 6.7 g/mol of thiosulfate (13). The relatively little data which exist for Y_{H_2S} (aerobic, thiobacilli) indicate values which are only two-thirds to three-fourths of the yields for the same strain grown under comparable conditions on thiosulfate. Kelly (13) points out that this is puzzling, because complete oxidation of a molecule of either substrate should yield eight electrons to the electron transport chain, and because, for *Thiobacillus denitrificans* and *Thiomicrospira denitrificans*, there is evidence of equivalent yields on sulfide and thiosulfate when nitrate served as a terminal electron acceptor.

The Y_{H_2S} values presented here for *Beggiatoa* sp. are considerably higher than the limited data for thiobacilli. Kelly (13) has indicated two reasons to believe that Y_{H_2S} values of thiobacilli are too low. First, nonbiological oxidation of sulfide may have considerably decreased the quantity of substrate actually available to the bacteria. Secondly, the relatively high level of sulfide, up to 300 μm in short-term incorporation experiments, may have inhibited growth of thiobacilli. By growing *Beggiatoa* sp. in gradient cultures, both of these potential problems were avoided. Here, biological oxidation exceeded nonbiological oxidation by at least 3 orders of magnitude, and the sulfide concentration actually experienced by filaments did not appear to exceed 30 μM.

It should be noted that the values of Y_{H_2S} tabulated here for *Beggiatoa* sp. show considerable variance (95% confidence limits to the mean, 6.7 to 10.1 g mol^{-1}). It appears that this variation comes from uncertainty inherent in integrating sulfide profiles and measuring protein. By contrast, the three assumptions used to derive biomass values from protein data (Table 1) are unlikely to be inaccurate by more than a few percent (13, 18). Partial oxidation products (i.e., S^0 and

$S_2O_3^{2-}$) were undoubtedly generated in the medium by chemical oxidation during medium aging and early growth. Consumption of these compounds by *Beggiatoa* sp. between 60 and 247 h could inflate our computed yields, but only by a maximum of 7 to 10% (Table 1). Consumption of these compounds and the internal sulfur granules would certainly help to explain the observation that a 5-day period of linear growth was sustained despite a static or declining sulfide flux (Fig. 2A).

As an explanation for why the *Thiobacillus* $Y_{S_2O_3^{2-}}$ data might, instead, represent suboptimal growth, we note that these data ($\bar{x}$ = 6.7 g/mol) were from experiments where typically the oxygen concentration was atmospheric (13). *Beggiatoa* sp., on the other hand, was grown at oxygen concentrations which averaged 50- to 100-fold lower (19). High concentrations of oxygen are known to favor the oxygenase function of ribulose bisphosphate carboxylase/oxygenase and could help to explain the lower $Y_{S_2O_3^{2-}}$ values of thiobacilli (13). Further studies in gradient cultures and in thiosulfate supplemented cultures under microoxic conditions are necessary to address this point.

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